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Role of Zscan4 in secondary murine iPSC derivation mediated by protein extracts of ESC or iPSC

Yoo-Wook Kwon [a, b, 1], Jae-Seung Paek [a, 1], Hyun-Jai Cho [a, b], Choon-Soo Lee [a], Ho-Jae Lee [a], In-Hyun Park [c], Tae-Young Roh [d], Chang-Mo Kang [e], Han-Mo Yang [a, b], Young-Bae Park [b], Hyo-Soo Kim [a, b, f, *]

[a] National Research Laboratory for Stem Cell Niche, Seoul National University College of Medicine, Seoul 110-744, Republic of Korea
[b] Innovative Research Institute for Cell Therapy, Seoul National University Hospital, Seoul 110-744, Republic of Korea
[c] Department of Genetics, Yale School of Medicine, New Haven, CT 06420, USA
[d] Division of Molecular and Life Science, Pohang University of Science and Technology (POSTECH), Pohang, Republic of Korea
[e] Division of Radiation Effect, Korea Institute of Radiological & Medical Sciences, Seoul 139-706, Republic of Korea
[f] Molecular Medicine & Biopharmaceutical Sciences, Seoul National University, Seoul 110-744, Republic of Korea

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ABSTRACT

Previously, we found that the delivery of mouse ES (mES) cell-derived proteins to adult fibroblasts enables the full reprogramming of these cells, converting them to mouse pluripotent stem cells (protein-iPS cells) without transduction of defined factors. During reprogramming, global gene expression and epigenetic status such as DNA methylation and histone modifications convert from somatic to ES-equivalent status. mES cell extract-derived iPS cells are biologically and functionally indistinguishable from mES cells in its potential in differentiation both in vitro and in vivo. Furthermore, these cells show complete developmental potency. However, the efficiency of generating iPS by treatment with extract from mES cells is still low. In this report, we demonstrated that protein extracts of mouse iPS cells that were previously generated by mES cell extract treatment were able to reprogram somatic cells to become ES-like cells (secondary protein-iPS cells). We confirmed that fetal animals (E12.5) could be derived from these cells. Surprisingly, the efficiency of forming Oct4-positive colonies was remarkably improved by treatment of somatic cells with mouse iPS cell extract in comparison to treatment with mES cell extract. By screening the genes differentially expressed between mouse iPS and mES cells, Zscan4, which is known to enhance telomere elongation and stabilize genomic DNA, was identified as a strong candidate to promote efficiency of reprogramming. Interestingly, treatment with protein extracted from mES cells overexpressing Zscan4 enhanced formation of Oct4-positive colonies. Our results provide an efficient and safe strategy for reprogramming somatic cells by using mouse iPS cell extract. Zscan4 might be a key molecule involved in the demonstrated improvement of reprogramming efficiency.
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1. Introduction

Embryonic stem (ES) cells can be maintained in culture with self-renewing division and pluripotency, and have the ability to differentiate into three germ layers. Clinicians and investigators have focused research efforts on the potential applications of human ES cells in treating diseases such as Parkinson's disease, spinal cord injury and organ failure. In addition, human ES cells may be used to investigate the mechanisms of diseases, as well as in experimental applications for drug screening and toxicology. However, there are disadvantages to using human ES cells in research, including ethical controversy and immune rejection following transplantation in patients. To overcome these hurdles, autologous or customized pluripotent stem cells from somatic cells have been intensively studied.

Yamanaka and his colleagues developed induced pluripotent stem cells (iPS cells) by introducing defined factors such as Oct4, Sox2, Klf4 and c-Myc into somatic cells [1]. iPS cells are promising

* Corresponding author. Innovative Research Institute for Cell Therapy, Seoul National University Hospital, 28 Yongon-dong, Chongno-gu, Seoul 110-744, Republic of Korea. Tel.: +82 2 2072 2226; fax: +82 2 766 8904.
E-mail address: hyosoo@snu.ac.kr (H.-S. Kim).
[1] These authors contributed equally to this work.

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to clinicians who seek a feasible system for cell-based therapy and regenerative medicine. However, not only does the delivery system of defined factors usually use retrovirus or lentivirus which can result in tumorigenicity, but one of the four factors, c-Myc itself is also a potent oncogene [2,3]. Much effort has been made to reduce the tumorigenic potential of iPS cells [4–11]. Recently, two groups reported the generation of iPS cells by treating cells with recombinant proteins [10,11]. However, Ding and his colleagues used not only proteins, but also chemicals such as valproic acid (VPA), and Kim's group treated cells more than six times with significant large amount of proteins in order to reprogram somatic cells. Therefore, these inefficient protocols require further optimization.

Previously we achieved full reprogramming of primary culture adult somatic cells with a single transfer of ES cell-derived extract proteins, without additional chemical treatment or repeated protein treatments. We demonstrated that cells reprogrammed via treatment with proteins extract from ES cells had characteristics very similar to conventional ES cells, including global gene expression pattern, in vitro and in vivo differentiation potential, contribution to chimeras and in vivo developmental ability [12]. Here, we investigated whether a single treatment with a protein extract from iPS cells could similarly reprogram adult somatic cells. We found that the protein extract from primary iPS cells was able to more efficiently dedifferentiate adult somatic cells to the pluripotent state, which could then re-differentiate into three germ layers in vitro and in vivo. These iPS cells also contributed to tetraploid complementation, generating embryo.

We found that extract from iPS cells was able to generate secondary iPS cells efficiently, suggesting a feasible model in production of secondary iPS cells by treatment with specific factors in protein complexes from primary reprogrammed cells. By screening factors related to reprogramming efficiency, we identified Zscan4 (zinc finger and SCAN domain-containing protein 4), which reactivates early embryonic genes and promotes genomic stability during reprogramming, as an effective enhancer in iPS cell generation.

These results suggest an alternative method to overcome many disadvantages of previous reprogramming methods that utilize defined factors, such as tumorigenicity, low efficiency and complicacy. Furthermore, this protocol does not require embryos or oocytes and thus entirely avoids major ethical issues.

2. Materials and methods

2.1. Generation of secondary iPS cells using protein extract of primary iPS cells and Zscan4-overexpressing mES cells

To generate iPS cells by treatment of somatic cells with protein extract from previously-produced iPS cells or Zscan4-overexpressing mES cells, we prepared cell extracts from these two cell types and transferred them to somatic cells after reversible permeabilization using streptolysin-O (Sigma–Aldrich) or digitonin according to the detailed protocol described in our previous study [12,13]. Typically, 20–35 mg/ml of proteins were used to induce pluripotent stem cells. The primary colonies were reseeded on an STO feeder layer and sub-cultured every 5 days. Cells from passage 5 to 7 were used for further experiments.

2.2. Mice and cell culture

For the preparation of primary skin fibroblasts, C57BL/6 and FVB mice and Oct4-promoter-driven GFP mice (Jackson Laboratory) were used. For teratoma formation experiments, non-obese diabetic/severe combined immuno-deficient (NOD/SCID) mice (Jackson Laboratory) were used. For all animal experiments, we followed the policies of Institutional Animal Care and Use Committee (IACUC) of the Clinical Research Institute in Seoul National University Hospital, Korea.

Skin fibroblasts were primarily cultured from the dermis of 8-week-old mice (Oct4-promoter-GFP-sFB). To exclude contamination of feeder cell genomic DNA, RNA and proteins, all samples were prepared after plating ES cells or iPS cells on 0.1% gelatin-coated tissue culture dishes in feeder free conditions. Detail protocols were described previously [12].

2.3. Genomic DNA PCR

Genomic DNA was prepared by using the DNeasy Blood & Tissue Kit (Qiagen). Microsatellite markers, which were developed at the Whitehead Institute-Massachusetts Institute of Technology Center for Genome Research, were applied to amplify genomic DNA from C57 and FVB mouse strains [30]. Primer sequences were described in Supplemental Method Table 1.

2.4. Alkaline phosphatase and immuno-cytochemical staining

An Alkaline Phosphatase Detection Kit (BCIP/NBT Substrate System, Dako) was used for alkaline phosphatase (ALP) staining. For immunocytochemistry experiments, colonies of secondary iPS cells were fixed with 4% paraformaldehyde and blocked with 1% BSA and 0.1% Triton X. Staining was performed by incubation overnight at 4°C with anti-SSEA1 and anti-Oct4 antibodies (Santa Cruz Biotechnology). Nuclei were counterstained with 4,6-diamindino-2-phenylindole(DAPI). Images were obtained by confocal microscope (LSM 710 Meta, Zeiss).

