

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

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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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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; DNAMEC, 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 glyceraldehyde-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	
<i>OCT3/4</i>	Forward: GAAACCCACACTGCAGCAGA Reverse: TCGCTTGCCCTTCTGGCG
<i>SOX2</i>	Forward: GGGAAATGGGAGGGGTGCAAAGAGG Reverse: TTGCGTGAGTGTGGATGGGATTGGTG
<i>NANOG</i>	Forward: CTCAGCTACAAACAGGTGAAGAC Reverse: TCCCTGGTGGTAGGAAGAGTAAA
<i>REX1 (ZFP42)</i>	Forward: CAGATCCTAAACAGCTCGCAGAAT Reverse: GCGTACGCAAATTAAGTCCAGA
<i>TERT</i>	Forward: CGTACAGGTTTCACGCATGTG Reverse: ATGACGCGCAGGAAAAATGT
<i>GAL</i>	Forward: AAACAATATCATGCGCACAATCA Reverse: GGGCACCGGCCTCTTT
<i>LEFTY2</i>	Forward: GCTCAGATGCTGAGCTCTAGTAGGA Reverse: GAAACTCCCAGCTGAAAATGTGT
<i>GAPDH</i>	Forward: CCACTCCTCCACCTTTGACG Reverse: ATGAGGTCCACCACCCTGTT
Endoderm-specific genes	
<i>SOX17</i>	Forward: AGCCAAGGGCGAGTCCCGTA Reverse: GCCTTCCACGACTTGCCCAGC
<i>CXCR4</i>	Forward: CTGCACCTGTCAGTGGCCGA Reverse: TTGGTGGCGTGGACGATGGC
<i>WNT3</i>	Forward: TCCTCGGCGCCTCTTCTAAT Reverse: CTGTGAGCCCAGAGATGTGT
<i>CD117 (KIT)</i>	Forward: ATCAAGCACAATGGCACGG Reverse: AAGGAGTGAACAGGGTGTGG
<i>GATA3</i>	Forward: CTGGCTCGCAGAATTGCA Reverse: TGGGTACGGCAGAATAAAACG
<i>GATA4</i>	Forward: GTTTTTCCCCTTTGATTTTTGATC Reverse: AACGACGGCAACAACGATAAT
<i>GATA6</i>	Forward: GGATTGTCCTGTGCCAACTGT Reverse: GGTTCACCCTCGGCGTTT
<i>HNF1B</i>	Forward: AACCACCGAAGAGGAAGCAA Reverse: TCGCATCAGTTTGTTCGATGA
<i>HNF4A</i>	Forward: CACCTGATGCAGGAACATATGG Reverse: CTGTCCGTTGCTGAGGTGAGT
<i>AFP</i>	Forward: GTTGCCAACTCAGTGAGGACAA Reverse: TGATACATAAGTGTCCGATAATAATGTCA
Pancreas-specific genes	
<i>PDX1</i>	Forward: GATACTGGATTGGCGTTGTTTG Reverse: TCCCAAGGTGGAGTGCTGTAG
<i>HNF6</i>	Forward: TGCGCAACCCCAAACC Reverse: TCCACATCCTCCGGAAGGT
<i>NKX6.1</i>	Forward: GCCTGTACCCCTCATCAAGGA Reverse: AAGTGGGTCTCGTGTGTTTCTC
<i>INS</i>	Forward: ACGAGGCTTCTTCTACACACCC Reverse: TCCACAATGCCACGCTTCTGCA
<i>PAX6</i>	Forward: GCTTACCATGGCAAATAACCT Reverse: GGCAGCATGCAGGAGTATGA
<i>SOX9</i>	Forward: CCCATGTGGAAGGCAGATG Reverse: GAAGGTAACTGCTGGTGTCTGA

(continued)

Table 1. (Continued)

