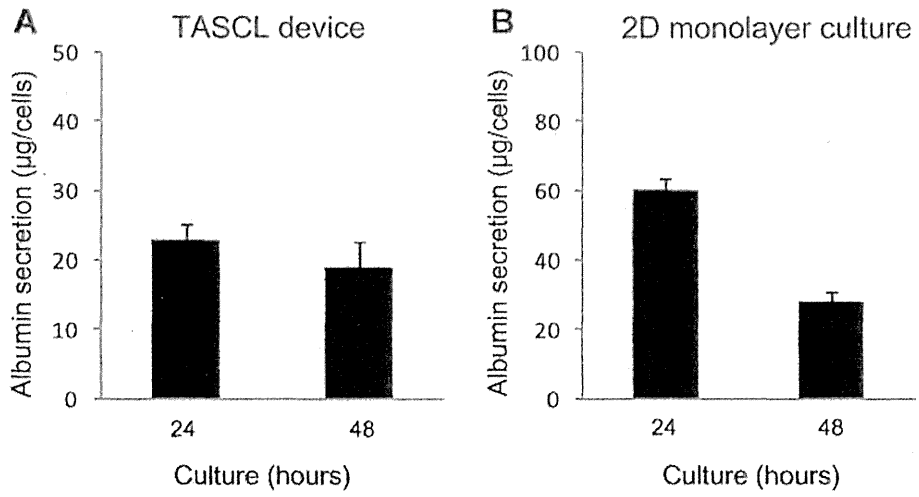

Figure 5. Albumin secretion in primary mouse hepatocyte constructs created using the TASCL device. The level of albumin synthesis was measured after 24 h of accumulation. (A) Hepatocytes were incubated on the TASCL device for 24 h. (B) Hepatocytes were incubated on the collagen-coated dishes for 24 h. The data represent the mean and SD of three independent experiments.

cell constructs was easily changed to a 3D environment. In this experiment, the size of individual microwells in the TASCL device was different (Fig. 1). Therefore, this device can be used as a screening tool to create uniform hepatocyte constructs. The combinatorial TASCL device described in this report can be used quickly and simply, and it will be useful for preparing hepatocyte constructs. In the future, we will report an application of the TASCL device taking into consideration properties of primary hepatocytes.

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Potential Factors for the Differentiation of ESCs/iPSCs Into Insulin-Producing Cells

Takako Tsugata,* Naruo Nikoh,* Tatsuya Kin,† Issei Saitoh,‡ Yasufumi Noguchi,§ Hideo Ueki,¶ Masami Watanabe,¶ Andrew M. James Shapiro,† and Hirofumi Noguchi*#

*Natural and Environmental Sciences Program, The Open University of Japan, Chiba, Japan
†Clinical Islet Transplant Program, University of Alberta, Edmonton, Alberta, Canada
‡Division of Pediatric Dentistry, Graduate School of Medical and Dental Science, Niigata University, Niigata, Japan
§Department of Socio-environmental Design, Hiroshima International University, Hiroshima, Japan
¶Department of Urology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan
#Department of Regenerative Medicine, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan

The low efficiency of in vitro differentiation of human embryonic stem cells (ESCs) or human induced pluripotent stem cells (iPSCs) into insulin-producing cells thus creates a crucial hurdle for the clinical implementation of human pluripotent stem cells (PSCs). In this study, we investigated the key factors for the differentiation of PSCs into insulin-producing cells. We obtained microarray data of HUES8 and HUES6 from two GeneChips (GPL3921: Affymetrix HT Human Genome U133A Array, GPL570: Affymetrix Human Genome U133 Plus 2.0 Array) in a database of GEO (NCBI), since HUES8 can differentiate into pancreatic cells, while HUES6 hardly demonstrates any differentiation at all. The genes with more than fourfold higher expressions in HUES8 compared to HUES6 included *RPS4Y1*, *DDX3Y*, *EIF1AY*, *GREM1*, *GATA6*, and *NLGN4Y*. Since there were four genes, *RPS4Y1*, *DDX3Y*, *EIF1AY*, and *NLGN4Y*, on the Y chromosome and HUES8 was a male cell line and HUES6 was a female cell line, we excluded these genes in this study. On the other hand, genes with more than fourfold higher expressions in HUES6 compared to HUES8 included *NLRP2*, *EGR1*, and *SMC3*. We next compared iPSCs derived from pancreatic cells (PiPSCs) and iPSCs derived from fibroblasts (FiPSCs). PiPSCs differentiated into insulin-producing cells more easily than FiPSCs because of their epigenetic memory. The gene expressions of *GREM1*, *GATA6*, *NLRP2*, *EGR1*, and *SMC3* in PiPSCs and FiPSCs were also investigated. The expression level of *GREM1* and *GATA6* in PiPSCs were higher than in FiPSCs. On the other hand, *EGR1*, which was lower in HUES8 than in HUES6, was predictably lower in PiPSCs than FiPSCs, while *NLRP2* and *SMC3* were higher in PiPSCs than FiPSCs. These data suggest that the expression of *GATA6* and *GREM1* and the inhibition of *EGR1* may be important factors for the differentiation of PSCs into insulin-producing cells.

Key words: Embryonic stem cells (ESCs); Induced pluripotent stem cells (iPSCs); *GATA6*; *GREM1*; *EGR1*

INTRODUCTION

Human embryonic stem cells (ESCs) are capable of differentiation into cells from the three embryonic germ layers that constitute the body and, therefore, have been used for regenerative medicine research at many institutions (12,21). However, the use of ESCs remains controversial for ethical reasons,which hinders the clinical uses of ESCs, since ESCs are derived from the inner cell mass of mammalian blastocysts (18). Recently, induced pluripotent stem cells (iPSCs) were generated from somatic cells by the transfection of several transcription factors to human somatic fibroblasts (20). This technical breakthrough has significant implications, since they provide a solution for overcoming the ethical issues associated with ESC derivation from embryos (10).

It has been reported that different human ESC lines express similar markers in the undifferentiated state (11), but marked differences have been observed in the differentiation propensity among various human ESC lines (16). Osafune et al. reported the differentiation potential of 17 human ESC lines, and the Harvard University embryonic stem cell line 8 (HUES8) was found to be the best for pancreatic differentiation, while the HUES3 cell line was the best for cardiomyocyte generation (16). Moreover, HUES8 and HUES6 were the best and the worst cell lines, respectively, for pancreatic differentiation among the

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Address correspondence to Hirofumi Noguchi, M.D., Ph.D., Department of Regenerative Medicine, Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan. Tel/Fax: +81-98-895-3331;
E-mail: noguchih@med.u-ryukyu.ac.jp *or* noguchih2006@yahoo.co.jp

17 identified human ESC lines. Therefore, differences in the gene expressions between HUES8 and HUES6 might be related to the differentiation of the pancreas from PSCs.

On the other hand, it has recently been shown that, following the reprogramming of mouse/human iPSCs, an epigenetic memory is inherited from parental cells, which affects the differentiation capacity of iPSC lines (14,15,17). It has also been shown that the epigenetic memory predisposes iPSCs derived from pancreatic β-cells to differentiate more readily into insulin-producing cells (1). These findings demonstrate that the iPSC phenotype may be influenced by their cells of origin, thereby suggesting that differences in the gene expression between iPSCs derived from pancreatic cells (PiPSCs) and those derived from fibroblasts (FiPSCs) may be related to their differentiation potential.

In this study, we investigated the factors required for the differentiation of PSCs into insulin-producing cells. We first compared the microarray data of the HUES8 and HUES6 cell lines and selected several genes and next compared PiPSCs and FiPSCs with the expressions of the genes that had been selected in the first study.

MATERIALS AND METHODS

Statistical Analysis of Microarray Data

The microarray data of HUES6 (female) and HUES8 (male) were extracted from the homepage of GEO (http://www.ncbi.nlm.nih.gov/geo/). The first set was Affymetrix HT Human Genome U133A Array (platform_id:GPL3921) of HUES6 (geo_accession: GSM 637760) and HUES8 (geo_accession: GSM 637761). The second set was Affymetrix Human Genome U133 Plus 2.0 Array (platform_id:GPL570) of HUES6 (geo_accession: GSM462819, GSM462820, GSM462821) and HUES8 (geo_accession: GSM 310860, GSM 310861, GSM 3108662). The factors were selected based on differences in the gene expressions of more than four times between HUES8 and HUES6 (only GPL570 is a value of $p<0.05$) using a Bioconductor (9). A data analysis was then performed using the R statistics software package (http://www.r-project.org/).

Generation of iPSCs From Human Pancreatic Cells

Pancreatic cells (>80% islets) from a human donor (female, aged in her 40s), which were isolated at the University of Alberta as previously described (19) and shipped to Japan, were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (Thermo Scientific, Kanagawa, Japan), and 0.5% penicillin (Sigma-Aldrich, St. Louis, MO, USA). The cells (1×10^5 cells) were transduced with sendai viral vectors (20 multiplicity of infection; DNAVEC, Ibaraki, Japan) encoding the four pluripotency-inducing factors: octamer-binding transcription factor 4 [*OCT4*; also known as pituitary-specific positive transcription factor 1 octamer-binding transcription factor 1 Unc-86 (POU) class 5 homeobox 1(*POU5F1*)], sex-determining region Y box 2 (*SOX2*), Kruppel-like factor 4 (*KLF4*), and v-myc avian myelocytomatosis viral oncogene homolog (*cMYC*). Two days after transduction, the cells were replated on resistant SNL (Sandos Inbred Mice embryo-derived and thioguanine and ouabain cells transformed by neomycin resistance and murine leukemia inhibitory factor) feeder layer (Cosmo Bio Co., Ltd., Tokyo, Japan). The next day, the medium was replaced with a standard ESC culture medium (Repro CELL, Kanagawa, Japan). Three to 4 weeks after transduction, about 10 colonies were visible. The colonies were picked up and then were transferred on an SNL feeder. The cultures were manually passaged at a 1:3–1:6 split ratio every 5–7 days.

Cell Culture

PiPSCs (induced cells described above) and FiPSCs (201B7, RIKEN BioResource Center, Japan) were maintained on an SNL feeder layer in DMEM-F12 (Sigma-Aldrich), 2 mM L-glutamine (Nacalaitesque, Kyoto, Japan), 1:100 dilution of nonessential amino acid (Life Technologies), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 5 ng/ml basic fibroblast growth factor (bFGF) (Repro CELL), and penicillin/streptomycin (Sigma-Aldrich). For passaging, iPSC colonies were dissociated with dissociation solution for human ESCs/iPSCs (Riken CDB, Kobe, Japan) and split at a ratio between 1:3 and 1:6.

Alkaline Phosphatase Staining

Alkaline phosphatase staining was performed on 15 colonies in 35-mm dishes using the Vector Blue Alkaline Phosphatase Substrate Kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions.

Karyotype Analysis

Chromosomal G-band analyses were performed at the Nihon Gene Research Laboratories, Japan. At least 20 metaphases were analyzed.