2.5. DNA microarray and mRNA expression analysis

We used mouse whole-genome BeadChips (illumina) for analyzing global gene expression. We prepared RNA from cells according to the instructions provided by the manufacturer. For scatter plots of gene expression by mES cells, C57 sFB and C57 secondary iPS cells, three groups were expressed into images through GCOS software. To analyze differentially expressed genes (DEG) among mES cells, C57 sFB, and C57 secondary iPS cells, we performed a one-way ANOVA test and measured with Benjamin & Hochberg FDR (False Discovery Rate) for enhancing significant test in multiple testing. Significance level was determined by less than FDR 5% and p-value 0.01. Total RNA from mES, C57 sFB, primary and C57 secondary iPS cells was isolated using RNeasy Mini Kit columns as described by the manufacturer (Qiagen). Quantitative real-time RT-PCR (qRT-PCR) was done with SYBR Green master mix (Roche, Hague RD, IN) using Applied Biosystems 7500 Fast Real-Time PCR system. Primer sequences were described in Supplemental Method Table 2.

2.6. Epigenetic analysis

To assess the methylation status of Oct4 and Nanog promoters, we performed bisulfite sequencing as described previously [31,32]. For analyzing the histone modification of Oct4 and Nanog promoters, we performed chromatin immunoprecipitation (ChIP) assays using the Chromatin Immunoprecipitation Assay Kit (Upstate). 1×10^6 cells were fixed and sonicated, and cell lysates were incubated in the presence of antibodies including anti-trimethyl histone 3 lysine 4 (H3K4, Abcam), anti-trimethyl histone 3 lysine 27 (H3K27, Upstate), anti-acetyl-Histone H3 (Ach-H3, Upstate) or anti-IgG antibody [1,31,32]. Samples were separated by Protein A Agarose/Salmon Sperm DNA (50% slurry, Upstate). We performed conventional semi-quantitative RT-PCR and real-time PCR with

these samples as described previously [12]. Primer sequences were described in Supplemental Method Tables 3, 4.

2.7. In vitro and in vivo differentiation

To evaluate the in vitro differentiation potential of iPS cells, we cultured cells in suspension conditions to generate embryoid bodies, which were then incubated on and attached to 0.1% gelatin-coated tissue culture dishes. Markers for the three germ layers were observed by immunocytochemical staining. To investigate the in vivo differentiation potential, 1×10^7 secondary iPS cells were subcutaneously injected into the backs of NOD/SCID mice and analyzed histologically.

2.8. Tetraploid complementation

To assess the in vivo developmental potential of the reprogrammed cells, tetraploid blastocyst complementation experiment was performed (Macrogen) [21,33]. Diploid FVB strain of secondary iPS cells was injected into B6D2F1 (BDF-1) tetraploid blastocysts. At E12.5, embryos were harvested and genotype was determined by D6Mit012.

2.9. Western blot analysis

Total protein was isolated from mES, His tag Zscan4 overexpressing mES, primary iPS (FVB sFB) with RIPA lysis buffer (Santa Cruz Biotechnology), supplemented with complete protease inhibitor cocktail (PIC,Santa Cruz Biotechnology) and 1 mM DTT. Lysates were incubated on ice for 30 min and mixed every 10 min. Protein concentration was determined by BSA assay. Proteins were separated on 8% gradient SDS–PAGE gels using transfer buffer (1 M Tris, 192 mM glycine, 40% acrylamide, 10% APS, and 10% SDS). The membranes were blocked with Tween-Tris-buffered saline (TBST: 25 mM Tris, 137 mM NaCl, 2.7 mM KCl, 0.2% Tween), 5% dry milk (SantaCruz) and 5% FBS. The same buffer was used for primary and secondary antibody incubations. Antibodies used for western blotting were Zscan4 (1:1000, millipore), His-tag (1:1000, abcam), α-tubulin (1:2500, SantaCruz). Goat anti-rabbit IgG-HRP (1:2000, SantaCruz) was used as second antibody. Membrane detection is confirmed by Bio imaging analyzer.

3. Results

3.1. Generation of pluripotent stem cells from somatic cells by treatment with protein extracts of iPS cell

To generate reprogrammed cells by treatment with iPS cell extracts, we prepared proteins from the iPS cells that were derived from somatic cells after treatment with ES cell extract (FVB background, "primary" iPS cells). We diluted iPS cell-derived extract proteins with culture media and introduced this mixture into fibroblasts (sFBs) obtained from an FVB background or C57 background mouse by a reversible permeabilization method using streptolysin-O or digitonin [12,13]. The brief timeline for reprogramming by treatment with iPS cell extracts is presented in Fig. 1a. To determine the efficacy of transfer of extracts into the cells, we used Texas Red-conjugated dextran and observed fluorescence inside the cells (Fig. S1). Previously, we reported that pluripotent cell extract-mediated reprogramming is not dependent on genetic material, but dependent on protein. When we treated the iPS cell extract with DNase or RNase prior to introduction, we could still obtain GFP-positive ES cell-like colonies from Oct4-GFP sFBs. However, when we heated the extract prior to transfer, the heat-inactivated extract could not induce the formation of any colony, suggesting that the protein is the active component for induction of reprogramming [12]. Based on this information, we treated 1×10^6 sFBs with about 25 mg/ml of iPS cell extract at day 0. Small colonies started to appear from day 4 to 7. On day 7, we trypsinized whole cells in the culture dish, including colonies, and reseeded the cells onto feeder STO cells. We observed numerous ES-like colonies between Day 19 and 25 (Fig. 1b). We summarized all iPSC clones what we generated with ES or iPS extracts (Table S1). We expanded the ES-like cells by subculturing every 5 days as previously described [12,14]. To emphasize that these cells were induced by treatment with extracts from primary iPS cells, we named these ES-like cells as "secondary" iPS cells. In this report, the secondary iPS cells that correspond to experimental data are generated from C57 skin fibroblasts (sFBs). Additional reproducible model of secondary iPS cells using a different origin was generated using skin fibroblasts with FVB background, and its experimental data are shown in the Supplemental Figures. Next, we confirmed the origin of these cells by performing genomic DNA PCR with unique microsatellite markers (Fig. 1c). The secondary iPS cells that were generated from C57 strain sFBs by treatment with primary iPS cell extract from FVB strain, showed the same genetic background as C57 fibroblasts, demonstrating that there was no cell contamination by primary iPS cells. It was confirmed under the reverse experimental design: generating FVB secondary iPS cells after treatment of FVB fibroblasts with iPS cell extract from C57 strain. To completely rule out the possibility of the contamination of ES or primary iPSC, we prepared the recipient somatic cells from Oct4 promoter GFP or actin-promoter GFP transgenic mouse. We succeeded in generating secondary and also primary iPSC from Oct4 promoter GFP MEF (clone #3 & 7 in Table S1, Figs. S9, S13) or Oct4 promoter GFP skin fibroblasts (clone #8 in Table S1, Fig. S14) but failed in generating complete iPSC from cFB of actin-promoter GFP mouse (clone #4 in Table S1, Fig. S10).

3.2. In vitro characterization of secondary iPS cells

To investigate whether the secondary iPS cells have characteristics of ES cells, we analyzed alkaline phosphatase activity in ES cells, primary iPS cells, and secondary iPS cells. All three pluripotent cells were positive for alkaline phosphatase, whereas somatic cells were negative (Fig. 2a, Fig. S2a). We performed immunocytochemistry to examine whether ES cell-specific markers were expressed in the secondary iPS cells. Oct4 was highly expressed in the nucleus, and SSEA1 was stained in the surface of secondary iPS cells (Fig. 2b, Fig. S2b). Typical markers of pluripotency including Nanog, Oct4, Sox2 and ERas were highly expressed in secondary iPS cells. The expression pattern of c-Myc and Klf4 was also similar in both ES cells and iPS cells (Fig. 2c). Using DNA microarray analysis, we compared the global gene expression profiles of primary iPS cells, secondary iPS cells, C57 mouse ES (mES) cells, sFBs and E14 mES cells whose extract could not generate iPS cells in contrast to C57 mES cells that could [12]. On a scatter plot, secondary iPS cells showed a very different pattern of global gene expression than the original somatic cells, but a very similar pattern to mES cells and primary iPS cells (Fig. 2d, Fig. S2c). Dnmt3b, which is thought to function in de novo methylation, rather than maintenance methylation, was upregulated in all four pluripotent stem cells in contrast to somatic cells [12]. Other epigenetic modulators, such as histone methyl-transferase 1 (HMT1) and histone deacetylase 6 (HDAC6), were also increased in mES cells and iPS cells. Moreover, Sirt1 that is sirtuin (silent mating type information regulation 2 homology) and can deacetylate proteins including p53, showed higher expression in secondary iPS cells and E14 mES cells than in somatic cells [15,16].