Gene	Sequences (5' to 3')
Genes selected by a microarray analysis	
<i>GREM1</i>	Forward: CCCCCCGCCAGACAAG Reverse: TTGCACCAGTCTCGCTTCAG
<i>EGR1</i>	Forward: TTTACAGTCTTGGTGCCTTTT Reverse: TCCCTCACAATTGCACATGTC
<i>NLRP2</i>	Forward: CTTTGAGGAAACCACCTGTGCAA Reverse: AACTGAACGGAGGGATGGAA
<i>SMC3</i>	Forward: TTGCTCTGATTTTTGCCATTCA Reverse: CATCCAGAGCCTGGTCAATTC

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-phosphate 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, α foeto-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 endoderm-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 endoderm-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
<i>OCT3/4 (POU5F1)</i>	<i>WNT3</i>	<i>INSULIN</i>
<i>SOX2</i>	<i>CD117</i>	<i>PDX1</i>
<i>NANOG</i>	<i>GATA4</i>	<i>PAX6</i>
<i>REX1 (ZFP42)</i>	<i>SOX17</i>	<i>NKX6.1</i>
<i>LEFTY2</i>	<i>HNF4</i>	<i>SOX9</i>
<i>TERT</i>	<i>CXCR4</i>	<i>HNF6</i>
<i>GAL</i>	<i>HNF1B</i>	
	<i>AFP</i>	
	<i>GATA6</i>	
	<i>GATA3</i>	

were no ESC-specific genes, endodermal-specific genes, or pancreas-specific genes out of the 49 genes on GPL3921 or out of the 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).