Quantitative RT-PCR

Total RNA was prepared with the RNeasy Micro Kit (Qiagen, Tokyo, Japan) and treated with RNase free DNase (Qiagen). A total of 500 ng RNA was used for a reverse transcription reaction using the QuantiTect Reverse Transcription Kit (Qiagen). qRT-PCR was performed on the Real-time PCR System (ABI7000) using SYBR Green (both from Life Technologies). The expression levels were normalized to the glycelaldehyde-3-phosphate dehydrogenase (GAPDH). The specific primers are listed in Table 1.

Table 1. Primer Sequences of Quantitative RT-PCR

Gene	Sequences (5′ to 3′)
ESC-specific genes	
OCT3/4	Forward: GAAACCCACACTGCAGCAGA
	Reverse: TCGCTTGCCCTTCTGGCG
SOX2	Forward: GGGAAATGGGAGGGGTGCAAAAGAGG
	Reverse: TTGCGTGAGTGTGGATGGGATTGGTG
NANOG	Forward: CTCAGCTACAAACAGGTGAAGAC
	Reverse: TCCCTGGTGGTAGGAAGAGTAAA
REX1 (ZFP42)	Forward: CAGATCCTAAACAGCTCGCAGAAT
	Reverse: GCGTACGCAAATTAAAGTCCAGA
TERT	Forward: CGTACAGGTTTCACGCATGTG
	Reverse: ATGACGCGCAGGAAAAATGT
GAL	Forward: AAACAATATCATGCGCACAATCA
	Reverse: GGGCACCGGCCTCTTT
LEFTY2	Forward: GCTCAGATGCTGAGCTCTAGTAGGA
	Reverse: GAAACTCCCAGCTGAAAATGTGT
GAPDH	Forward: CCACTCCTCCACCTTTGACG
	Reverse: ATGAGGTCCACCACCCTGTT
Endoderm-specific genes	
SOX17	Forward: AGCCAAGGGCGAGTCCCGTA
	Reverse: GCCTTCCACGACTTGCCCAGC
CXCR4	Forward: CTGCACCTGTCAGTGGCCGA
	Reverse: TTGGTGGCGTGGACGATGGC
WNT3	Forward: TCCTCGGCGCCTCTTCTAAT
	Reverse: CTGTGAGCCCAGAGATGTGT
CD117 (KIT)	Forward: ATTCAAGCACAATGGCACGG
	Reverse: AAGGAGTGAACAGGGTGTGG
GATA3	Forward: CTGGCTCGCAGAATTGCA
	Reverse: TGGGTACGGCAGAATAAAACG
GATA4	Forward: GTTTTTTCCCCTTTGATTTTTGATC
	Reverse: AACGACGGCAACAACGATAAT
GATA6	Forward: GGATTGTCCTGTGCCAACTGT
	Reverse: GGTTCACCCTCGGCGTTT
HNF1B	Forward: AACCACCGAAGAGGAAGCAA
	Reverse: TCGCATCAGTTTGTTCGATGA
HNF4A	Forward: CACCTGATGCAGGAACATATGG
	Reverse: CTGTCCGTTGCTGAGGTGAGT
AFP	Forward: GTTGCCAACTCAGTGAGGACAA
	Reverse: TGATACATAAGTGTCCGATAATAATGTCA
Pancreas-specific genes	
PDX1	Forward: GATACTGGATTGGCGTTGTTTG
	Reverse: TCCCAAGGTGGAGTGCTGTAG
HNF6	Forward: TGCGCAACCCCAAACC
	Reverse: TCCACATCCTCCGGAAGGT
NKX6.1	Forward: GCCTGTACCCCTCATCAAGGA
	Reverse: AAGTGGGTCTCGTGTGTTTTCTC
INS	Forward: ACGAGGCTTCTTCTACACACCC
	Reverse: TCCACAATGCCACGCTTCTGCA
PAX6	Forward: GCTTCACCATGGCAAATAACCT
	Reverse: GGCAGCATGCAGGAGTATGA
SOX9	Forward: CCCATGTGGAAGGCAGATG
	Reverse: GAAGGTTAACTGCTGGTGTTCTGA

(*continued*)

Table 1. (Continued)

Gene	Sequences (5′ to 3′)
Genes selected by a microarray analysis	
GREM1	Forward: CCCCCCGCCAGACAAG
	Reverse: TTGCACCAGTCTCGCTTCAG
EGR1	Forward: TTTCACGTCTTGGTGCCTTTT
	Reverse: TCCCTCACAATTGCACATGTC
NLRP2	Forward: CTTTGAGGAAACCACTGTGCAA
	Reverse: AACTGAACGGAGGGATGGAA
SMC3	Forward: TTGCTCTGATTTTTGCCATTCA
	Reverse: CATCCAGAGCCTGGTCAATTTC

OCT3/4, octamer-binding transcription factor 3/4 [also known as pituitary-specific positive transcription factor 1 octamer-binding transcription factor 1 Unc-86 (POU) class 5 homeobox 1(POU5F1)]; SOX2: sex-determining region Y box 2; REX1, reduced expression protein 1 (Zinc finger protein 42; ZFP42); TERT, telomerase reverse transcriptase; GAL:, galanin/galanin-message-associated peptide (GMAP) pre-propeptide; LEFTY2, left-right determination factor 2; GAPDH, glyceraldehyde 3-phospahte dehydrogenase; CXCR4, chemokine C-X-C motif receptor 4; WNT3, wingless-type MMTV integration site family, member 3; CD117, cluster of differentiation 117 (also known as v-kit Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog, KIT); GATA3, guanine–adenine–thymine–adenine-binding protein 3; HNF1B, hepatocyte nuclear factor 1B; AFP, αfeto-protein; PDX1, pancreatic and duodenal homeobox 1; NKX6.1, NK6 homeobox 1; INS, insulin; PAX6, paired box 6; GREM1, Gremlin 1 [also known as differential screening-selected gene aberrative in neuroblastoma (DAN) family bone morphogenetic protein (BMP) antagonist]; EGR1, early growth response 1; NLRP2, nucleotide-binding domain and leucine-rich repeat containing gene (NLR) family, pyrin domain containing 2; SMC3, structural maintenance of chromosomes 3.

RESULTS

Comparison of the Gene Expressions of Human ESC Lines by the Microarray Data

Osafune et al. reported the differentiation potential of 17 human ESC lines, and the male HUES8 and female HUES6 were found to be the best and the worst lines, respectively, for pancreatic differentiation (16). We obtained the microarray data of HUES8 and HUES6 from two GeneChips (GPL3921: Affymetrix HT Human Genome U133A Array, GPL570: Affymetrix Human Genome U133 Plus 2.0 Array) in a database of GEO (NCBI). Seventy-two genes on GPL3921 and 133 genes on GPL 570 were expressed more than fourfold higher in HUES8 than in HUES6. The matched genes of these two chips were ribosomal protein S4, Y-linked 1 (*RPS4Y1*), DEAD (Asp-Glu-Ala-Asp) box helicase 3, Y-linked (*DDX3Y*), eukaryotic translation initiation factor 1A, Y-linked (*EIF1AY*), Gremlin 1 [*GREM1* also known as differential screening-selected gene aberrative in neuroblastoma (DAN) family bone morphogenetic protein (BMP) antagonist], guanine–adenine–thymine–adenine-binding protein 6 (*GATA6*), and neuroligin 4, Y-linked (*NLGN4Y*). Since four of these genes, *RPS4Y1*, *DDX3Y*, *EIF1AY*, and *NLGN4Y*, are on the Y chromosome, and HUES8 is a male cell line, while HUES6 is a female cell line, we excluded these genes in the following study. We also investigated ESC-specific genes, endoderm-specific genes, and pancreas-specific genes as shown in Table 2 (2,3,4,20,22) in 72 genes on GPL3921 and 133 genes on GPL 570. There were no ESC-specific genes, three endodermal-specific genes (*GATA3*, *GATA4*, and *GATA6*), and one pancreas-specific gene (paired box 6; *PAX6*) out of the 72 genes on GPL3921. There were two ESC-specific genes [*SOX2* and galanin/galanin-message-associated peptide (GMAP) pre-propeptide (*GAL*)], two endodermal-specific genes (*SOX17* and *GATA6*), and no pancreas-specific genes in 133 genes on GPL 570 (Fig. 1A).

Forty-nine genes on GPL3921 and 191 genes on GPL 570 were expressed more than fourfold higher in HUES6 than in HUES8. The matched genes of these two chips were nucleotide-binding domain and leucine-rich repeat containing gene (NLR) family, pyrin domain containing 2 (*NLRP2*), early growth response 1 (*EGR1*), and structural maintenance of chromosomes 3 (*SMC3*). We also investigated the ESC-specific genes, endoderm-specific genes, and pancreas-specific genes as shown in Table 2 (2,3,4,20,22) in 49 genes on GPL3921 and 191 genes on GPL 570. There

Table 2. Human ESC-Specific Genes, Endoderm-Specific Genes, Pancreas-Specific Genes

ESC-Specific Genes	Endoderm-Specific Genes	Pancreas-Specific Genes
OCT3/4 (POU5F1)	*WNT3*	*INSULIN*
SOX2	*CD117*	*PDX1*
NANOG	*GATA4*	*PAX6*
REX1 (ZFP42)	*SOX17*	*NKX6.1*
LEFTY2	*HNF4*	*SOX9*
TERT	*CXCR4*	*HNF6*
GAL	*HNF1B*	
	AFP	
	GATA6	
	GATA3	

were no ESC-specific genes, endodermal-specific genes, or pancreas-specific genes out of the 49 genes on GPL3921 or out ofthe 191 genes on GPL 570 (Fig. 1B).

Generation of iPSCs From Human Pancreatic Cells

Epigenetic memory has been shown to predispose iPSCs derived from pancreatic β-cells to differentiate more readily into insulin-producing cells (1). These findings suggest that differences in the gene expression between PiPSCs and FiPSCs may be related to their differentiation potential. In this study, 201B7 cells (20) were used as FiPSCs. To generate PiPSCs, human pancreatic cells (more than 80% islets) were cultured and then transduced with four sendai viral vectors encoding the reprogramming factors *OCT4*, *SOX2*, *KLF4*, and *c-MYC*. Four weeks after transduction, 10 colonies were visible. The colonies were picked up and then were transferred on an SNL feeder (Fig. 2A). After three to four passages, the PiPSC lines showed a typical ESC-like morphology (Fig. 2B), and they could be maintained beyond 20 passages (Fig. 2C). PiPSCs expressed alkaline phosphatase (Fig. 2D) and divided actively without showing any growth inhibition (Fig. 2E).

Karyotype of PiPSCs

In some cases, chromosomal abnormalities have been shown to exist in human ESC lines and human iPSC lines. We investigated the karyotype of PiPSCs. PiPSCs exhibit a normal diploid karyotype of 46XX chromosomes (Fig. 3).