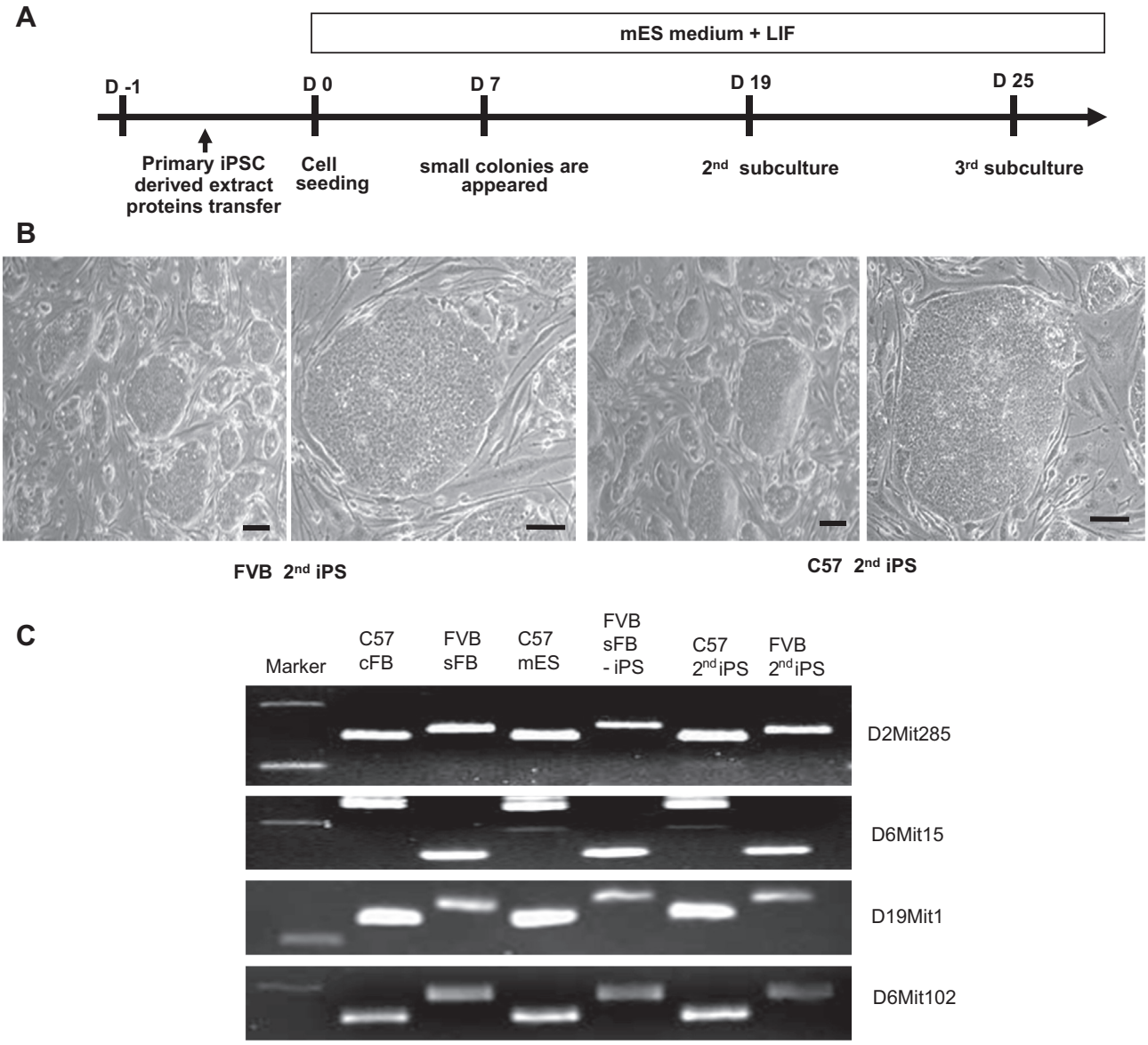


Fig. 1. Generation of dedifferentiated cells with iPS cell extracts. (A) Timeline for the induction of dedifferentiation of somatic cells by treatment with protein cell extracts from primary iPS cells of FVB background. About 20 colonies are obtained on day 7. (B) Phase contrast morphology and fluorescent imaging of secondary iPS cells generated from FVB-background skin fibroblast (FVB secondary iPS) and from C57-background sFBs (C57 secondary iPS). (C) Genomic DNA PCR using unique microsatellite markers to confirm the origin of the secondary iPS cells.

During dedifferentiation or maintenance of stemness, the epigenetic modifications on promoters of stemness genes are very important [17]. Bisulfite genomic sequencing demonstrated that the CpGs in the promoters of Oct4 and Nanog were mostly unmethylated in both mES cells and secondary iPS cells, but mostly methylated in sFBs (Fig. 2f, Fig. S2d). In order to evaluate histone modifications after reprogramming, we performed chromatin immunoprecipitation (ChIP) analysis with antibodies detecting trimethylation of histone H3 at lysine 4 (H3K4), histone H3 at lysine 27 (H3K27) or acetylation of histone 3 [1,18]. ChIP assay showed that the promoters of Oct4 and Nanog had increased trimethylation of H3K4 but decreased trimethylation of H3K27 in mES cells and secondary iPS cells. Furthermore, acetylation of these promoters in mES cells and secondary iPS cells were increased. However, the histone modification pattern of somatic cells was completely opposite to that of mES cells and secondary iPS cells (Fig. 2g, Fig. S2e). These results demonstrate that epigenetic modifications, including DNA methylation and histone modifications, in secondary iPS cells were very similar to mES cells but completely different from somatic cells, indicating that secondary iPS cells are completely reprogrammed in regards to epigenetic modifications.

3.3. Differentiation potential of secondary iPS cells

We determined the differentiation capacity of the secondary iPS cells by the embryoid body (EB)-based spontaneous differentiation method. Typical EB-like morphology was observed in secondary iPS cells at 5 days after suspension culture (Fig. 3a). EBs were then cultured on a 0.1% gelatin-coated dish. After 7–10 days, these cells expressed markers of three germ layers, including ectodermal (glial fibrillary acidic protein), mesodermal (α-smooth muscle actin) and endodermal (α-fetoprotein) proteins (Fig. 3b). Next, we injected the secondary iPS cells into NOD/SCID mice to investigate the in vivo pluripotent potential and differentiation capability. Four weeks

