Stem Cell Research 15 (2015) 481–494

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

E-Ras improves the efficiency of reprogramming by facilitating cell cycle progression through JNK–Sp1 pathway

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ARTICLE INFO

Article history:
Received 29 April 2015
Received in revised form 26 August 2015
Accepted 14 September 2015
Available online 16 September 2015

Keywords:
E-Ras
ES cell
Cell proliferation
Cell cycle
Reprogramming

ABSTRACT

We have previously shown that pluripotent stem cells can be induced from adult somatic cells which were exposed to protein extracts isolated from mouse embryonic stem cells (mESC). Interestingly, generation of induced pluripotent stem (iPS) cells depended on the background of ES cell lines; possible by extracts from C57, but not from E14. Proteomic analysis of two different mES cell lines (C57 and E14) shows that embryonic Ras (E-Ras) is expressed differently in two mES cell lines; high level of E-Ras only in C57 mESC whose extracts allows iPS cells production from somatic cells. Here, we show that E-Ras augments the efficiency in reprogramming of fibroblast by promoting cell proliferation. We found that over-expression of E-Ras in fibroblast increased cell proliferation which was caused by specific up-regulation of cyclins D and E, not A or B, leading to the accelerated G1 to S phase transition. To figure out the common transcription factor of cyclins D and E, we used TRANSFAC database and selected SP1 as a candidate which was confirmed as enhancer of cyclins D and E by luciferase promoter assay using mutants. As downstream signaling pathways, E-Ras activated only c-Jun N-terminal kinases (JNK) but not ERK or p38. Inhibition of JNK prevented E-Ras-mediated induction of pSP1, cyclins D, E, and cell proliferation. Finally, E-Ras transduction to fibroblast enhanced the efficiency of iPS cell generation by 4 factors (Oct4/Klf4/Sox2/C-myc), which was prevented by JNK inhibitor. In conclusion, E-Ras stimulates JNK, enhances binding of Sp1 on the promoter of cyclins D and E, leading to cell proliferation. E-Ras/JNK axis is a critical mechanism to generate iPS cells by transduction of 4 factors or by treatment of mESC protein extracts.

1. Introduction

ES cells are pluripotent cells derived from the inner cell mass (ICM) of the blastocyst at least an early post-implantation stage (Evans and Kaufman 1981) and are capable of dividing and self-renewing for extended periods (Loebel et al. 2003). These properties made ES cells can be used for treating Alzheimer's disease, Parkinson's disease, and other degenerative diseases (Thomson et al. 1998). However, application of ES cells to treatment has major hurdles, such as ethical issue and rejection problem after transplantation (Colman and Burley 2001). One solution to circumvent these problems is the generation of pluripotent cells by reprogramming somatic cells. In 2006, Yamanaka has generated ES cell-like cells, which are named iPS cells, from mouse fibroblast with four transcription factors (Oct3/4, Sox2, C-myc and Klf4) (Takahashi and Yamanaka 2006). Nonetheless, there are still considerations, such as cost, safety, and efficiency in generating iPS cells. Therefore, to resolve these issues, new methods are investigated, such as reducing the number of defined factors (Kim et al. 2008) and using non-viral inducers (Huangfu et al. 2008; Shi et al. 2008; Xu et al. 2008) or proteins (Cho et al. 2010; Zhou et al. 2009) or direct conversions (Han et al. 2014). In our previous study, iPS cells were generated from fibroblast by treating them with mES cells protein extract (Cho et al. 2010). Notably, only cell extracts from C57 mES cells were able to generate iPS cells from fibroblasts, but extracts from E14 mES cells were not (Cho et al. 2010). Through proteomic analysis of two different mES cell lines to find the key proteins associated with reprogramming, we noticed proteomic contrast between C57 and E14 mES cells. The expression of E-Ras in C57 mES cells was significantly higher than in E14 mES cells (Jin et al. 2011). Therefore, we hypothesized that E-Ras influences the reprogramming efficiency. Knowledge about molecular mechanisms of reprogramming will make reprogramming efficiency better. Recent studies have identified signaling pathways which play