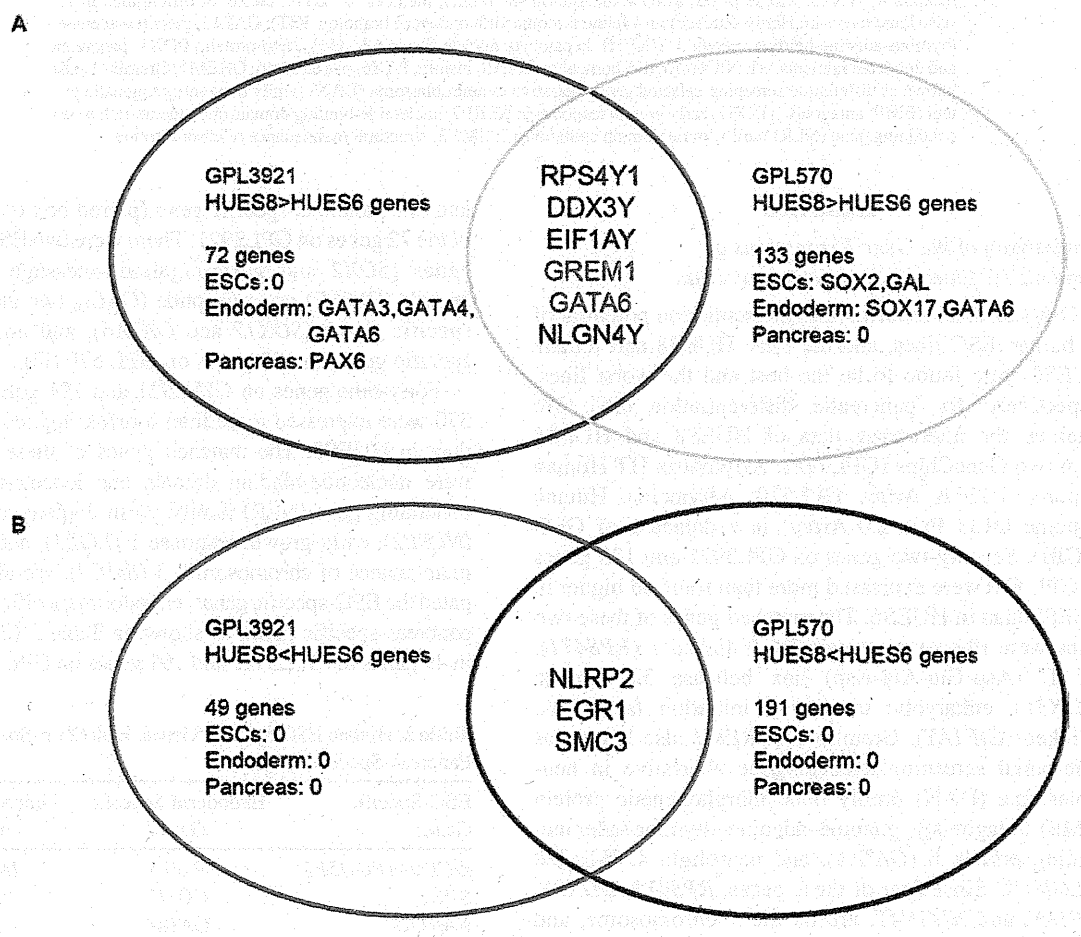

Figure 1. Microarray analysis. (A) Genes that are expressed more than fourfold higher in HUES8 compared to HUES6 in two gene chips (GPL3921 and GPL570). (B) Genes that are expressed more than fourfold higher in HUES6 compared to HUES8. ESCs, embryonic stem cells; GATA3, guanine–adenine–thymine–adenine-binding protein 3; PAX6, paired box 6; RPS4Y1, ribosomal protein S4, Y-linked 1; DDX3Y, DEAD (Asp-Glu-Ala-Asp) box helicase 3; EIF1AY, eukaryotic translation initiation factor 1A, Y-linked, GREM1, Gremlin 1 [also known as differential screening-selected gene aberrative in neuroblastoma (DAN) family bone morphogenetic protein (BMP) antagonist]; NLGN4Y, neuroligin 4, Y-linked; SOX2, sex-determining region Y box 2; GAL, galanin/galanin-message-associated peptide pre-propeptide (GMAP); NLRP2, nucleotide-binding domain and leucine-rich repeat containing gene (NLR) family, pyrin domain containing 2; EGR1, early growth response 1; SMC3, structural maintenance of chromosomes 3.

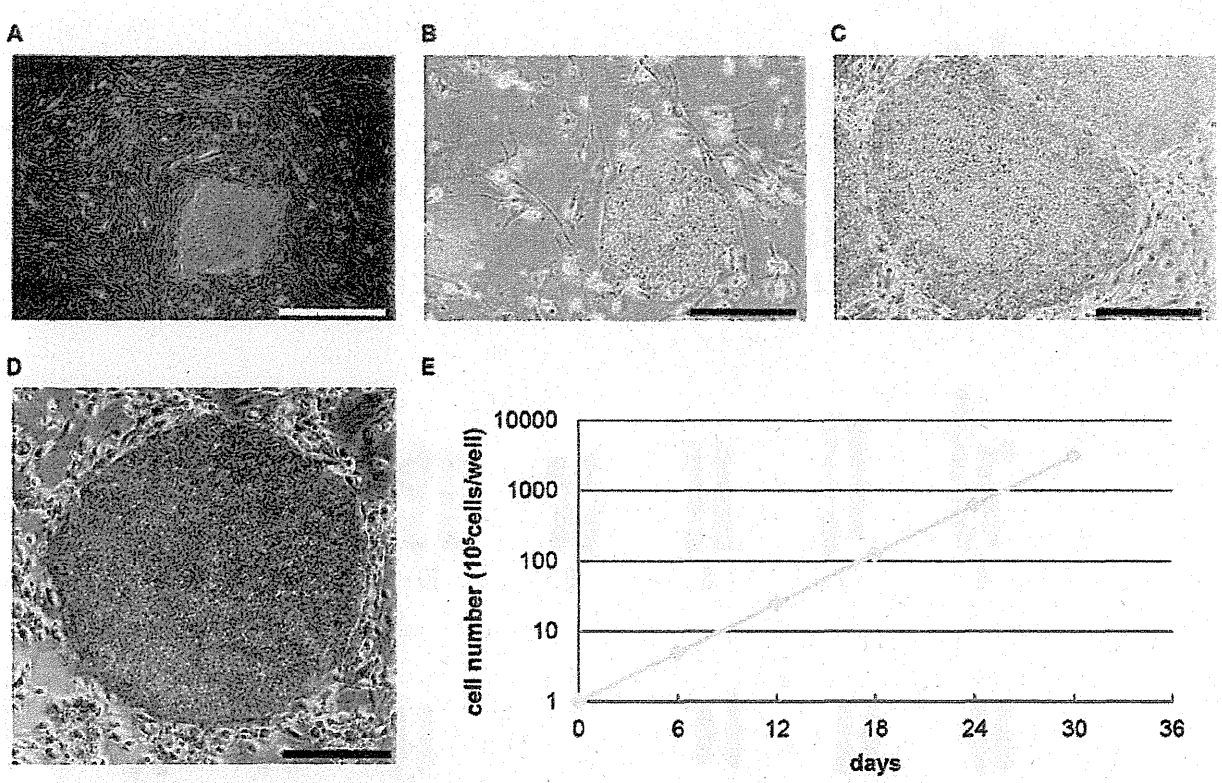

Figure 2. Generation of iPSCs from human pancreatic cells.(A) PiPSCs; passage 0. (B) PiPSCs; passage 4. (C) PiPSCs; passage 20. (D) Alkaline phosphatase staining of PiPSCs (passage 20). Scale bars: 500 μm. (F) Growth curve of PiPSCs.

Expression of ESC-Specific Genes, Endoderm-Specific Genes, and Pancreas-Specific Genes in PiPSCs and FiPSCs

An expression analysis of ESC-specific genes, endoderm-specific genes, and pancreas-specific genes (Table 2) in PiPSCs and FiPSCs was conducted using quantitative RT-PCR (qRT-PCR). ESC-specific genes, endoderm-specific genes, and pancreatic-specific genes are shown in Table 2. The expression of ESC-specific genes in PiPSCs was relatively lower than that in FiPSCs, except for the reduced expression protein 1 (*REX1* also known as zinc finger protein 42; *ZFP42*) gene (Fig. 4A). For endoderm-specific genes, the expressions of wingless-type mouse mammary tumor virus (MMTV) integration site family, member 3 (*WNT3*), cluster of differentiation 117 (*CD117*, also known as v-kit Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog, *KIT*), and *GATA4*, which are known to be early endodermal markers in PiPSCs, were relatively lower than those observed in FiPSCs, while the expression of other endodermal-specific genes in PiPSCs was relatively higher than that in FiPSCs. In particular, the expression of *GATA3* in PiPSCs was about nine times higher than that in FiPSCs (Fig. 4B). The expression of pancreas-specific genes in PiPSCs was relatively higher than that of FiPSCs (Fig. 4C). These data suggest that, following the reprogramming of PiPSCs, an epigenetic memory is thus inherited from the pancreatic cells.

Expression of Genes Selected by Microarray Analysis in PiPSCs and FiPSCs

An expression analysis of the genes selected by microarray analysis (Fig. 1) in PiPSCs and FiPSCs was conducted using qRT-PCR. *GREM1* and *GATA6* genes, which both had a higher expression in HUES8 than in HUES6 for both GPL3921 and GPL570, were expressed at higher levels in PiPSCs than FiPSCs (Fig. 5A). For three genes, in which the expression was higher in HUES8 than HUES6 only on GPL3921, *GATA3*, and *PAX6*, the expressions were relatively higher in PiPSCs than FiPSCs, while *GATA4* was lower in PiPSCs than FiPSCs (Fig. 5B). For three genes in which the expression was higher in HUES8 than HUES6 only on GPL570, *SOX17* was relatively higher in PiPSCs than FiPSCs, while *SOX2* and *GAL* were lower in PiPSCs than in FiPSCs (Fig. 5C).

For three genes in which the expression was lower in HUES8 than HUES6 on both GPL3921 and GPL570, *EGR1* was lower in PiPSCs than FiPSCs, while *NLRP2* and *SMC3* were unexpectedly higher in PiPSCs than FiPSCs (Fig. 6).