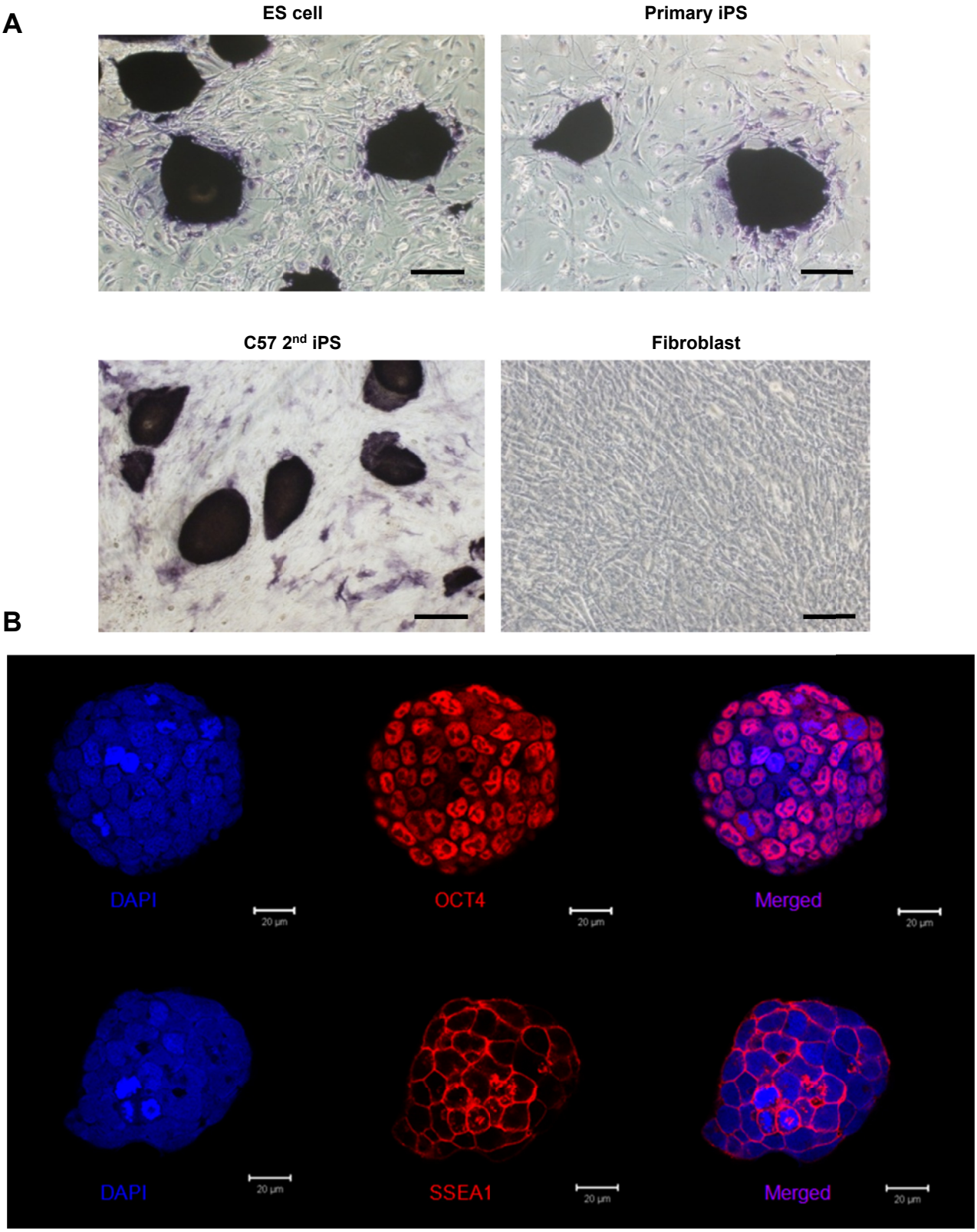
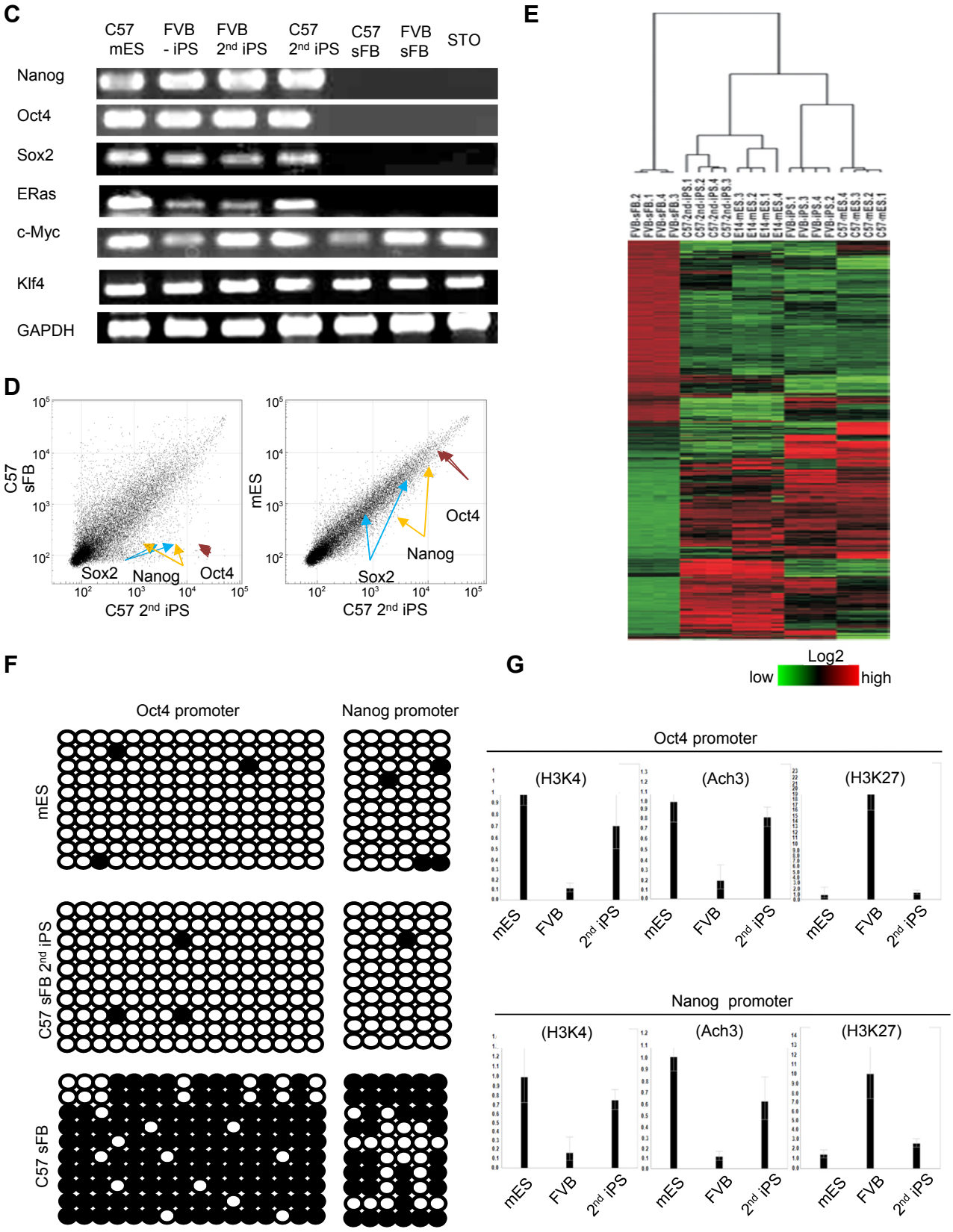


Fig. 2. In vitro characterization of secondary iPS cells compared to conventional ES cells. (A) Alkaline phosphatase staining of mES cell, primary iPS cells, C57 secondary iPS cells and adult skin fibroblasts. (B) Immunocytochemistry showed the expression of Oct4 and SSEA1 in C57 secondary iPS cells. (C) RT-PCR analysis of pluripotency genes in mES cells, primary iPS cells, secondary iPS cells, somatic cells and feeder cells (STO). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. (D) Scatter plots present the profound difference of global gene expression between secondary iPS cells and their original somatic cells, and mild difference between global gene expression by mES cells and secondary iPS cells. The red lines indicate a two-fold difference in log scale. (E) Hierarchical clustering was analyzed on 2082 differentially expressed genes. Red indicates upregulated genes whereas green indicates decreased gene expression. (F) Bisulfite sequencing of the Oct4 and Nanog promoters showed the change of DNA methylation status after reprogramming. Open circles indicate unmethylated CpG nucleotides and closed circles indicate methylated CpGs. (G) Chromatin Immunoprecipitation of trimethylated histone H3 lysine 4, trimethylated histone H3 lysine 27 and acetylated histone H3 showed a dramatic shift of histone modification in Oct4 and Nanog in secondary iPS cells from somatic cells to ES-like cells. Real-time PCR ($n = 3$, each) data were adjusted by GAPDH (ES cell = 1.0 as an arbitrary unit for H3K4, Ach3 and H3K27). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