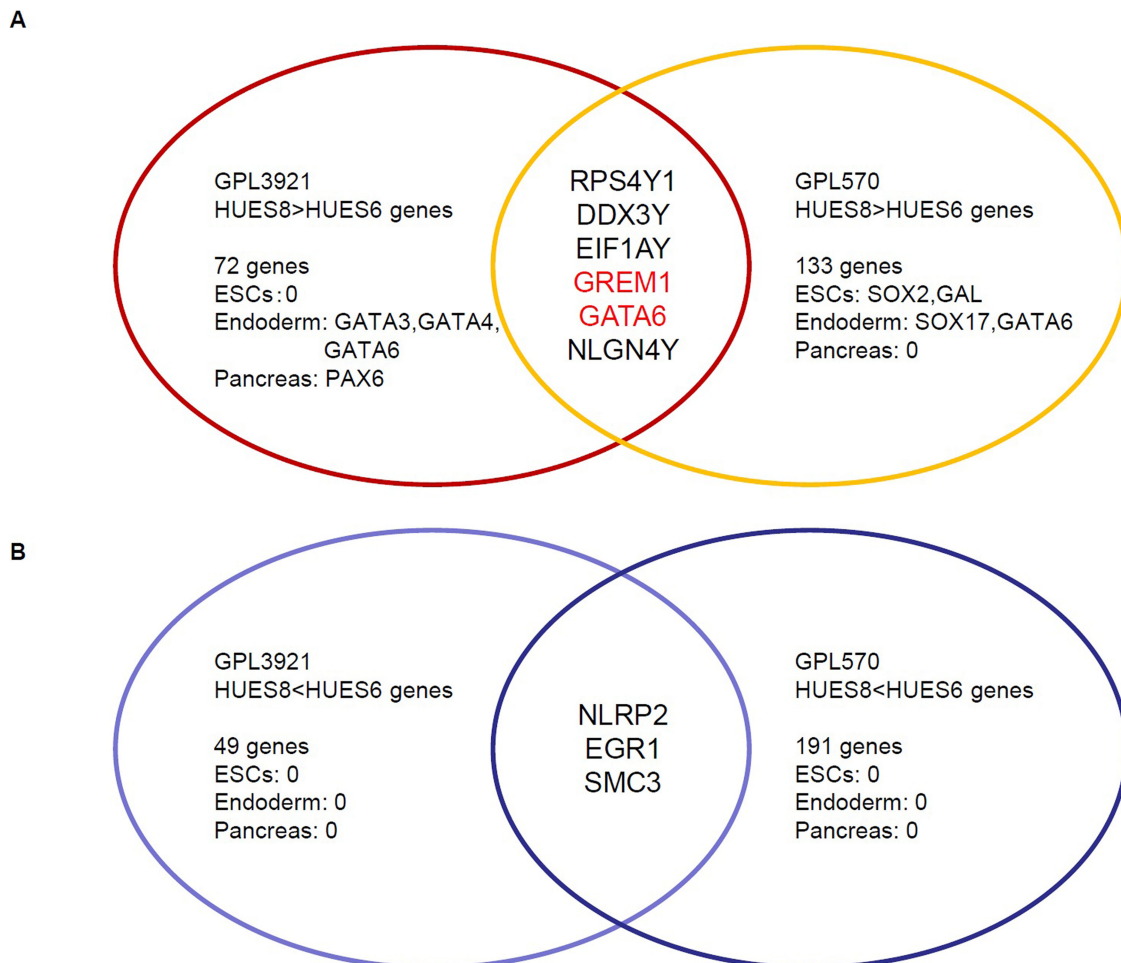


Figure 1. Microarray analysis. (A) Genes that are expressed more than fourfold higher in HUES8 compared to HUES6 in two gene chips (GPL3921 and GPL570). (B) Genes that are expressed more than fourfold higher in HUES6 compared to HUES8. ESCs, embryonic stem cells; GATA3, guanine-adenine-thymine-adenine-binding 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.