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http://dx.doi.org/10.1016/j.scr.2015.09.004

important roles in the reprogramming, including MAPK/ERK (Silva et al. 2008), p53-p21 (Li et al. 2009; Hong et al. 2009; Kawamura et al. 2009) and Wnt/β-catenin (Marson et al. 2008). However, the efficiency of iPSC generation is still low and the underlying mechanisms are largely unknown. E-Ras is expressed in undifferentiated mES cells, but not in adult mouse tissues and differentiated ES cells (Takahashi et al. 2003). Around 80–95% of E-Ras protein binds to GTP regardless of its membrane localization (Takahashi et al. 2003; Zhang et al. 1993). Therefore, E-Ras can activate such signaling pathways constitutively, not like other Ras proteins (Yu et al. 2014). E-Ras activates phosphatidylinositol-3-OH kinase (PI3K) and promotes cell growth, but the cell cycle was not affected by E-Ras–PI3K pathway (Takahashi et al. 2003; Takahashi et al. 2005). In other words, there is other pathway that E-Ras affects the cell proliferation. In the previous studies, a high proliferation rate is required for reprogramming efficiency and maintenance of stem cell identity (Ruiz et al. 2011; Xu et al. 2013). Even though E-Ras was in the top 24-list of Yamanaka factor candidates (Takahashi and Yamanaka 2006), it has not been well studied in the context of reprogramming.

In this study, we revealed the new mechanism of E-Ras to enhance cell cycle progression and reprogramming. E-Ras activates JNK selectively, which facilitates binding of pSP-1 on the promoter of cyclins D and E genes, enhances cell cycle progression, and increases the efficiency of iPS cell generation from fibroblast. These findings suggest the tight association between cell proliferation and reprogramming, where E-Ras plays an important role.

2. Materials and methods

2.1. Cell culture

C57BL/6-background mES cells (accession #SCRC-1002; ATCC) and E14 mES cells (generously provided by Jeong Mook Lim, Seoul National University, Seoul, Korea) were grown on feeder layer of STO cells (5% CO_2, 37 °C). STO cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO) high glucose supplemented with 10% Fetal Bovine Serum (FBS; GIBCO), 1% Antibiotic-Antimycotic (GIBCO) on