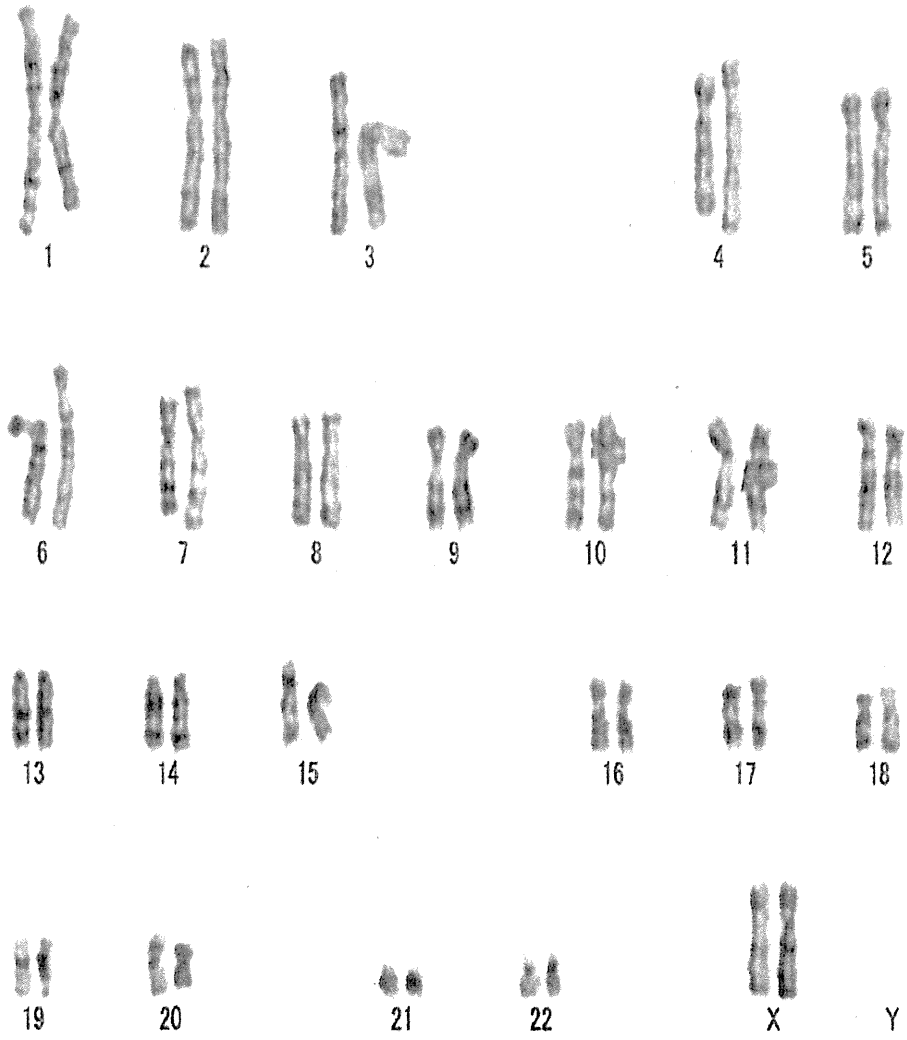

Figure 3. Karyotype of PiPSCs. PiPSCs exhibit a normal diploid karyotype of the 46XX chromosomes.

These data suggest that expression of GREM1 and GATA6 and the suppression of EGR1 may explain why these cells can be differentiated to insulin-producing cells more readily.

DISCUSSION

Based on a report regarding the differences in the differentiation induction efficiency among 17 ESC lines (16), we analyzed the microarray data ofthe HUES8 and HUES6 cell lines. The data that we obtained suggest that a high expression of GREM1 and GATA6, and the suppression of EGR1, NLRP2, and SMC3 might be important factors for the differentiation of ESCs into pancreatic cells. We next analyzed the expression of these genes in PiPSCs and FiPSCs based on epigenetic memory of iPSCs (1). The data for iPSCs suggest that a high expression of GREM1 and GATA6 and the suppression of EGR1 could play an important role in the differentiation of iPSCs into pancreatic cells. GATA6 is well known to play an important role in the development of the pancreas (5,13). On the other hand, there are few reports that have investigated the relationship between GREM1 and pancreas development. Dodge et al. analyzed the gene expression profiles of three different stages during in vitro islet generation: namely, the initial adherent, expanded, and differentiated stages. In the expanded and differentiated stages, the expression of the *GREM1* gene was upregulated (6). GREM1 is a member of a BMP (osteogenic protein) antagonist family. Since the BMP signal is important for liver development, its inhibition by GREM1 might therefore be an important factor for pancreas development.

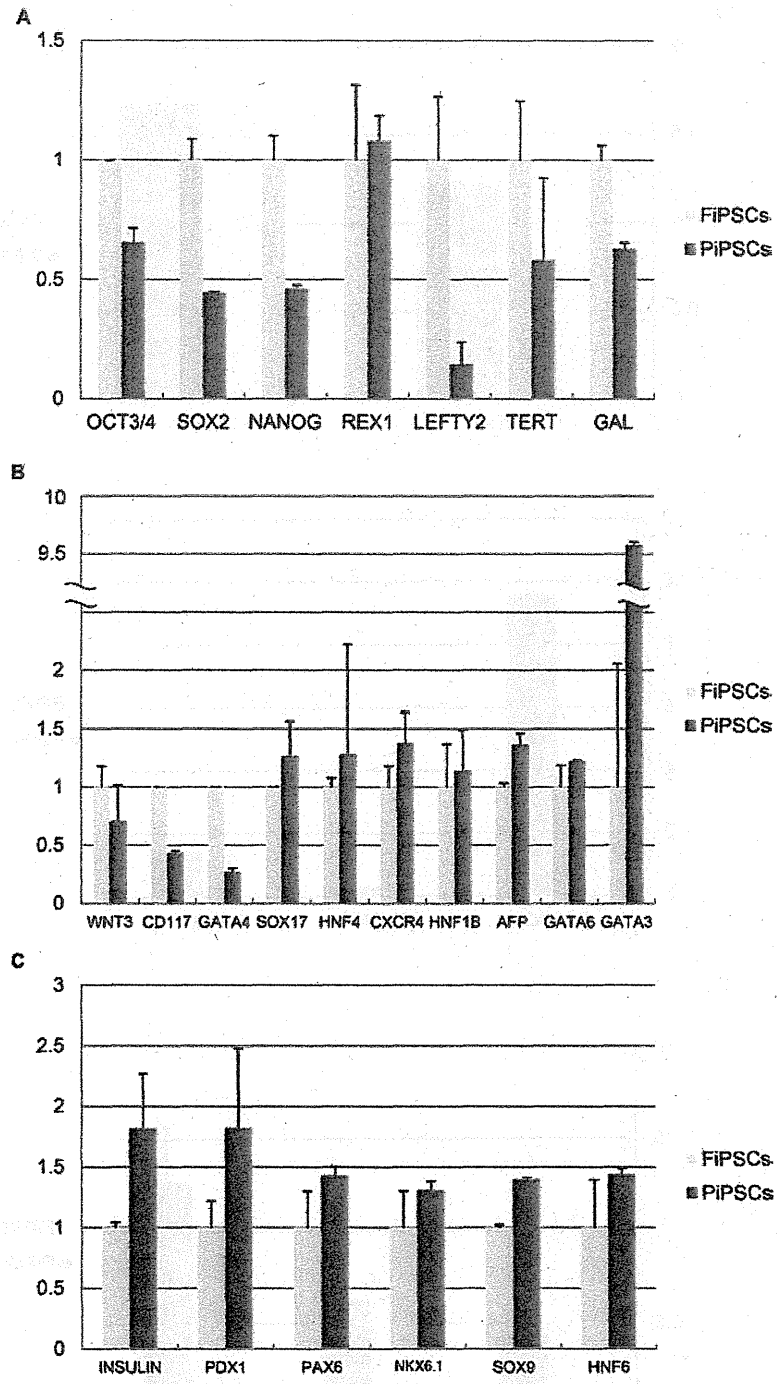

Figure 4. Gene expression in FiPSCs and PiPSCs. (A) A quantitative RT-PCR analysis of ESC-specific genes in FiPSCs and PiPSCs. (B) A quantitative RT-PCR analysis of endodermal-specific genes in FiPSCs and PiPSCs. (C) A quantitative RT-PCR analysis of pancreas-specific genes in FiPSCs and PiPSCs. The data are expressed as the genes-to-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) ratio, with that of FiPSCs arbitrarily set at 1 (n=3). OCT3/4, octamer-binding transcription factor 3/4 [also known as pituitary-specific positive transcription factor 1 octamer-binding transcription factor 1 Unc-86 (POU) class 5 homeobox 1(POU5F1)]; REX1, reduced expression protein 1 (Zinc finger protein 42; ZFP42); LEFTY2, left-right determination factor 2; TERT, telomerase reverse transcriptase; WNT3, wingless-type MMTV integration site family, member 3; CD117, cluster of differentiation 117 (also known as v-kit Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog, KIT); HNF4, hepatocyte nuclear factor 4; CXCR4, chemokine C-X-C motif receptor 4; AFP, αfeto-protein; PDX1, pancreatic and duodenal homeobox 1; NKX6.1, NK6 homeobox 1.