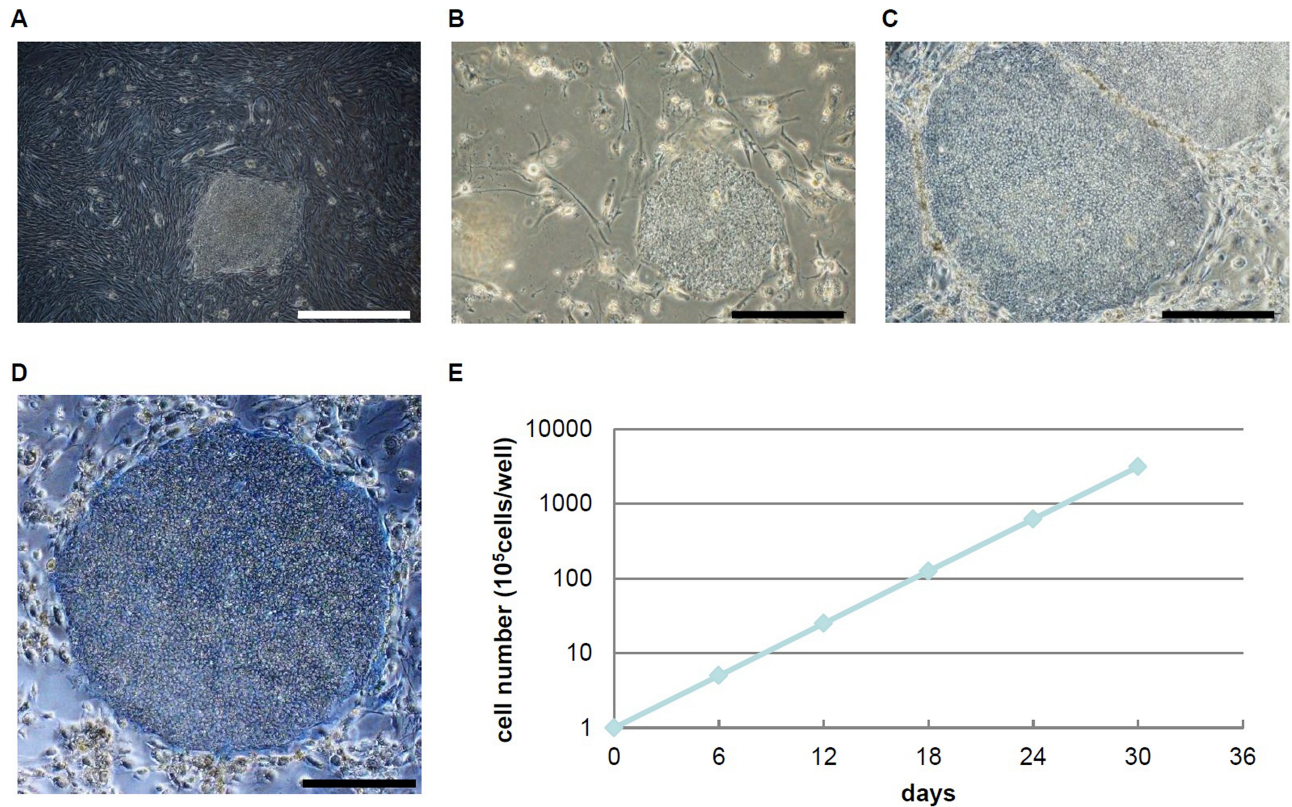


Figure 2. Generation of iPSCs from human pancreatic cells. (A) PiPSCs; passage 0. (B) PiPSCs; passage 4. (C) PiPSCs; passage 20. (D) Alkaline phosphatase staining of PiPSCs (passage 20). Scale bars: 500 μ m. (E) Growth curve of PiPSCs.

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).