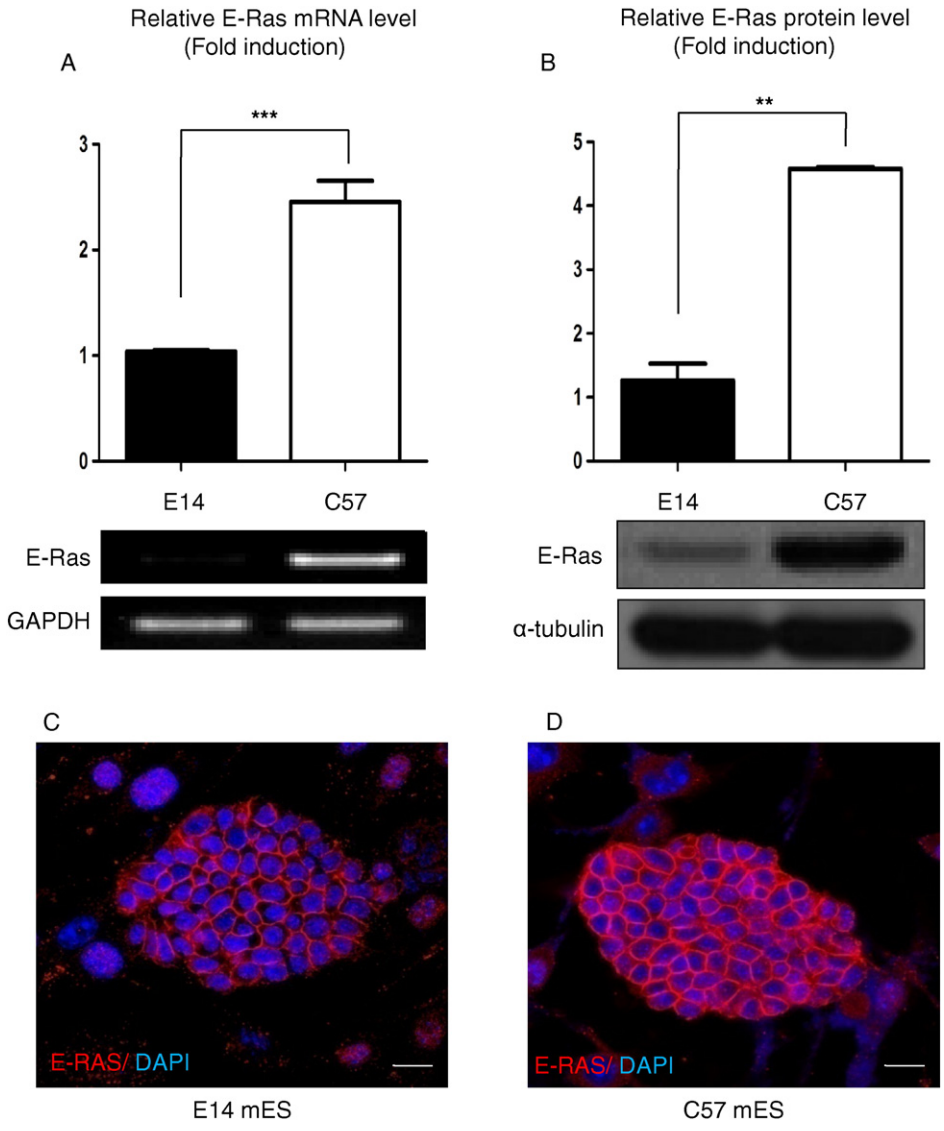


Fig. 1. Difference of E-Ras expression between C57 and E14 mES cells. Total RNA and protein were isolated from E14 mES cells and C57 mES cells. (A) E-Ras mRNA level was measured by real-time RCR and conventional RT-PCR. Level of mRNA from E14 mES cells was normalized to a value of 1. mRNA level of E-Ras was higher in C57 mES cells than in E14 mES cells. ***$p < 0.0001$. (B) Protein level of E-Ras was detected by Western blot analysis and bands were quantified by Image J software. E-Ras protein level was higher in C57 mES cells. **$p < 0.001$. (C)(D) Immunofluorescence images showed that E-Ras localized at the cell membrane. Higher E-Ras expression (red) was detected in C57 mES cells than in E14 mES cells. Scale bars = 20 μm. Abbreviations: mES cells, mouse embryonic stem cells; E-Ras, Embryonic Ras; and DAPI, 4′, 6-diamidino-2-phenylindole.

0.1% gelatin-coated plate. STO feeder cells were treated with mitomycin C (10 μg/ml medium, sigma) for 2.5 h to block mitotic activity and seeded on 0.1% gelatin-coated plate one day before subculturing mES cells. mES cells were cultured in DMEM (GIBCO) supplemented with 10% Fetal Bovine Serum (FBS; GIBCO), MEM NEAA (GIBCO), 1 mM 2-Mercaptoethanol (Sigma), 1% penicillin (100 IU/ml, GIBCO) and streptomycin (50 μg/ml, GIBCO). In ES media, Leukemia Inhibitory Factor (LIF, Chemicon) was added to mES cells media to maintain pluripotency. mES cells were passaged every 2–4 days.

2.2. Retroviral and lentiviral infection and iPS cell generation

E-Ras expression retroviral vector (pMXs-E-Ras; addgene) or mock vector (pMXs-GFP; addgene) was transfected into 293T cells using Lipofectamine 2000 (Invitrogen). The virus soup was taken a day after transfection and used to transduce NIH-3T3 cells with polybrene. NIH-3T3 cells, a mouse embryonic fibroblast cell line, and E-Ras over-expressed NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO) high glucose supplemented with 10% Fetal Bovine Serum (FBS; GIBCO), 1% Antibiotic-Antimycotic (GIBCO) on plate (5% CO_2, 37 °C). FUW-based lentiviral vectors (FUW-tetO-OSKM: Oct4/Sox2/Klf4/C-Myc and M2rtTA; addgene) were transfected into 293T cells using PEI (Polyethylenimine; Sigma). Viral medium was harvested at 48 h following transfection and filtered through 0.45-um pore filters. For concentration, viral supernatant was ultracentrifuged at 25,000 rpm for 1.5 h at 4 °C, and the pellets were resuspended in appropriate transduction medium. Virus soup was used to transduce mouse embryonic fibroblast (isolated from E 13.5 Oct3/4-promoter