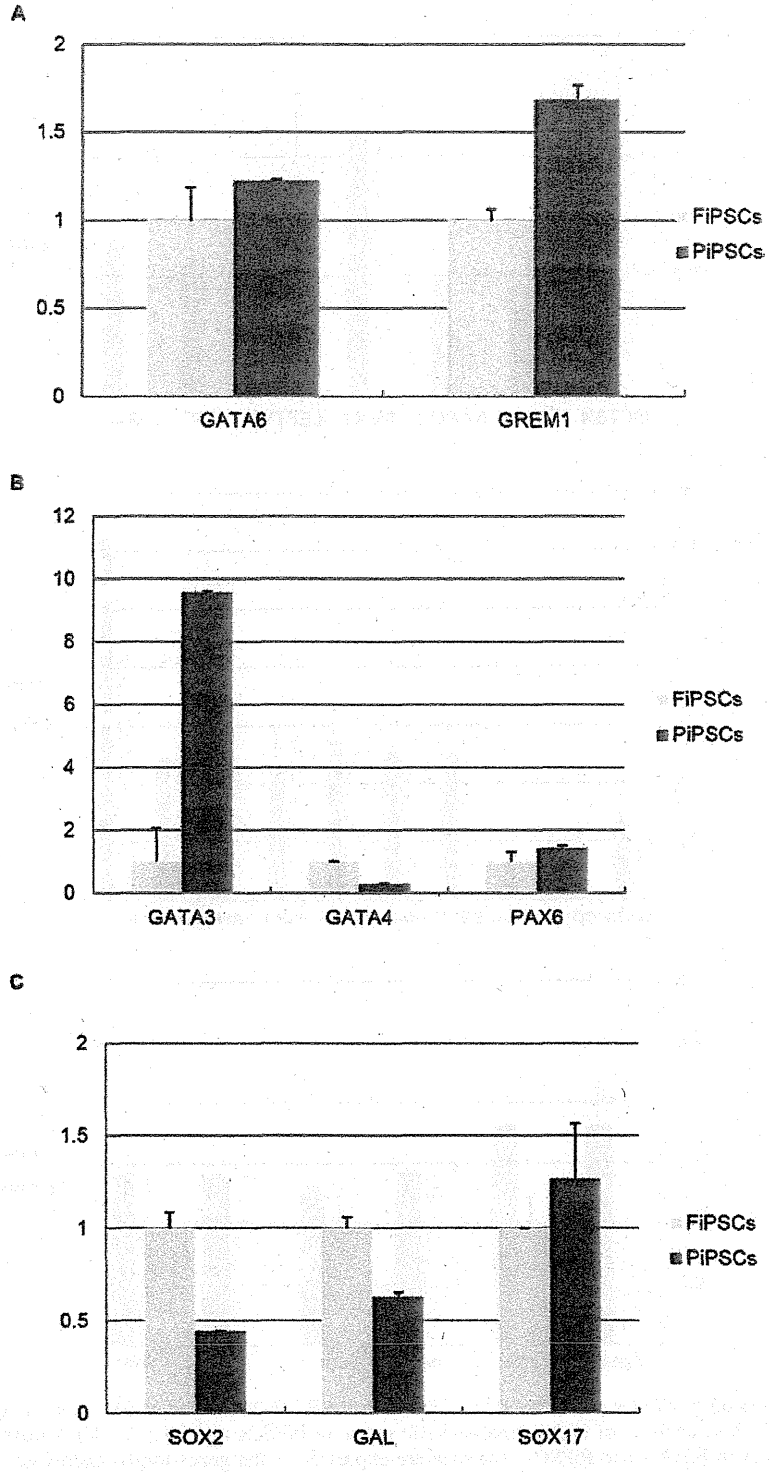

Figure 5. Expression analysis of the genes selected by a microarray analysis (HUES8>HUES6) in PiPSCs and FiPSCs.(A) A quantitative RT-PCR analysis of the GATA6 and GREM1 genes, in which the expression was higher in HUES8 than in HUES6 on both GPL3921 and GPL570, in FiPSCs and PiPSCs. (B) A quantitative RT-PCR analysis of GATA3, GATA4, and PAX6 genes, in which the expression was higher in HUES8 than HUES6 on only GPL3921, in FiPSCs and PiPSCs. (C) A quantitative RT-PCR analysis of SOX2, GAL, SOX17 genes, in which expression was higher in HUES8 than HUES6 on only GPL570, in FiPSCs and PiPSCs. The data are expressed as the genes-to-GAPDH ratio, with that of FiPSCs arbitrarily set at 1 ($n=3$).

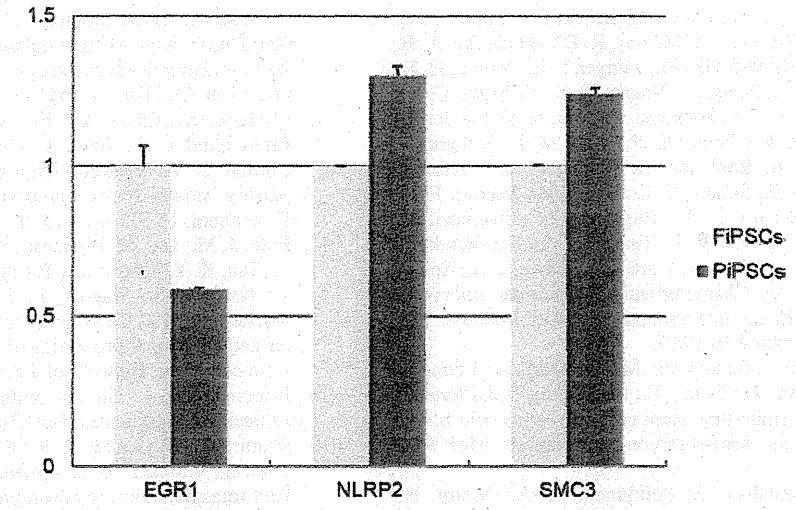

Figure 6. Expression analysis of the genes selected by a microarray analysis (HUES8<HUES6) in PiPSCs and FiPSCs. A quantitative RT-PCR analysis of EGR1, NLRP2, and SMC3 genes, in which the expression was lower in HUES8 than in HUES6 on both GPL3921 and GPL570, in FiPSCs and PiPSCs. The data are expressed as the genes-to-GAPDH ratio, with that of FiPSCs arbitrarily set at 1 (n=3).

The protein encoded by the *EGR1* gene belongs to the EGR family of C2H2-type zinc-finger proteins. It is a nuclear protein, and it functions as a transcriptional regulator. One group reported EGR1 to regulate the transcription of the insulin gene (7) and the *Pdx1* gene (8). Since the relationship between EGR1 and pancreas development is unknown, we therefore plan to investigate the role of EGR1 in pancreas development in a future study.

In conclusion, the expression of GREM1 and the inhibition of EGR1 might therefore be important factors in the differentiation of PSCs into insulin-producing cells. Further study of these genes is needed to generate insulin-producing cells with glucose sensitivity from ES/iPS cells.

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Induction of tissue-specific stem cells by reprogramming factors, and tissue-specific selection

H Noguchi*[,1,2,3,4], I Saitoh[5], T Tsugata[3], H Kataoka[6], M Watanabe[7] and Y Noguchi[8]

Although induced pluripotent stem (iPS) cells have significant implications for overcoming most of the ethical issues associated with embryonic stem (ES) cells, there are still several unresolved issues related to the use of iPS cells for clinical applications, such as teratoma formation. In this study, we were able to generate tissue-specific stem (induced tissue-specific stem; iTS) cells from the pancreas (iTS-P) or liver (iTS-L) by transient overexpression of reprogramming factors, combined with tissue-specific selection. The generation of iTS cells was easier than that of iPS cells. The iTS-P/iTS-L cells express genetic markers of endoderm and pancreatic/hepatic progenitors and were able to differentiate into insulin-producing cells/hepatocytes more efficiently than ES cells. Subcutaneous transplantation of both types of iTS cells into immunodeficient mice resulted in no teratoma formation. The technology used for the transient overexpression of reprogramming factors and tissue-specific selection may be useful for the generation of other tissue-specific stem cells, and the generation of iTS cells could have important implications for the clinical application of stem cells.
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Embryonic stem (ES) cells are capable of unlimited proliferation *in vitro*, while maintaining their potential to differentiate into cells from the three embryonic germ layers. Induced pluripotent stem (iPS) cells are generated from adult fibroblasts or other somatic cells, and are similar to ES cells in their morphology, gene expression, epigenetic status and *in vitro* differentiation. Mouse iPS cells give rise to adult chimeras and show competence for germline transmission.[1–7] This technical breakthrough has significant implications for overcoming the ethical issues associated with ES cell derivation from embryos. The generation of mouse iPS cells without the genomic integration of exogenous reprogramming factors by the repeated transfection of plasmids expressing Oct3/4, Sox2 (sex-determining region Y-box2), Klf4 and c-Myc,[8] and by using nonintegrating adenoviruses transiently expressing the four factors[9] has been reported. Moreover, the generation of human iPS cells without the genomic integration of exogenous reprogramming factors by plasmids expressing Oct3/4, Sox2, Klf4, c-Myc, Nanog, LIN28 and SV40LT,[10] or Oct3/4, Sox2, Klf4, L-Myc, LIN28 and p53 shRNA[11] has been shown. These reports provide strong evidence that insertional mutagenesis is not required for *in vitro* reprogramming. The production of iPS cells without viral integration addresses a critical safety concern for the potential use of iPS cells in regenerative medicine. However, the use of iPS cells for clinical therapies is hampered by their potential for tumor formation and the limited ability to generate pure populations of differentiated cell types *in vitro*.

Adult tissue-specific stem/progenitor cells could represent one of the alternative sources for the treatment of diseases. Several *in vitro* studies have shown that insulin-producing cells (IPC) can be generated from adult pancreatic ductal tissues.[12–14] The assessment of 83 human islet grafts transplanted using the Edmonton Protocol since 1999[15] showed that a significant positive correlation was observed between the number of islet progenitor (ductal-epithelial) cells transplanted and the long-term metabolic success, as assessed by an intravenous glucose tolerance test at ~2 years post-transplantation. Therefore, pancreatic duct/progenitor cells could become a new source of IPC. One of the most difficult, and yet unresolved issues, is how to isolate pancreatic 'stem' cells, which have self-renewal capacity. We and other groups have established mouse pancreatic stem cell lines using specific culture conditions.[16,17] One of our established pancreatic stem cell lines, HN#13, derived from the pancreatic tissue of an 8-week-old mouse without genetic manipulation could be maintained during repeated passages for more than 1 year without growth inhibition under specific culture conditions. The HN#13 cells do not have tumorigenic properties, and have normal chromosomes. The cells express

[1]Department of Surgery, Chiba-East National Hospital, National Hospital Organization, Chiba, Japan; [2]Department of Regenerative Medicine, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan; [3]Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; [4]Natural and Environmental Sciences Program, The Open University of Japan, Chiba, Japan; [5]Division of Pediatric Dentistry, Graduate School of Medical and Dental Science, Niigata University, Niigata, Japan; [6]Department of Primary Care and Medical Education, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; [7]Department of Urology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan and [8]Department of Socio-environmental Design, Hiroshima International University, Hiroshima, Japan
*Corresponding author: H Noguchi, Department of Surgery, Chiba-East National Hospital, National Hospital Organization, 673 Nitona-cho, Chuo-ku, Chiba 260-8712, Japan. Tel: +81 43 261 5171; Fax: +81 43 268 2613; E-mail: n.hirofumi@cehpnet.com or noguchih2006@yahoo.co.jp

Abbreviations: iPS, induced pluripotent stem; ES, embryonic stem; iTS, induced tissue-specific stem; iTS-P, iTS cells from the pancreas; iTS-L, iTS cells from the liver; Pdx1, the pancreatic and duodenal homeobox factor-1; iF, induced fibroblast-like; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; Sox, sex-determining region Y-box; Hnf, hepatocyte nuclear factor; DE, definitive endoderm; GTE, gut tube endoderm; PP, pancreatic progenitors; IPC, insulin-producing cells; NeoR, neomycin resistance; α1-AT, α1-antitrypsin; G6PC, glucose 6-phosphatase; Pecam1, platelet/endothelial cell adhesion molecule 1; Mixl1, Mix1 homeobox-like 1; Zic1, zinc finger protein of the cerebellum 1; EP, endodermal progenitor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum

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the pancreatic and duodenal homeobox factor-1 (Pdx1), one of the transcription factors of the β-cell lineage.[17] However, we were unable to isolate and culture mouse pancreatic stem cells from older donors[18] or pancreatic stem cells from human pancreatic tissue.[19] In the mouse study, pancreatic stem cells were isolated from the pancreata of all newborn mice examined. Meanwhile, pancreatic stem cells were isolated from only 10% of the pancreata of 8-week-old mice, not from the pancreata of any 24-week-old mice.[18] These data suggest that young donors have a larger number of pancreatic stem cells, whereas that older donors have few or virtually no pancreatic stem cells. These data also indicate that it is extremely difficult to isolate pancreatic stem cells from older donors.

In this study, we were able to generate tissue-specific stem (induced tissue-specific stem; iTS) cells from mouse pancreatic tissue (iTS-P) by transient overexpression of reprogramming factors combined with Pdx1 selection. Furthermore, we were also able to generate iTS cells from the mouse liver (iTS-L) by transient overexpression of reprogramming factors combined with HNF4α selection.