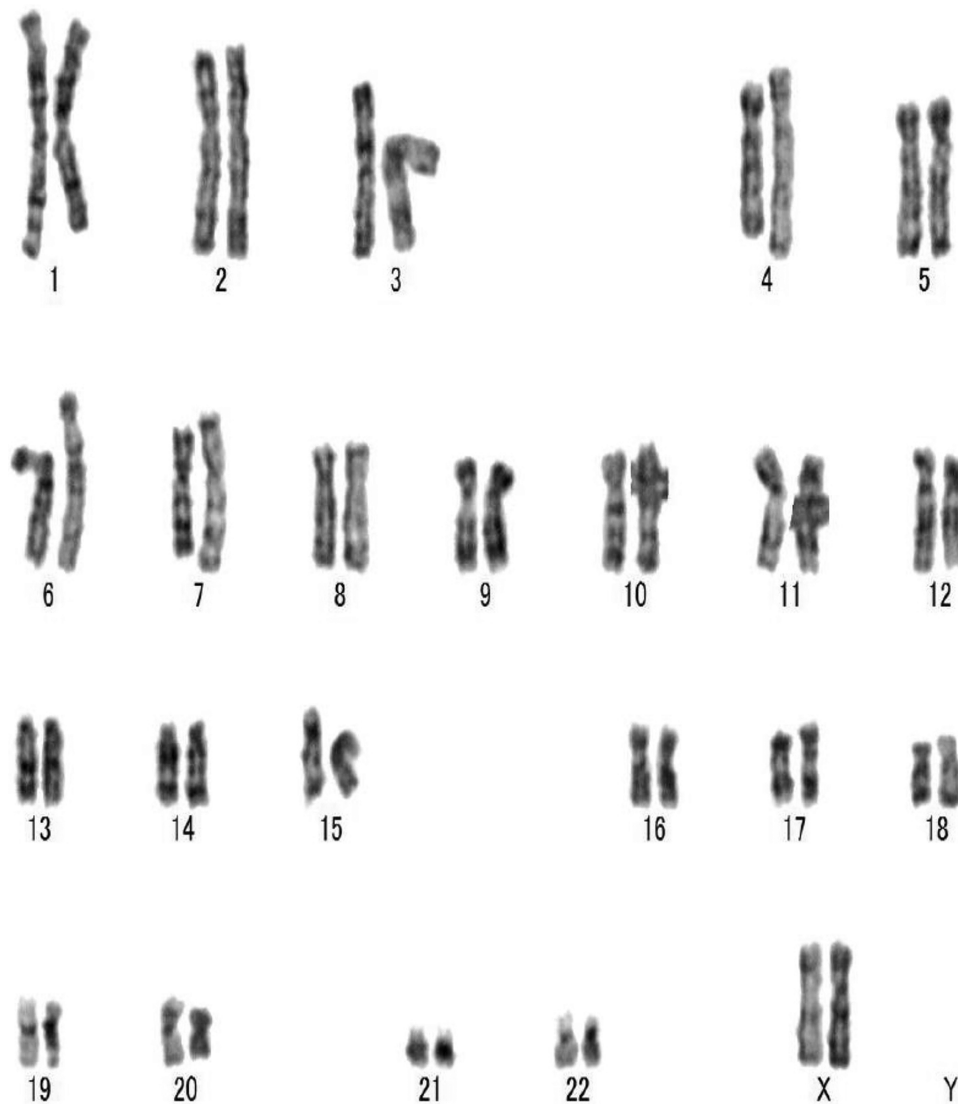


Figure 3. Karyotype of PiPSCs. PiPSCs exhibit a normal diploid karyotype of the 46XX chromosomes.

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 of the 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.