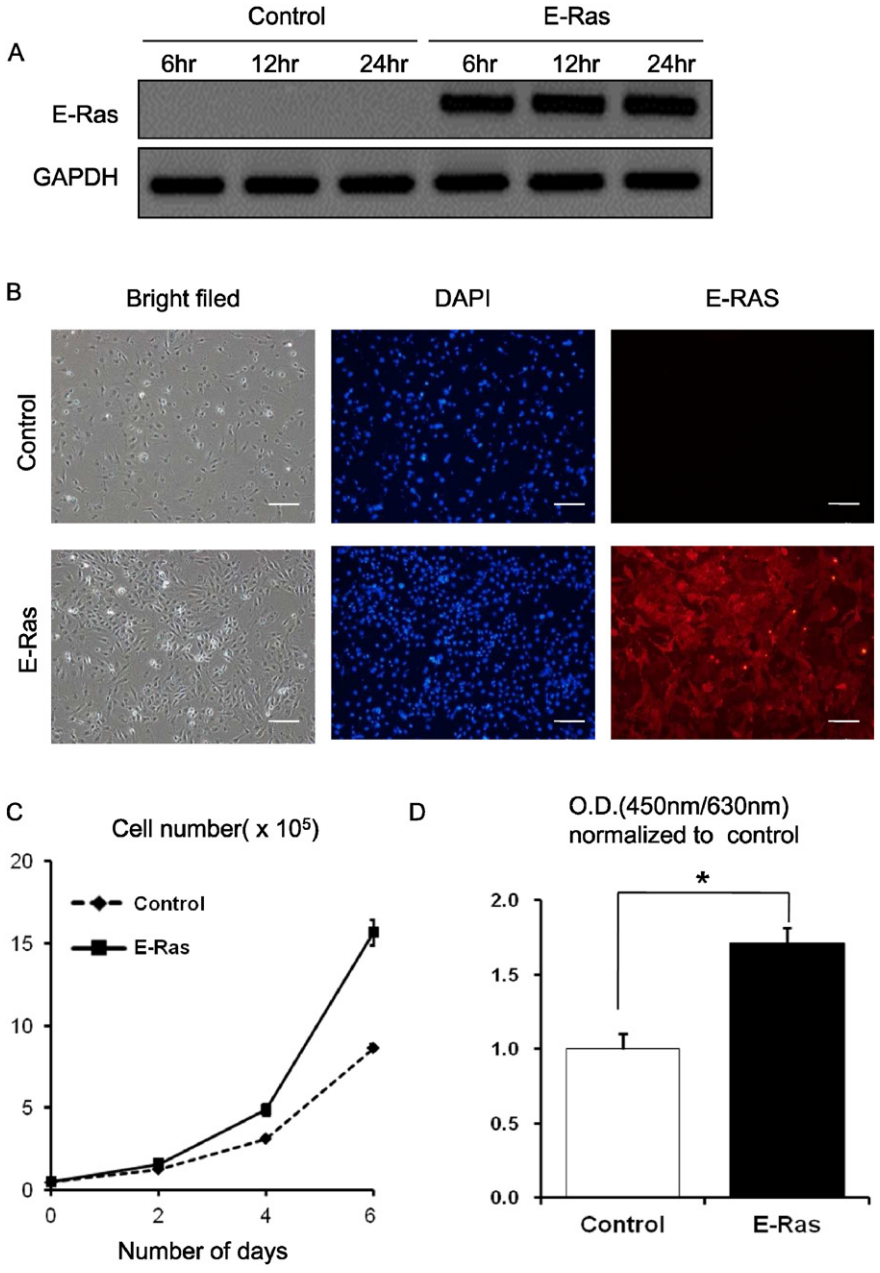


Fig. 2. E-Ras up-regulates cell proliferation. (A) E-Ras overexpression was confirmed by RT-PCR. (B) Immunostaining images showed increased proliferation activity in E-Ras over-expressed NIH-3T3 cells (red) in comparison to the control cells. Scale bars = 100 μm. (C) Cell growth was measured by the cell count, and the results showed increase in cell proliferation in E-Ras over-expressed cells compared with control cells. (D) Cell proliferation rate of E-Ras over-expressed NIH-3T3 cells and mock were measured by WST-1 assay. The results were expressed as percent of total number of cells and E-Ras over-expressed cells showed higher O.D. as compared the control cells. *$p < 0.005$. Abbreviations: O.D., Optical density.

GFP mouse embryos; MEF) with polybrene. 24 h after transduction, the mES cell medium was replaced. To inhibit JNK activity, MEFs were treated with JNK inhibitor. MEFs were seeded on MMC-treated STO feeders.

2.3. RNA preparation and PCR analyses

Total RNA were isolated from NIH-3T3 cells and E-Ras overexpressed NIH-3T3 cells by using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. 500 ng of total RNA was reverse-transcribed to cDNA using amfi Rivet cDNA synthesis premix (Gendepot).

Conventional reverse transcription (RT)-PCR was performed by using Maxime Premix (Intron) and quantitative real-time PCR was performed by using SYBR Green master mix (ROCHE) following the manufacturer's protocol. The primers for PCR analysis are listed in the Supplemental Table S1.