Results

Generation of iTS-P cells from mouse pancreatic tissue. We attempted to generate mouse iPS cells from older-donor pancreata by transfection of a single plasmid expressing Oct3/4, Sox2 and Klf4 with or without c-Myc. The three or four cDNAs were connected in this order with the 2A peptide and inserted into a plasmid containing the CMV or CAG[20] promoter (Supplementary Figure 1a). We transfected the OSKM plasmid (four factors) or OKS plasmid (three factors) into pancreatic tissue obtained from 24-week-old mice on days 1, 3, 5 and 7 (Figure 1a). We were able to generate only one colony of iPS cells from 24-week-old mouse pancreata using the OSKM plasmid during five experiments and were unable to generate iPS cells using the OKS plasmid throughout five experiments. However, we noticed that there were some cells that had self-renewing potential. The morphology of some cells was similar to that of mouse pancreatic stem cells, which we had previously established from young-donor pancreata without genetic manipulation.[17] We designated them 'induced tissue-specific stem (iTS-P) cells'. Morphology of the other cells was similar to that of fibroblast cells, which we designated 'induced fibroblast-like (iF) cells' (Figure 1b).

To evaluate the plasmid integration in these cells (passage 45), genomic DNA was amplified by polymerase chain reaction (PCR) with specific primers (Supplementary Figure 1a; Supplementary Table 1). Although the PCR detected plasmid incorporation into the host genome of some cells, no amplification of plasmid DNA was observed in several cells, such as iTS-P 3F-1 and iTS-P 4F-1 (Supplementary Figures 1b and c). Although we cannot formally exclude the presence of small plasmid fragments, these data show that some of the cells that have self-renewal capacity are most likely free from plasmid integration into the host genome.

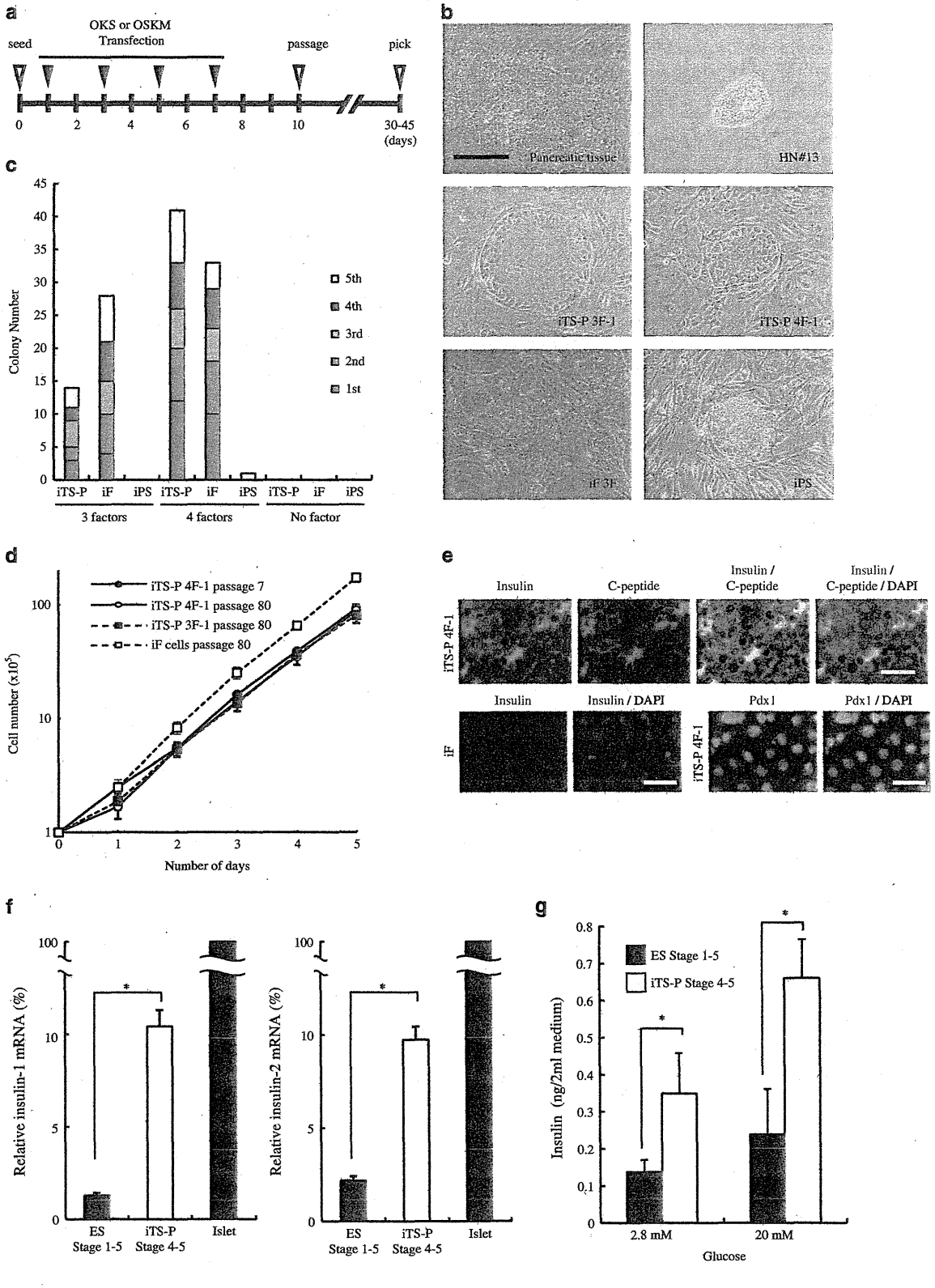
Generative efficacy and characterization of iTS-P cells. To evaluate the efficiency of generating iTS-P cells, the OSKM plasmid or OKS plasmid were transfected into pancreatic tissue from five different mice that were 24 weeks old (one experiment using one mouse). A total of 14 colonies of iTS-P cells were generated using the OKS plasmid during five experiments, and a total of 41 colonies of iTS-P cells were generated using the OSKM plasmid during five experiments, thus suggesting that the OSKM plasmid induces iTS-P cells more efficiently than OKS plasmid. Moreover, the generation of iTS-P cells was easier than that of iPS cells (Figure 1c). Mock transfection (no factors) did not yield any iTS-P or iF cells. There was plasmid integration in two clones of the 14 colonies generated using the OKS plasmid (14.3%) and in five clones of the 41 colonies generated using the OSKM plasmid (12.2%).

To investigate the gene expression in these cells, a reverse transcription PCR (RT-PCR) analysis of ES cell marker genes was performed. The high-level expression of the pluripotency markers Oct3/4, Sox2 and Nanog was limited to ES and iPS cells (Supplementary Figure 2). We next investigated the gene expression patterns of endodermal/pancreatic progenitor (PP) cell markers. Differentiated cells from ES cells (generated by a stepwise differentiation protocol that relies on intermediates thought to be similar to the cell populations present in the developing embryo)[21,22] were used as a positive control (Supplementary Figure 3a). The expression of marker genes of the definitive endoderm (DE; Sox17), gut tube endoderm (GTE; hepatocyte nuclear factor 4α; Hnf4α) and PP (Pdx1) was detected in iTS-P cells, which was similar to the patterns detected in our mouse pancreatic stem cell line, HN#13, but not iF, iPS or ES cells (Supplementary Figures 3b–g).

The iTS-P 3F-1/4F-1 cells continued to divide actively beyond passage 80 without changes in their morphology or growth activity (Figure 1d). To examine the teratoma formation and tumorigenic potential *in vivo*, iTS-P 3F-1/4F-1 cells (10^6–10^7 cells) at passage 45 were transplanted into nude or NOD/scid mice. No teratomas or tumors developed in the nude or NOD/scid mice that received iTS-P 3F-1/4F-1

Figure 1 Generation of iTS-P cells from mouse pancreatic tissue and differentiation of iTS-P cells into IPC. (**a**) Time schedules for the induction of 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 transfections. (**b**) The morphology of mouse pancreatic tissue, HN#13 cells, iTS-P 3F-1, iTS-P 4F-1, iF and iPS cells. Scale bars = 200 μm. (**c**) The colony numbers of iTS-P cells. The OSKM plasmid or OKS plasmid was transfected into pancreatic tissue from five different mice aged 24 weeks, and the number of colonies was counted after 30–45 days. '1st, 2nd,...' refers to the mouse number. (**d**) Growth curves of iTS-P cells (passage 45 and 80) and iF cells (passage 80). Error bars represent S.E. (**e**) Immunostaining of iTS-P 4F-1 cells (Pdx1) and IPC derived from iTS-P 4F-1 cells (insulin, C-peptide). Insulin staining of iF cells treated with the stepwise protocol was also performed. Scale bars = 50 μm. (**f**) The quantitative RT-PCR analysis of insulin genes in differentiated iTS-P 4F-1 cells. Differentiated cells derived from iTS-P 4F-1 cells (passage 45) by stage 4–5 and derived from ES cells by stage 1–5 were analyzed by quantitative RT-PCR. Isolated islets were used as positive control. The data are expressed as the insulin-to-β-actin ratio with that of the islets arbitrarily set at 100 ($n = 4$). Error bars represent S.E. (**g**) The insulin release assay. Differentiated iTS-P 4F-1 cells (passage 45) by the stage 4–5 protocol and derived from ES cells by the stage 1–5 protocol were stimulated with 2.8 and 20 mM D-glucose, and the amount of insulin released into the culture supernatant was analyzed by an ELISA. Error bars represent S.E. $^*P < 0.05$

cells during an observation period of at least 6 months, as is the case with HN#13 cells.[7] In contrast, the sites injected with 1×10^6 ES cells or iPS cells developed teratomas about 3 weeks after transplantation (Supplementary Figures 4a and b; Table 1). These data indicate that the iTS-P 3F/4F cells do not have the ability to form teratomas.



Differentiation of iTS-P cells into IPC. To determine whether iTS-P cells can be differentiated into IPC, we applied the stepwise differentiation protocol shown in Supplementary Figure 3a. The stepwise differentiation protocol relies on intermediates thought to be similar to cell populations present in the developing embryo.[21,22] ES cells differentiate into DE in stage 1; the DE cells differentiate into GTE in stage 2; the GTE cells differentiate into PP in stage 3; and PP cells differentiate into IPC in stages 4 and 5. As iTS-P 4F-1 cells express endodermal cell markers (PP cell markers), we included stages 4 and 5 of the induction protocol in the stepwise differentiation protocol. Differentiated cells from ES cells (stage 1–5) were used as a control. The iTS-P 4F-1 cells were differentiated into IPC (Figure 1e; Supplementary Figure 4c) more efficiently than ES cells or iPS cells derived from pancreatic tissue by the stepwise differentiation protocol (Figure 1f; Supplementary Figure 4d). The insulin-positive cells were C-peptide positive, thus excluding the possibility of insulin uptake from the media. 19.3 ± 2.3% of the differentiated cells were insulin/C-peptide positive. The iF cells were unable to be differentiated into IPC (Figure 1e). An RT-PCR analysis confirmed the expression of endocrine-specific gene products, such as Glut2, glucokinase, NeuroD, Pax4, Nkx2.2, glucagon and somatostatin (Supplementary Figure 5a). To evaluate whether the differentiated cells had glucose sensitivity, the differentiated cells from the iTS-P 4F-1 clone were exposed to low (2.8 mM) or high (20 mM) concentrations of glucose. The cells released about sixfold higher amounts of mouse insulin than an ES-derived population at both glucose concentrations (Figure 1g). The stimulation index was similar between the differentiated cells from iTS-P 4F-1 cells and ES cells (Supplementary Figure 5b).