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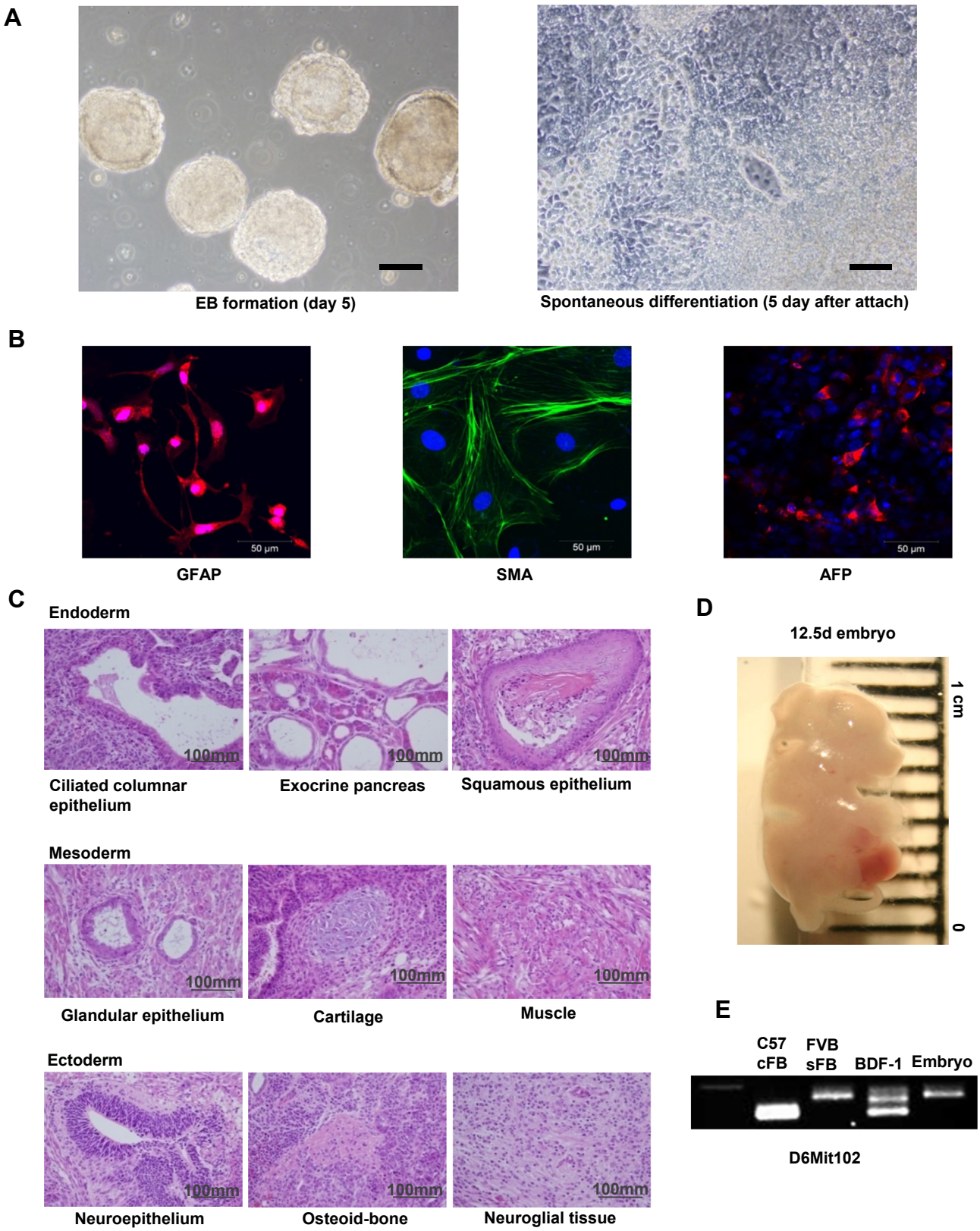

Fig. 3. Differentiation and developmental potential of secondary iPS cells. (A) In vitro embryoid body (EB) formation by suspension culture (left) and spontaneous induction of differentiation by attachment of EB onto gelatin-coated dishes (right). (B) Immunocytochemistry confirming in vitro differentiation into ectoderm, mesoderm and endoderm. GFAP, glial fibrillary acidic protein. SMA, α-smooth muscle actin. AFP, α-fetoprotein. DAPI (blue) for nuclei staining. (C) Well differentiated teratomas were observed in SCID mice injected with secondary iPS cells. Histology of teratomas by H&E staining. (D) The embryo (12.5 day) derived by tetraploid blastocyst complementation demonstrates the developmental potential of secondary iPS cells. (E) Genomic DNA PCR showed that the origin of the fetus is FVB background secondary iPS cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

later, we obtained teratomas containing tissues of all three germ layers, including exocrine pancreas (endoderm), muscle and cartilage (mesoderm), and neuroepithelium (ectoderm) from two different secondary iPS cells (Fig. 3c, Fig. S3). These results confirmed that the secondary iPS cells are comparable to mES cell in terms of in vitro and in vivo pluripotency.

To assess the developmental potential of secondary iPS cells, we performed a tetraploid blastocyst complementation experiment. This method is considered as the most stringent way to examine pluripotency and developmental capacity of stem cells (Eggan et al., 2001). It has been reported that iPS cells were able to produce viable mice through tetraploid complementation [19–21]. We introduced diploid FVB strain-secondary iPS cells into B6D2F1 (BDF-1) tetraploid blastocysts, found, and harvested embryos at E12.5 (Fig. 3d). We genotyped the embryos and confirmed that the embryos were positive for FVB marker and derived from the FVB secondary iPS cells (Fig. 3e).

3.4. Improvement of efficiency in generating iPS cells by iPS extract

In this study, we generated and fully characterized two different secondary iPS cell lines by treatment of somatic cells with primary iPS cell extracts. Interestingly, treatment with the extract of primary iPS cells generated more colonies than that of the mES cells did at the same time point (day 25 after introduction of proteins into the somatic cells, Fig. 4a–e). To investigate the key proteins involved in this improvement of efficiency, we compared the proteomics of C57-mES cells to that of primary iPS cells. However, as we recently reported, these two cell types show very similar patterns of protein expression [22]. Due to the sensitivity limit of Q-TOF LC–MS/MS, iTRAQ™ may not be able to identify all the proteomic differences between mES and iPS cell lines. Therefore, we analyzed our microarray data thoroughly with a focus on the genes related to reprogramming efficiency. It has recently been reported that the stabilization of genomic DNA and the rapid enhancement of telomere elongation improved the generation of iPS cells [23] and that telomeres were elongated by telomerase during reprogramming [24]. Accordingly, we selected telomere regulators that are involved in long-term genomic stability and telomere elongation and compared their expressions between mES and primary iPS cells (Fig. 5a). To our surprise, microarray data showed a higher expression of Zscan4 in primary iPS cells than in mES cells, whereas no differences in the expression level of telomere reverse transcriptase (TERT) and others. TERT expression or telomerase activity was actually lower in primary iPS cells than in mES cells in the real-time PCR of TERT or TRAP assay (Fig. S4a,b). Previous studies have reported that Zscan4 (zinc finger and SCAN domain containing 4) regulates telomere elongation and genomic stability in ES cells and that Zscan4-mediated telomere extension does not require telomerase [25]. Accordingly, we hypothesized that Zscan4 may contribute to the process of reprogramming by the primary iPS cell extract. In order to test this hypothesis, we confirmed higher expression of Zscan4 in the primary iPS cells using real time PCR for Zscan4, and found that expression of Zscan4 was again much higher in primary iPS cells than in mES cells (Fig. 5b). Additionally, telomere length correlates well with Zscan4 level in these two cell lines (Fig. 5c,d). We hypothesized that Zscan4 might be able to change the protein profile of mES cells, so that it would be more similar to that of the primary iPS cells, thus increasing the efficiency of generation of iPS cells by ES cell extract. To investigate this hypothesis, we induced the expression of Zscan4 in mES cells and whole extracts were delivered into Oct4-promoter GFP fibroblasts. Before we prepared extracts from Zscan4 overexpressing mES cells, we first confirmed that mRNA and protein of Zscan4 was highly expressed (Fig. 6a, Fig. S5a) and telomeres were elongated in these cells using qRT-PCR and western blot analysis (Fig. 6b). Next, to confirm whether the Zscan4 protein was delivered into somatic cells, we performed western blot analysis with Zscan4 or His antibody after treatment of extract from Zscan4 overexpressing mES cells. Because Zscan4 expression vector contains His-Tag, we could reconfirm the delivery of Zscan4 protein by His antibody. As shown Fig. 6c and Fig. S5b, much more amount of Zscan4 protein was detected in somatic cells which were treated with extract from Zscan4 overexpressing mES cells. Reprogramming efficiency was increased by treatment with extract from Zscan4 overexpressing mES cells, as evaluated by fluorescence-activated cell sorting (FACS) analysis of GFP-positive cells. Oct4-GFP sFBs treated with extract from Zscan4 overexpressing mES cells represented 2.3 times more GFP positive cells than those treated with extract from authentic mES cells (Fig. 6d). In contrast, extract derived from Zscan4 knockdown mESC could not generate iPSC (Fig. S6). These results support that Zscan4 is key molecule in increasing efficiency of iPS generation.

Next, we investigated the mechanism how Zscan4 could enhance the efficiency of iPSC generation. It has been reported that the combination of Yamanaka 4 factors and Zscan4 attenuated DNA damage response and markedly promoted the efficiency of iPSC generation through stabilizing the genomes, resulting in p53 downregulation [23]. So, we compared p53 level in fibroblasts after treatment of extract from authentic mES and Zscan4-overexpressing mES cells. As we expected, MEF treated with extract from Zscan4-overexpressing mES showed lower p53 expression as well as expression of bax and p21, p53 target genes, than MEF treated with extract from mES did (Fig. 7). Interestingly, MEF treated with iPSC extract showed very similar expression pattern of p53, bax, and p21 to MEF treated with Zscan4-overexpressing mES cell extract (Fig. S7). Thus, the higher expression of Zscan4 in iPSC extract might play an important role to enhance the reprogramming efficiency.