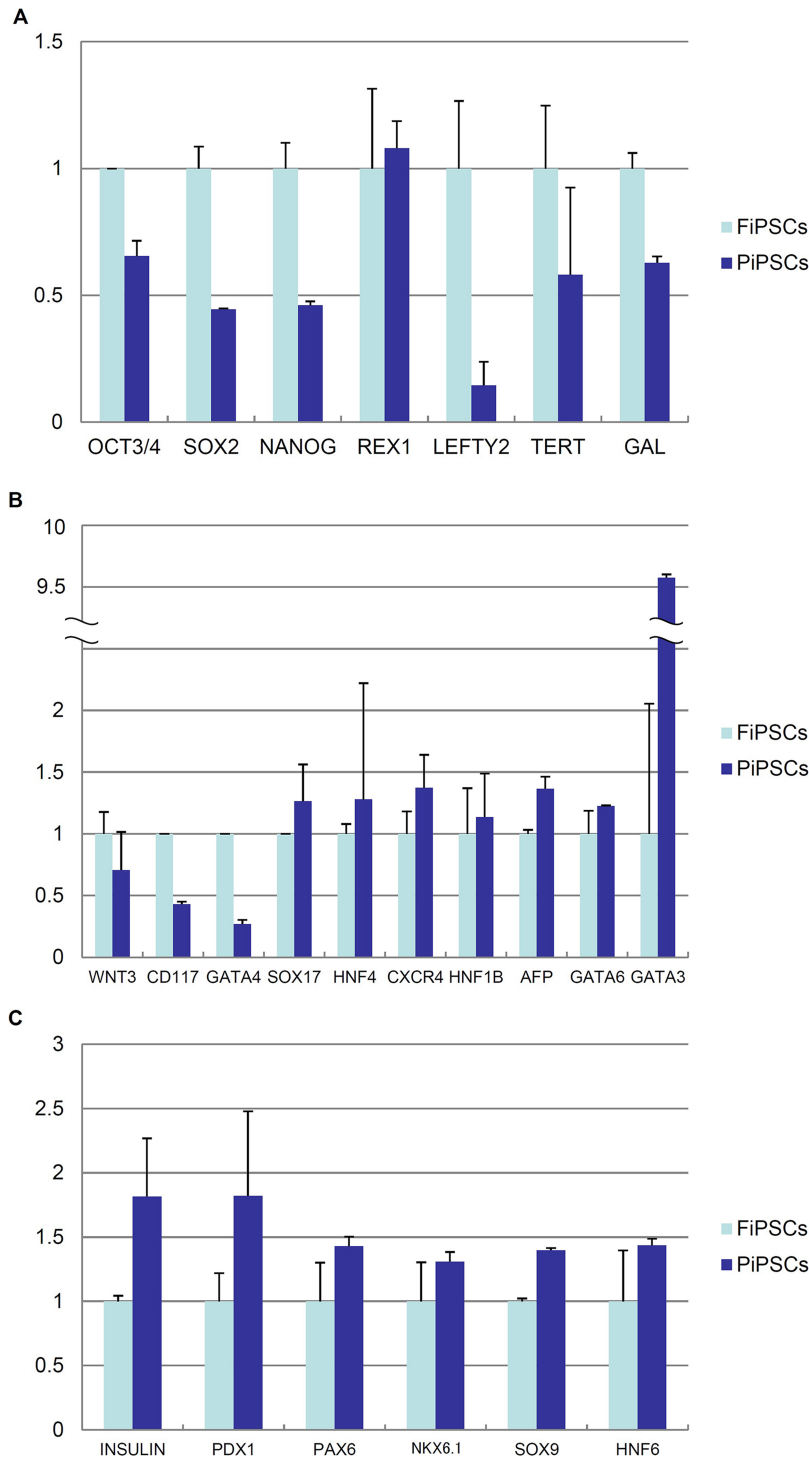


Figure 4. Gene expression in FiPSCs and PiPSCs. (A) A quantitative RT-PCR analysis of ESC-specific genes in FiPSCs and PiPSCs. (B) A quantitative RT-PCR analysis of endodermal-specific genes in FiPSCs and PiPSCs. (C) A quantitative RT-PCR analysis of pancreas-specific genes in FiPSCs and PiPSCs. The 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, α feto-protein; PDX1, pancreatic and duodenal homeobox 1; NKX6.1, NK6 homeobox 1.