Table 1 Teratoma formation

Cell type	Injected cell number	Mice bearing teratoma/total mice injected	Period (days)
ES	1×10^6	5/5 (nude mice)	60
ES-derived β-cells	1×10^6	2/5 (nude mice)	90
iPS-P	1×10^6	5/5 (nude mice)	60
iTS-P 4F-1	1×10^6	0/5 (nude mice)	180
iTS-P 4F-1	1×10^7	0/5 (NOD/scid mice)	180
iTS-P 3F-1	1×10^6	0/5 (nude mice)	180
iTS-P 4FP-1	1×10^7	0/5 (NOD/scid mice)	180
iTS-P 4FP-2	1×10^7	0/5 (NOD/scid mice)	180
iTS-P 4FP-4	1×10^7	0/5 (NOD/scid mice)	180
iTS-P 4FP-6	1×10^7	0/5 (NOD/scid mice)	180
iTS-P[a]-derived β-cells	1×10^6	0/5 (NOD/scid mice)	180
ES	1×10^6	5/5 (nude mice)	60
ES-derived hepatocytes	1×10^6	3/5 (nude mice)	90
iPS-L	1×10^6	5/5 (nude mice)	60
iTS-L 4F-1	1×10^7	0/5 (nude mice)	180
iTS-L 4F-2	1×10^7	0/5 (NOD/scid mice)	180
iTS-L[b]-derived hepatocytes	1×10^6	0/5 (NOD/scid mice)	180

Abbreviation: ES, embryonic stem
[a]iTS-P 4FP-1
[b]iTS-L 4F-1

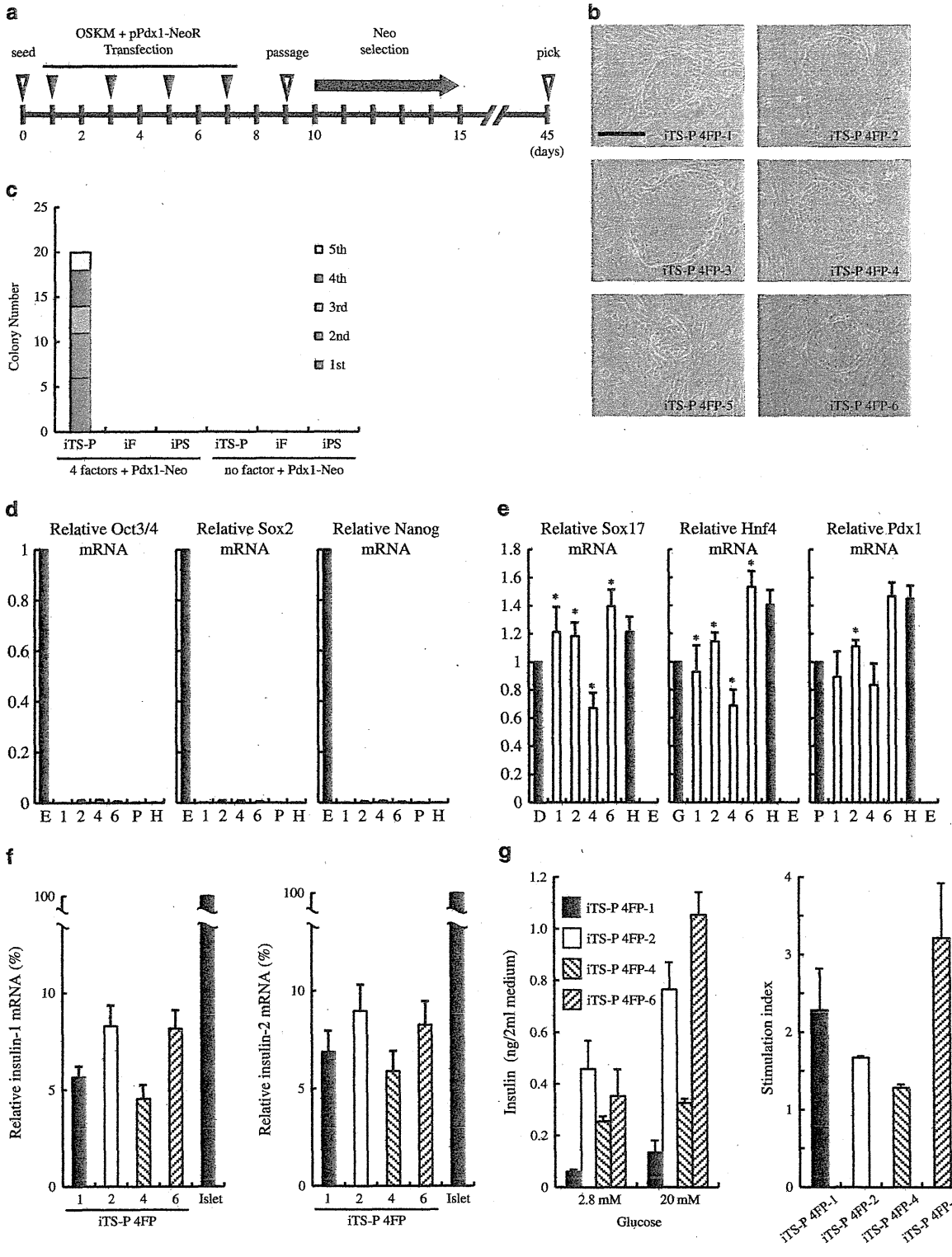
Generation of iTS-P cells by the expression plasmid and Pdx1 selection. We next attempted to achieve the efficient selection of iTS-P cells, as there was a large number of iF cells also included in the above experiments. As iTS-P 4F-1 cells expressed the Pdx1 transcription factor at both the mRNA (Supplementary Figure 3g) and protein level (Figure 1e), we used a plasmid containing a neomycin resistance (NeoR) gene that was driven by the Pdx1 promoter (Supplementary Figure 6a). We transfected the OSKM plasmid and pPdx1-NeoR plasmid together into the pancreatic tissue obtained from a 24-week-old mouse on days 1, 3, 5 and 7 (Figure 2a), and G418 was added to the ES culture media from day 10 to 15 to select Pdx1-expressing cells. We obtained multiple colonies (iTS-P 4FP-1 to -6; Figure 2b) that had self-renewal capacity and were morphologically similar to iTS-P 4F-1 cells. There were no fibroblast-like colonies in this experiment. To evaluate the plasmid integration in these cells (passage 45), genomic DNA from these cells was amplified by PCR with the primers indicated in Supplementary Figure 1a. Although PCR detected plasmid incorporation into the host genome of some cells, no amplification of plasmid DNA was observed in iTS-P 4FP-1, -2, -4 or -6 cells (Supplementary Figure 6b).

Generative efficacy and characterization of iTS-P 4FP cells. To evaluate the efficiency of generating iTS-P 4FP cells, the OSKM plasmid and pPdx1-NeoR plasmid were transfected into pancreatic tissue from five different mice that were 24 weeks old (one experiment using one mouse). A total of 20 colonies of iTS-P cells were generated using the OSKM plasmid and pPdx1-NeoR plasmid during five experiments (Figure 2c). As a total of 41 colonies of iTS-P 4F cells were generated using only the OSKM plasmid during five experiments, the efficiency of iTS-P cell generation

Figure 2 Generation of iTS-P 4FP cells by expression plasmid and Pdx1 selection. (**a**) Time schedules for the induction and selection of iTS-P cells with the plasmid. Open arrowheads indicate the timing of cell seeding, passaging and colony pickup. Solid arrowheads indicate the timing of transfection. Selection by neomycin was performed from day 10 to day 15. (**b**) The morphology of the iTS-P 4FP-1 to -6 cells. Scale bars = 200 μm. (**c**) The colony number of iTS-P 4FP cells. The OSKM plasmid and pPdx1-NeoR plasmid were transfected into pancreatic tissue from five different mice aged 24 weeks, and the number of colonies was counted after 30–45 days. '1st, 2nd,...' refers to the mouse number. (**d**) Quantitative RT-PCR analysis of ES cell marker genes in iTS-P 4FP cells. ES cells, pancreatic tissue and HN#13 cells were used as controls. The data are expressed as the genes-to-β-actin ratio, with that of the differentiated cells arbitrarily set at 1 ($n = 4$). E, ES cells; 1, iTS-P 4FP-1 cells; 2, iTS-P 4FP-2 cells; 4, iTS-P 4FP-4 cells; 6, iTS-P 4FP-6 cells; P, pancreatic cells (90% > islets); H, HN#13 cells. Error bars represent S.E. (**e**) Quantitative RT-PCR analysis of endodermal/pancreatic cell marker genes in iTS-P 4FP cells. ES cells, HN#13 cells and cells differentiated from ES cells (DE, GTE and PP cells) were used as controls. The data are expressed as the genes-to-β-actin ratio, with that of the differentiated cells arbitrarily set at 1 ($n = 4$). 1, iTS-P 4FP-1 cells; 2, iTS-P 4FP-2 cells; 4, iTS-P 4FP-4 cells; 6, iTS-P 4FP-6 cells; H, HN#13 cells; E, ES cells; D, DE cells; G, GTE cells; P, PP cells. Error bars represent S.E. *$P < 0.05$ compared with ES cells. (**f**) The quantitative RT-PCR analysis of insulin genes in differentiated iTS-P 4FP cells. Differentiated cells derived from iTS-P 4FP-1, -2, -4 and -6 cells (passage 45) by the stage 4–5 protocol were analyzed by quantitative RT-PCR. Isolated islets were used as positive control. Data are expressed as the insulin-to-β-actin ratio, with that of the islets arbitrarily set at 100 ($n = 4$). Error bars represent S.E. (**g**) The results of the insulin release assay. Differentiated iTS-P 4FP-1, -2, -4 and -6 cells (passage 45) by the stage 4–5 protocol were stimulated with 2.8 and 20 mM D-glucose, and the amount of insulin released into the culture supernatant was analyzed by ELISA. Error bars represent S.E.

decreased when the pPdx1-NeoR was also used. This may have been due to the relatively lower concentration of OSKM plasmid per cell due to the addition of pPdx1-NeoR. There was plasmid integration in three clones of the 20 colonies generated (15.0%).

To investigate the gene expression profile in these cells, a RT-PCR analysis of ES cell marker genes, endodermal marker genes and pancreatic marker genes was performed (Figures 2d, e and 3). No teratomas or tumors developed in the NOD/scid mice receiving iTS-P 4FP-1, -2, -4 and -6 cells



(1×10^7) at either stage during an observation period of at least 6 months (Table 1). These data indicate that the iTS-P 4FP cells express endodermal markers, and do not form teratomas.