4. Discussion

Previously, we reported that the introduction of ES cell-derived proteins into adult fibroblasts generated pluripotent stem cells (primary iPS cells) with characteristics very similar to conventional ES cells in terms of epigenetic modifications and global gene expression. These primary iPS cells are fully-dedifferentiated cells with the ability to re-differentiate into three germ layers both in vitro and in vivo. Especially, these protein-derived iPSCs are well differentiated into dopaminergic neuron and transplantation of neural precursor cells derived from these iPSCs showed improved function in Parkinson's disease rat model [26].

In this study, we demonstrated that extracts from primary iPS cells that were generated from somatic cells by delivery of ES cell extracts were also effective in the induction of somatic cells to pluripotent stem cells (secondary iPS cells). Furthermore, the efficiency to generate iPS cells was better with extract of primary iPS cells than with that of mES cells. The underlying mechanism for this difference in efficiency was the difference in expression of Zscan4 between primary iPS and mES cells.

In our previous study, we analyzed extracts of two mES cell lines with different genetic backgrounds (E14 or C57 strain). We found that the induction of pluripotent stem cells was possible only with extracts from C57 strain mES cells, but not with extracts from E14 mES cells. We investigated the proteomic differences between these two different strains of ES cells using iTRAQ™ and Mass/Mass experiments. We found that there are many proteomic differences between these two ES cell lines. Interestingly, ribosomal protein levels were higher in C57 strain mES cells than in E14 mES cell lines. These results indicate that a large capacity of protein synthetic

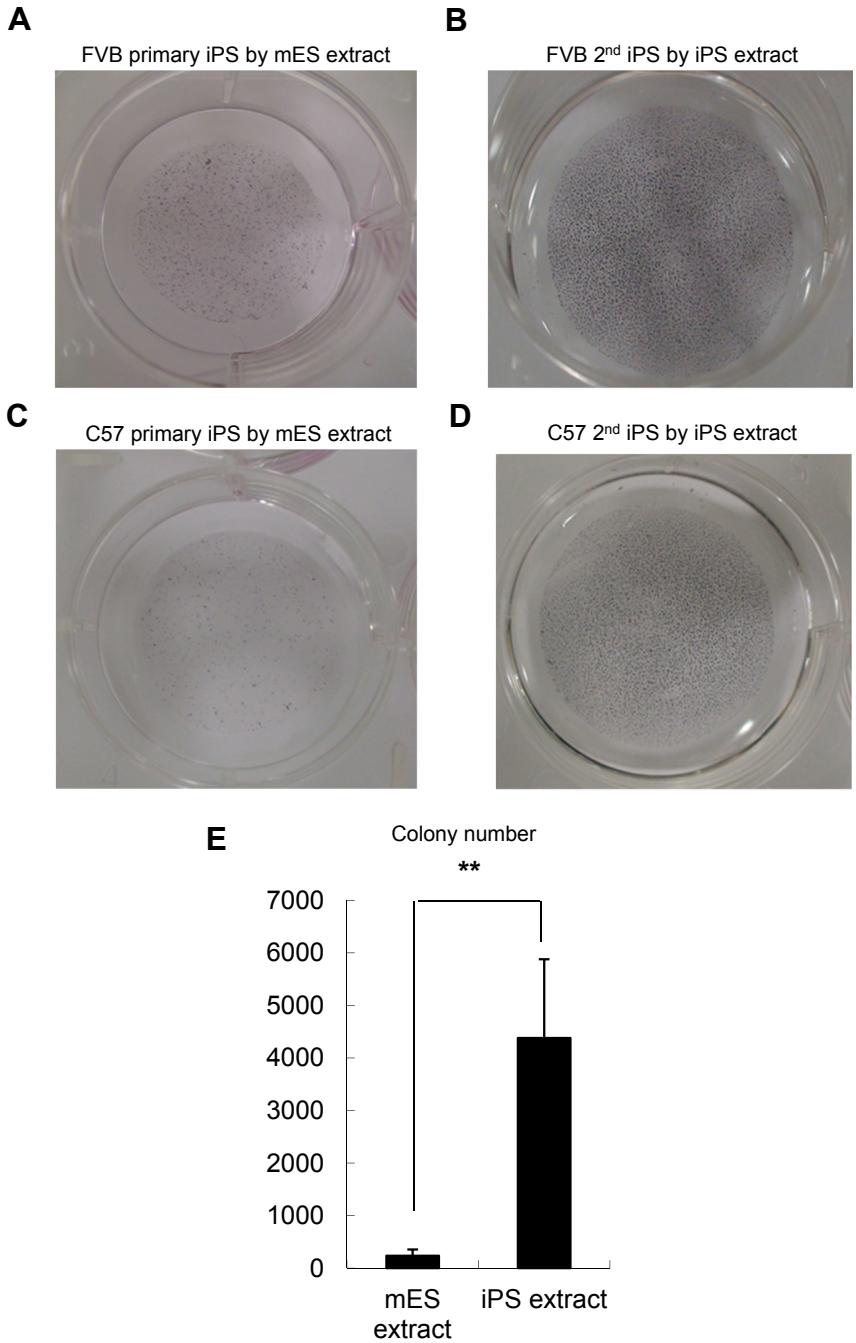

Fig. 4. Comparison of reprogramming efficiency between mES and iPS extracts. (A–D) Alkaline phosphatase staining of primary iPS cells induced by mES extract (A, C) and secondary iPS cells induced by iPS extract (B, D). (E) A higher number of colonies were induced by iPS extract than by mES extract. Asterisk (**) indicates a significant difference at P value < 0.01 (t-test).

machinery may be required for the initiation of reprogramming [22].

In this study, we found that primary iPS cells are closer to C57 mES cells, whereas secondary iPS cells are closer to E14 mES cells in hierarchical clustering of gene expression presented in the microarray. Additionally, one of the epigenetic modulators Sirt1 was more highly expressed in primary iPS cells and C57 mES cells whose extracts can generate secondary iPS cells than in E14 mES cells whose extract cannot generate iPS cells. Thus, Sirt1 may be one of the key epigenetic regulators during protein-based reprogramming. Based on these results, we suggest that extract of secondary iPS cells cannot induce somatic cells into tertiary iPS cells because they have similar characteristics to E14 mES cells (Fig. 2e). Further studies will be required to examine the possibility of generating "tertiary" iPS cells using protein extracts of secondary iPS cells whose gene expression patterns are close to E14 mES cells.

We observed that extract from iPS cells showed higher efficiency in generating iPS cells from somatic cells than extract from ES cells did. To investigate the key molecules involved in this improvement of efficiency, we compared the genomics and proteomics of C57 mES cells and primary iPS cells. However, these two