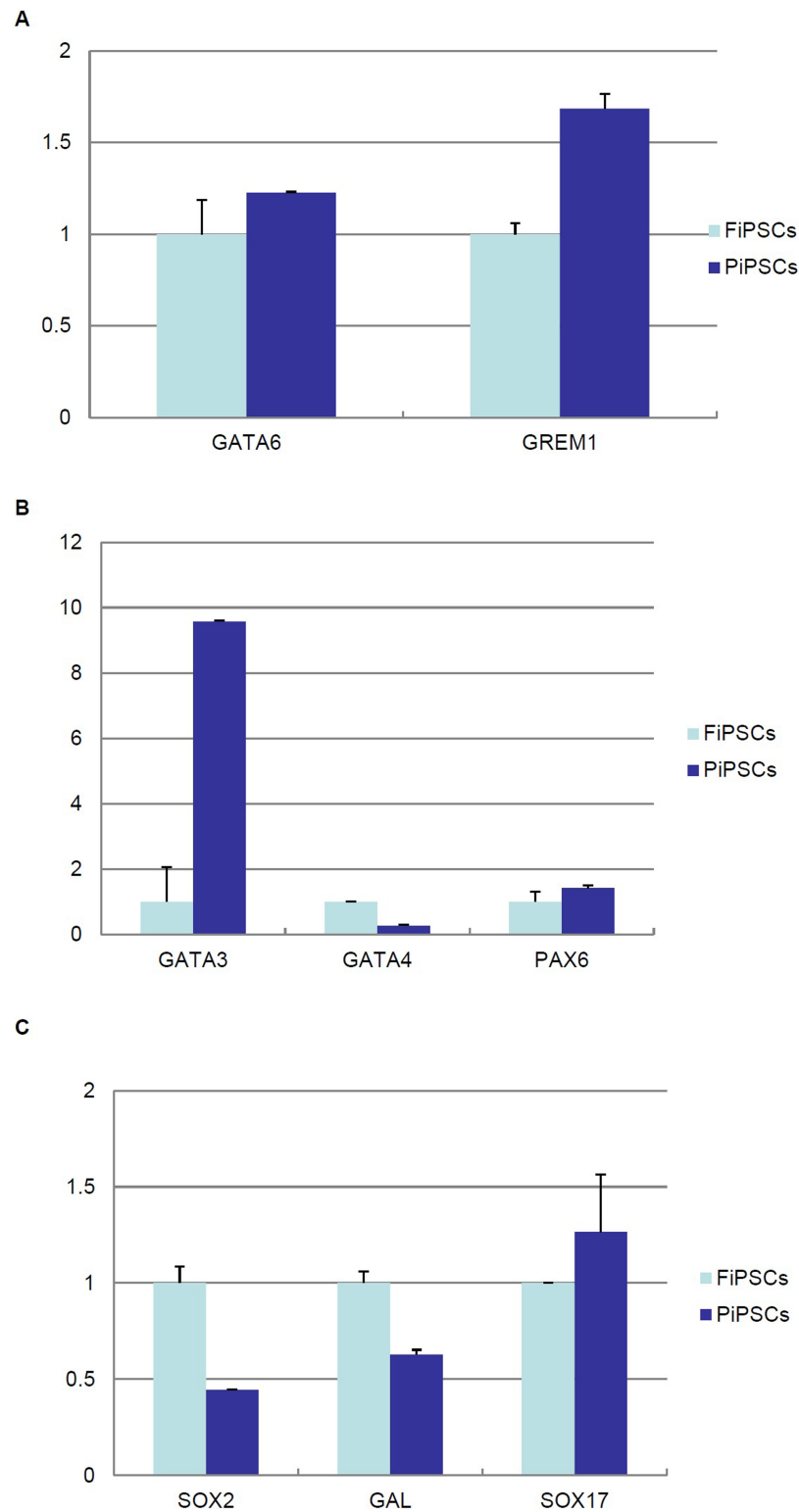


Figure 5. Expression analysis of the genes selected by a microarray analysis (HUES8>HUES6) in PiPSCs and FiPSCs. (A) A quantitative RT-PCR analysis of the GATA6 and GREM1 genes, in which the expression was higher in HUES8 than in HUES6 on both GPL3921 and GPL570, in FiPSCs and PiPSCs. (B) A quantitative 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 ($n=3$).

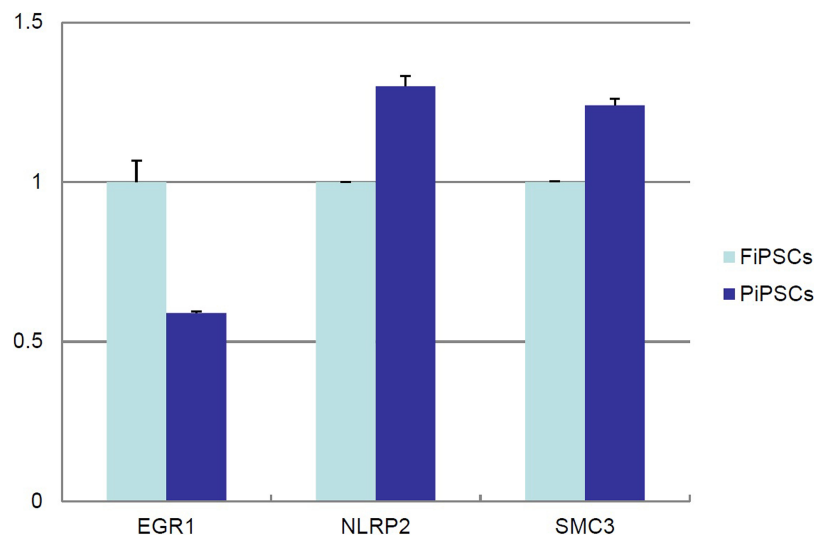


Figure 6. Expression analysis of the genes selected by a microarray analysis (HUES8<HUES6) in PiPSCs and FiPSCs. A quantitative RT-PCR analysis of EGR1, NLRP2, and SMC3 genes, in which the expression was lower in HUES8 than in HUES6 on both GPL3921 and GPL570, in FiPSCs and PiPSCs. The data are expressed 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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