Differentiation of iTS-P 4FP cells into IPC. To determine whether they could differentiate into IPC, we applied the stage 4–5 protocol from the stepwise differentiation protocol (shown in Supplementary Figure 3a) to the iTS-P 4FP cells. All of the iTS-P 4FP clones without plasmid integration were differentiated into IPC by the stage 4–5 protocol (Figure 2f; Supplementary Figure 7). $20.0 \pm 1.4\%$ of the differentiated cells were insulin/C-peptide positive on immunostaining and expressed endocrine-specific gene products (Supplementary Figure 8). All of the differentiated cells from iTS-P 4FP-1, -2, -4 and -6 cells released mouse insulin on exposure to both the low and high concentrations of glucose (Figure 2g), although the amount of insulin produced differed among the cells. The stimulation index was also different among the clones. These data suggest that the pPdx1-NeoR plasmid can efficiently select iTS-P cells, but the differentiation ability of the cells into IPC depends on the individual clone.

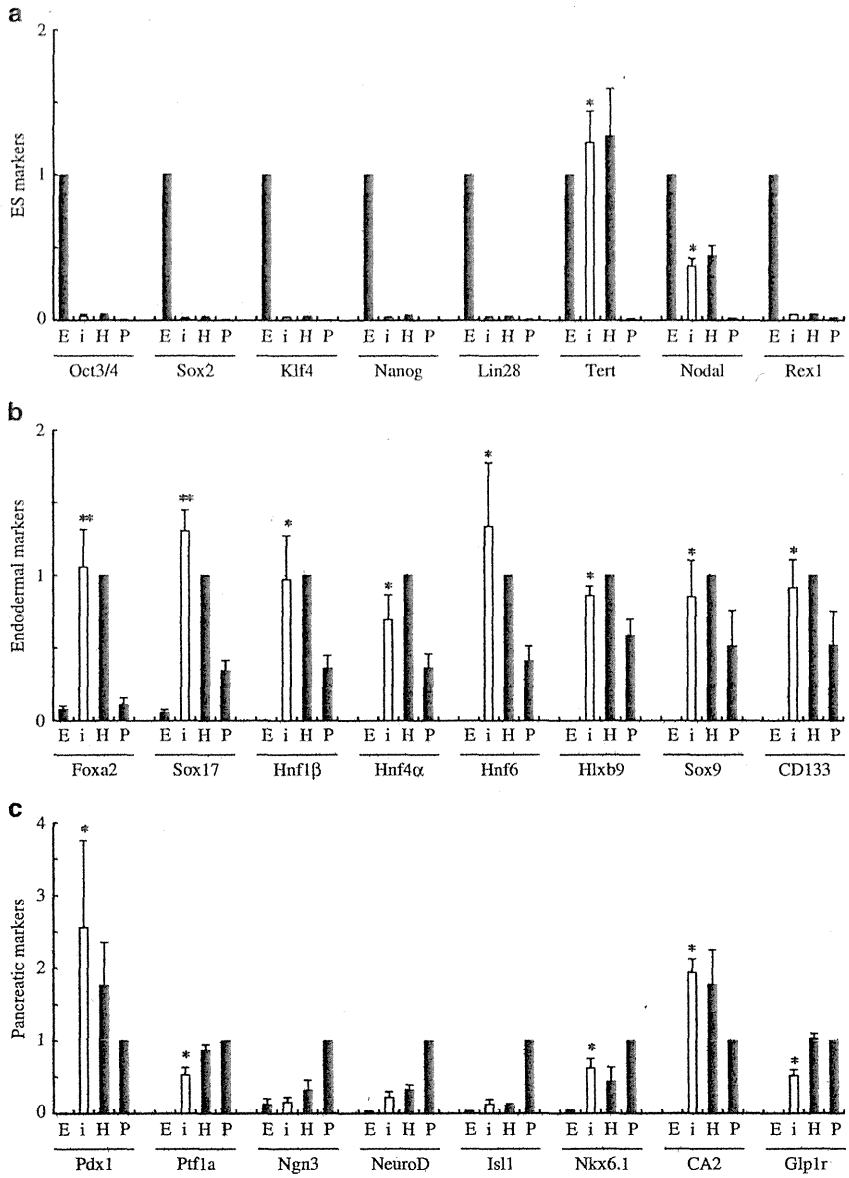


Figure 3 Quantitative RT-PCR analysis of ES and endodermal/pancreatic cell marker genes in iTS-P 4FP cells. (**a**) The quantitative RT-PCR analysis of ES cell marker genes in iTS-P 4FP cells. ES cells were used as control. $^*P<0.05$ compared with pancreatic cells. (**b**) The quantitative RT-PCR analysis of endodermal cell marker genes in iTS-P 4FP cells. HN#13 cells were used as control. $^*P<0.05$ compared with ES cells. $^{**}P<0.05$ compared with ES cells or pancreatic cells. (**c**) The quantitative RT-PCR analysis of pancreatic cell marker genes in iTS-P 4FP cells. Pancreatic cells (90% > islets) were used as control. $^*P<0.05$ compared with ES cells. E, ES cells; i, iTS-P 4FP-1 cells; H, HN#13 cells; P, pancreatic cells (90% > islets). The data are expressed as the genes-to-β-actin ratio, with that of the control cells arbitrarily set at 1 ($n=4$). The error bars represent S.E.

Generation of iTS-L cells from mouse liver cells. We next attempted to generate iTS cells from mouse liver cells by transient overexpression of reprogramming factors, combined with HNF4α selection. The OSKM plasmid was transfected into liver cells (>95% hepatocyte) from 24-week-old mice on days 1, 3, 5 and 7 (Figure 4a). We were able to generate four colonies of iPS cells during five experiments. The iTS-L cells were selected by a quantitative RT-PCR analysis of HNF4α (Figure 4b). A total of 58 colonies of iTS-L cells were generated during five experiments (one experiment using one mouse), thus suggesting that the generation of iTS-L cells is easier than that of iPS cells (Figures 4c and d).

We evaluated the plasmid integration in these cells (passage 45). No amplification of plasmid DNA was observed in several cells, such as iTS-L 4F-1 (Supplementary Figure 9a). There was plasmid integration into seven clones of 58 colonies generated using the OSKM plasmid (12.1%).

Characterization of iTS-L cells. To investigate the gene expression in these cells, an RT-PCR analysis of ES cell marker genes and endodermal marker genes was performed (Figure 4e; Supplementary Figures 9b, c and 10a). The iTS-L 4F-1 cells continued to divide actively beyond passage 80 without changes in their morphology or growth activity (Figure 4f). No teratomas or tumors developed in the nude mice that received iTS-L 4F-1 cells (1×10^7) at passage 45 during an observation period of at least 6 months. In contrast, sites injected with 1×10^6 ES cells developed teratomas about 3 weeks after transplantation (Supplementary Figure 10b; Table 1).

Differentiation of iTS-L cells into hepatocytes. To determine whether iTS-L cells can be differentiated into hepatocytes, we applied the differentiation protocol reported previously.[23] The iTS-L cells were successfully differentiated into albumin-producing cells (Figure 4g). A quantitative RT-PCR analysis confirmed the expression of hepatocyte-specific gene products, such as α1-antitrypsin (α1-AT), glucose 6-phosphatase (G6PC) and albumin. The iTS-L 4F-1 cells were differentiated into hepatocytes more efficiently than ES cells or iPS cells derived from liver cells (Figures 5a and b; Supplementary Figure 10c). Other clones (iTS-L 4F-2 and iTS-L 4F-3) were also differentiated into hepatocyte (Supplementary Figure 10d), although the level of albumin mRNA differed among the cells. These data suggest that iTS-L cells can be differentiated into hepatocytes, but the differentiation ability depends on the individual clone.

Restriction of iTS cell developmental potential. To determine whether the developmental potential of iTS-P/iTS-L cells was restricted to pancreas/liver lineages, respectively, cultures were induced using the conditions established to drive ES cells toward hepatocytes, IPC, neuroectoderm[24] or mesoderm.[23] The upregulation of albumin mRNA was limited to iTS-L cells (Figure 5b) and the upregulation of insulin mRNA was limited to iTS-P cells (Figure 5c). Moreover, the upregulation of the mesoderm markers platelet/endothelial cell adhesion molecule 1 (Pecam1) or Mix1 homeobox-like 1 (Mixl1; Figure 5d), and neuroectoderm markers zinc finger protein of the cerebellum 1 (Zic1) or Sox1 (Figure 5e), was not observed in either the iTS-P or iTS-L cells, thus suggesting that iTS cells are committed to a specific type of tissue development.

Discussion

The iPS technology has significant implications for overcoming most of the ethical issues associated with ES cell derivation from embryos. However, the iPS cells still have some ethical issues, because they have similar or the same potency as ES cells, such as issues posed by human cloning. When we focus on the treatment of patients, differentiated tissue is needed. Although islet/liver transplantation is an efficient strategy for the treatment of diabetes/liver failure,[25] it is circumscribed by the limited and irregular supply of cadaveric donors and the risks of immunosuppressant therapy. In this study, we induced pancreatic/liver stem cells from mouse pancreatic tissue/liver cells by transient overexpression of reprogramming factors and tissue-specific selection. The iTS-P/iTS-L cells were able to differentiate into IPC/hepatocytes more efficiently than ES cells. On the other hand, the iTS-P/iTS-L cells did not differentiate into mesoderm or neuroectoderm. As the iTS-P/iTS-L cells are pancreas/liver-specific stem cells, the use of these cells seems to have fewer ethical concerns than ES cells and even iPS cells, because there are no concerns regarding their potential use to generate germ cells, and no need to destroy an embryo. Moreover, the iTS-P/iTS-L cells do not form teratomas. The ES/iPS cells have a risk for teratoma formation, even after transplantation of differentiated cells derived from ES/iPS cells, due to possible contamination with undifferentiated cells. This is one of the advantages of iTS-P/iTS-L cells compared with ES/iPS cells in terms of their potential clinical use. On the other hand, we performed transplantation of undifferentiated iTS-P cells in normal nude mice and nude mice with diabetes; however, there were few/no IPC in the grafts. We also performed transplantation of undifferentiated iTS-L cells in normal nude mice and nude mice with liver failure (90% hepatectomy); however, there were few/no albumin-producing cells in the grafts. These data suggest that undifferentiated iTS cells themselves cannot be used to treat the disease and that establishing differentiational protocols are important for clinical application.

Our control study using a plasmid with no factors showed no existing adult pancreatic stem cell population in the 24-week-old mice (Figure 1c). Therefore, iTS-P cells were definitively induced by three or four factors. It has recently been shown that, following the reprogramming of mouse/human iPS cells, an epigenetic memory is inherited from the parental cells.[26–30] It was also shown that the epigenetic memory predisposes iPS cells derived from pancreatic β cells to differentiate more readily into IPC.[31] These findings demonstrate that the iPS cell phenotype may be influenced by their cells of origin, and suggest that their skewed differentiation potential may prove useful in the generation of differentiated cell types that are currently hard to produce from ES/iPS cells for the treatment of human diseases. The iTS-P/iTS-L cells have to be cells that inherit numerous components of epigenetic memory from