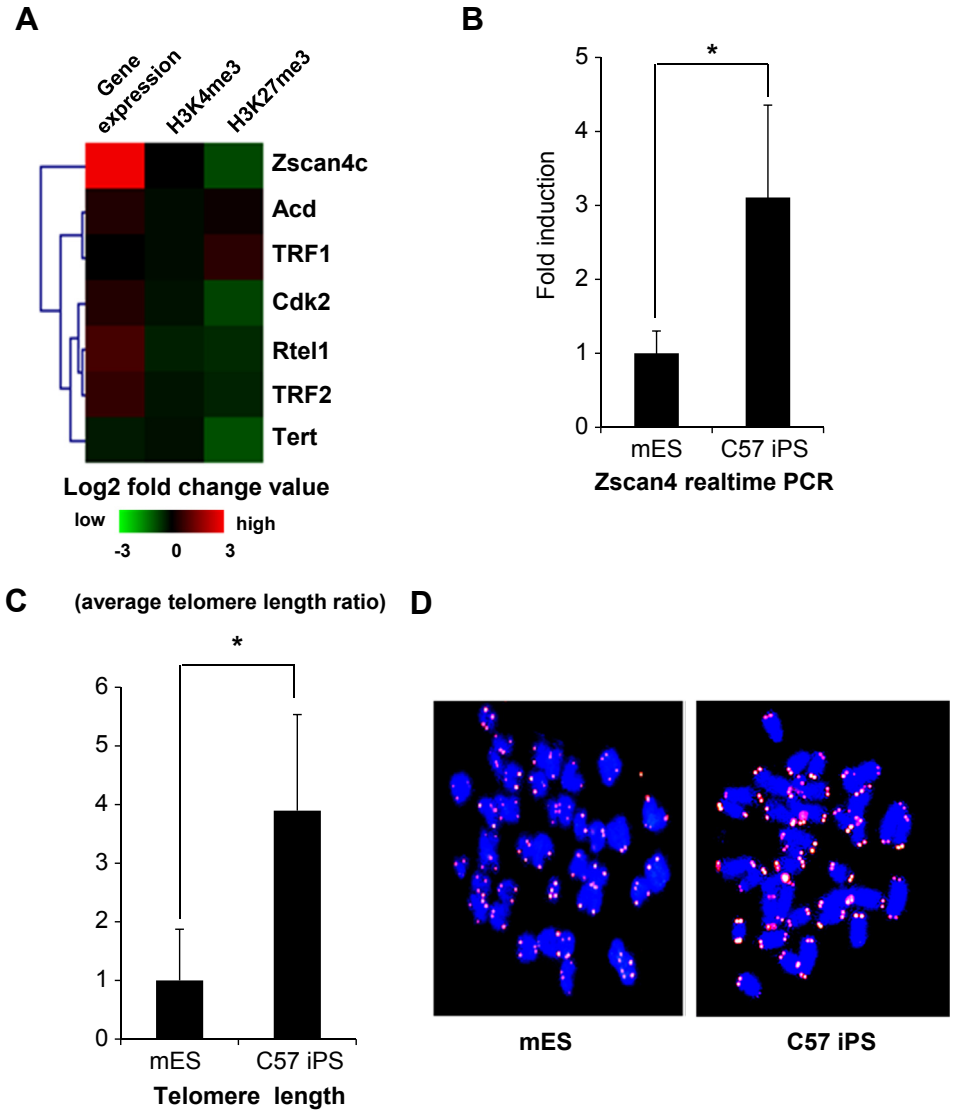

Fig. 5. Telomere elongation in protein-derived iPS cells. (A) Heat map for gene expression and histone modification of the iPS cells vs mES cells (The log2 ratio fold changes of all indicated genes relative to mES cells). (B) The gene expression of Zscan4 in mES cells and iPS cells was verified by real-time PCR. Expression level was adjusted by GAPDH (mES cell = 1.0 as an arbitrary unit). Asterisk (*) indicates a significant difference at P value < 0.05 (t-test). (C) Comparison of the average telomere length ratios (mean ± standard error) between mES cells and C57 iPS cells. Asterisk (*) indicates a significant difference at P value < 0.05 (t-test). (D) Representative image of FISH signal in mES cells (left) compared to iPS cells (right).

cell types showed very similar patterns of gene and protein expression. Only 13 proteins were upregulated in primary iPS as compared to C57 mES cells, including heat shock protein beta-1 (HspB1) and anamorsin (cytokine-induced apoptosis inhibitor 1). These data are well correlated with a recent report that small differences in gene expression do exist between various ES and iPS cell lines. But these differences do not consistently distinguish ES cells from iPS cells [27].

In this study, we screened the genes related to reprogramming efficiency and found that Zscan4, which is known to promote genomic stability and to elongate telomeres, may be the key factor in improving reprogramming efficiency during the generation of secondary iPS cells. It has recently been reported that viral transduction of Zscan4 increased efficiency of iPS generation in combination with Yamanaka's reprogramming factors (Oct4, Sox2, Klf-4 and c-Myc) and that maintenance of genomic stability through Zscan4 promotes the generation of high quality of iPS cells [23,25]. Accordingly, we assume that extract from high quality iPS cells enhanced the efficiency to generate secondary iPS cells. Further studies will be required to evaluate the detailed mechanisms, including how Zscan4 is induced during protein-derived iPS generation. To explore the mechanism by which Zscan4-overexpressing mES cell protein extract enhances the efficiency of iPS generation, we are analyzing proteomic changes in mES cells that are induced to express high levels of Zscan4. This information will help us develop high efficiency methods to produce high quality iPS cells via protein introduction.

Cell-cycle acceleration and inhibition of the p53/p21 pathway resulted in enhancing efficiency of reprogramming [28,29]. In our study, Zscan4 decreased p53 and p21 level (Fig. 7) and increased expression of cyclinD1, which controls cell cycle G1/S transition (Fig. S8). These data suggest that Zscan4 could promote reprogramming through accelerating cell cycle and processing non-stochastic reprogramming.

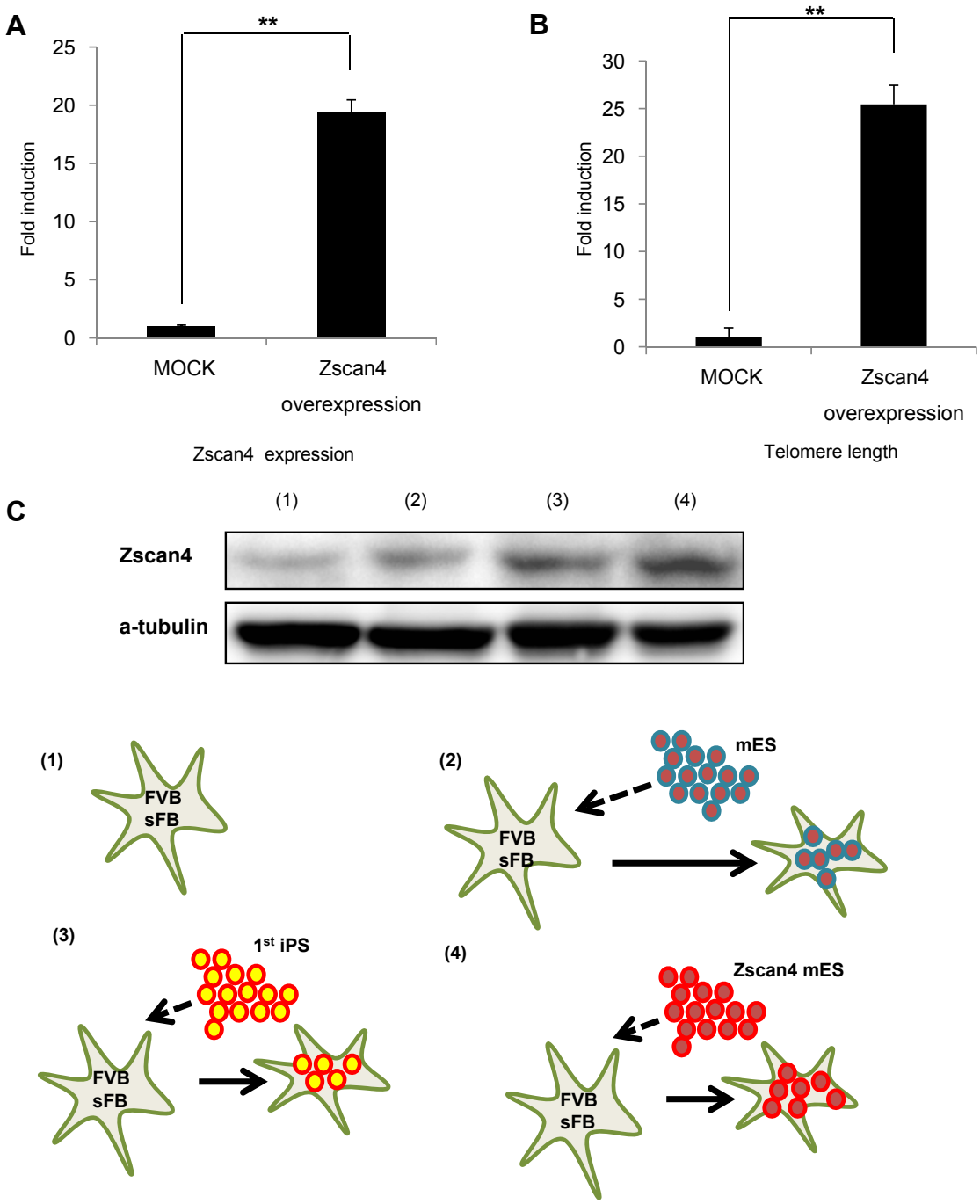

Fig. 6. Zscan4 enhanced reprogramming efficiency. (A) Overexpression of Zscan4 following transfection of the Zscan4 gene into mES cells was confirmed by real-time PCR. Asterisk (**) indicates a significant difference at P value < 0.01 (t-test). (B) Overexpression of Zscan4 in mES cells promotes elongation of telomeres. Data are presented as mean ± SEM. The symbol ** denotes high statistical significance ($P < 0.01$). (C) Zscn4 was detected by western blot analysis. Proteins are prepared in FVB sFB after delivery of extracts from (2) mES, (3) primary iPSC, (4) Zscan4 overexpressing mES cells respectively. Lane (1) shows the Zscan4 level in FVB sFB without treatment of extract. (D) Extracts from Zscan4-overexpressing mES cells improves reprogramming efficiency. GFP positivity represents the activation of Oct4 promoter. GFP positive cells are observed 15 days after delivery of proteins. FACS analysis was performed to enumerate GFP positive cells. It was confirmed that extract from Zscan4-overexpressing mES cells increased the number of Oct4-GFP positive cells.

5. Conclusions

We developed a protein-based reprogramming method without genetic, chemical or embryonic materials. By this approach we are able to avoid the ethical issues raised by the use of embryos for reprogramming. It is also very simple to introduce proteins into the somatic cells without repeated introduction. As described in our previous study [12], the protein-based reprogramming approach is safe for the induction of pluripotent cells. All chimeric mice developed from protein-based iPS cells have lived for more than a year in good condition without any tumor. We also demonstrated here that extracts from fresh primary iPS cells are more effective in generating iPS cells than extracts from passaged ES cells are. Zscan4 that is expressed higher in primary iPS than in mES cells is very important molecule in efficient reprogramming. However, we still do not know whether extracts from human iPS cells can generate

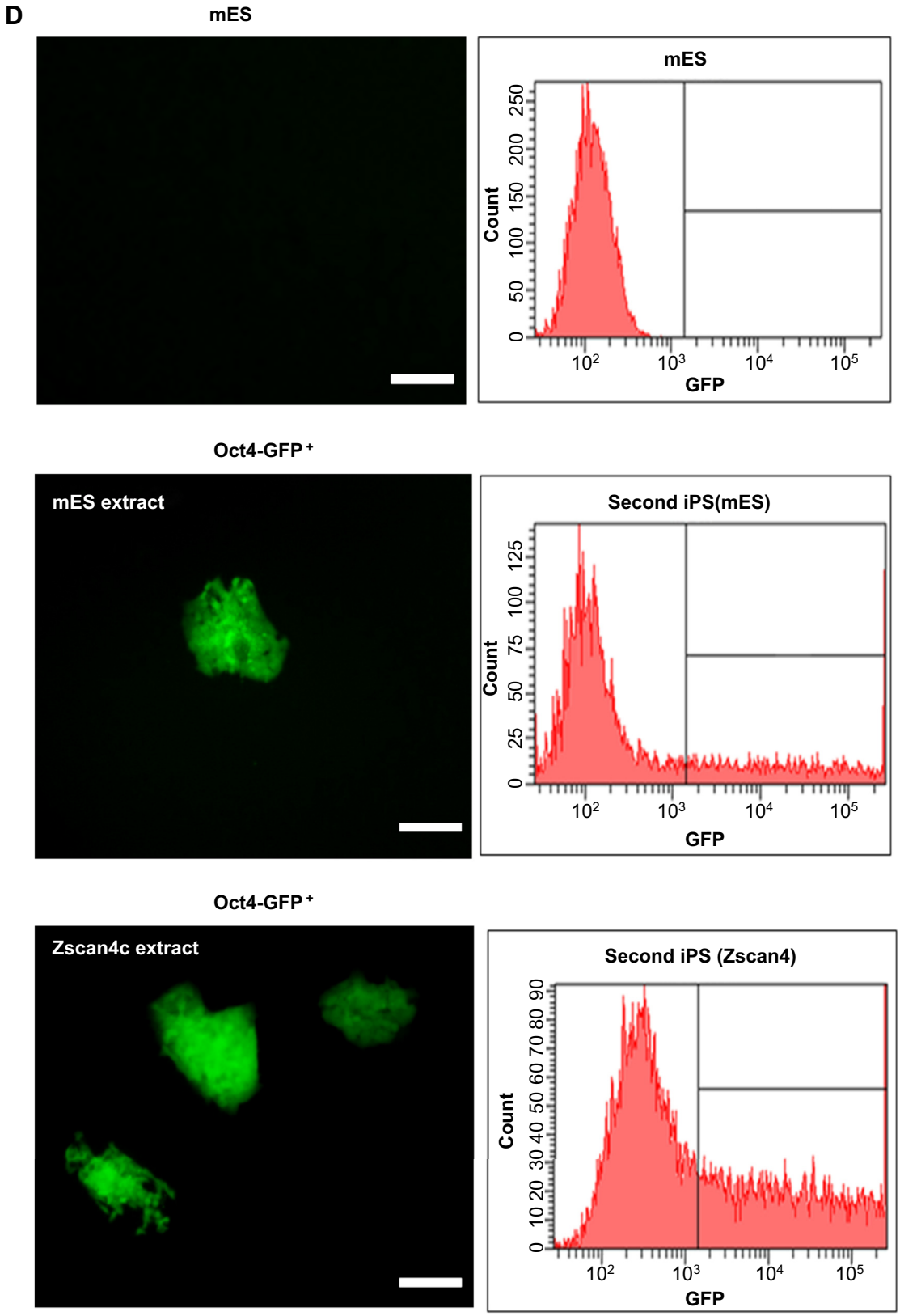

Fig. 6. (*continued*).

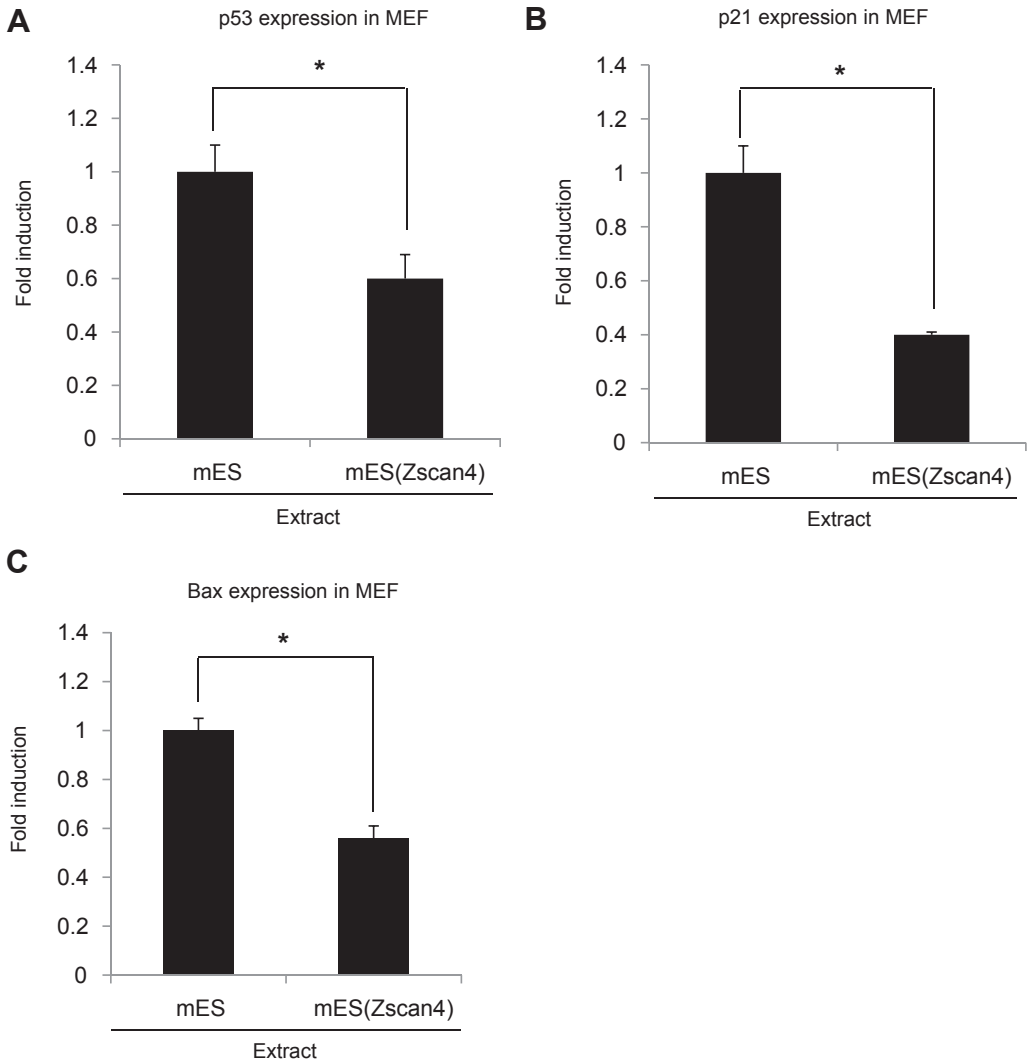

Fig. 7. Zscan4 decreased the expression level of p53, p21 and Bax. mRNA was prepared from FVB sFB after treatment of extract from mES (left) and Zscan4 overexpressing mES (right) cells. Gene expression level was determined by real-time PCR. Asterisk (*) indicates a significant difference at P value < 0.05 (t-test).

pluripotent cells from human somatic cells. If it is possible to successfully generate human iPS cells using this protocol, this may be the preferable strategy for clinical applications.

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The authors have no competing financial interests to declare.

Appendix A. Supplementary material

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biomaterials.2015.03.031.

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