2.4. Confocal immunofluorescence

Cells were prepared on μ-Dish (ibidi) 35 mm and fixed using cold 4% formaldehyde for 15 min at room temperature. After blocking for

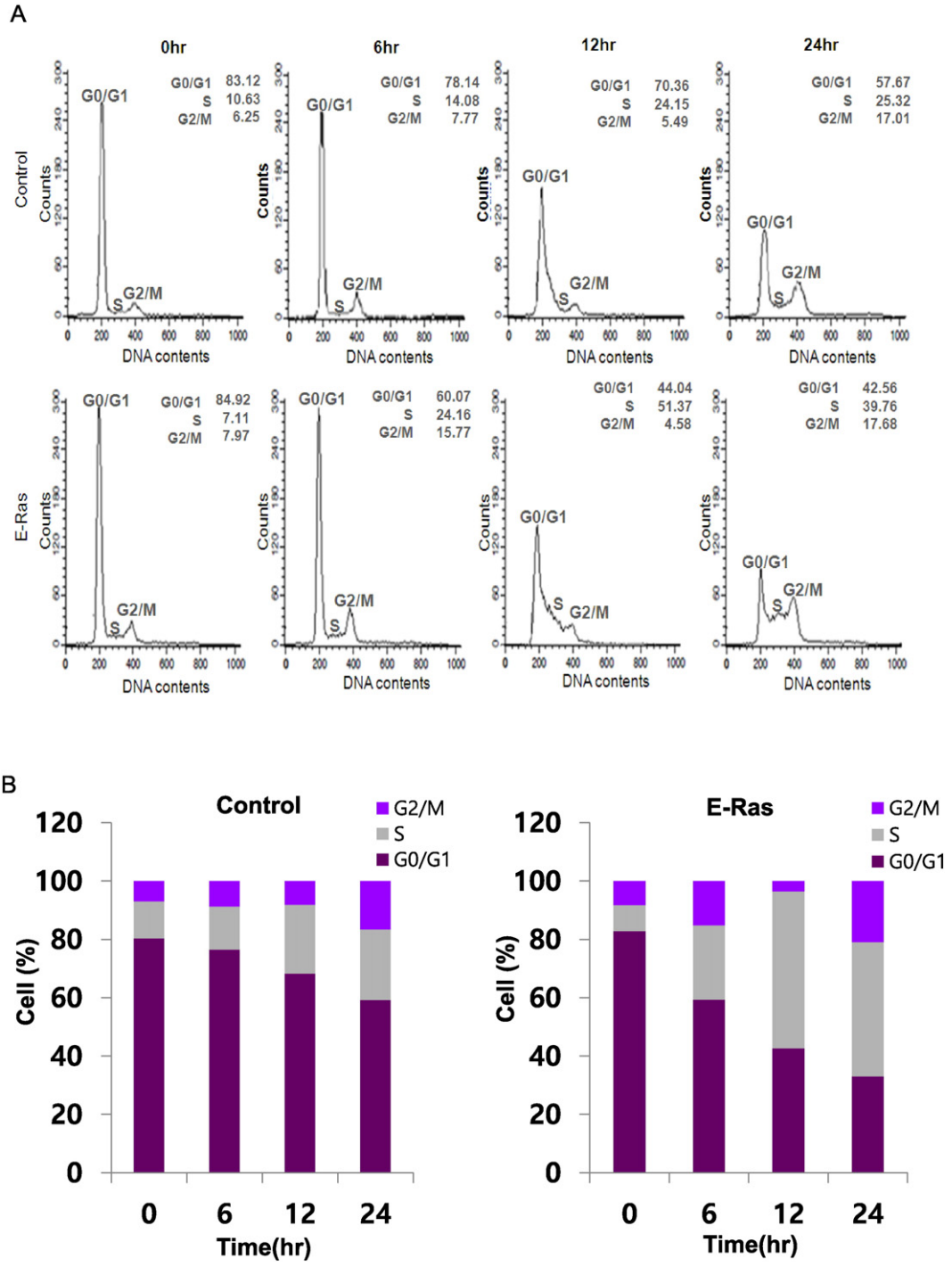


Fig. 3. Effect of E-Ras on cell cycle progression. (A) FACS analysis of cell cycle progression in E-Ras over-expressed cells and control cells. To synchronize cell cycle, each cell was serum starved for 48 h and forced to enter into G1 phase after treating with 10% serum media, and we measured DNA contents every 6 h. The results showed that cell cycle of E-Ras over-expressed cell was more rapidly progressed contrast to cell cycle of control cells. Results were representative of 3 independent experiments. (B)(C) Mean percentage of cell populations in G_0/G_1, S and G_2/M phases. All experiments were performed in triplicate to assess consistency of response. Abbreviations: FACS, Fluorescence-activated cell sorting.

C

Control

		Cell cycle distribution(%)			
Trials	Phase	0hr	6hr	12hr	24hr
N:1	G0/G1	83.12	78.14	70.36	57.67
	S	10.63	14.09	24.15	25.32
	G2/M	6.25	7.77	5.49	17.01
N:2	G0/G1	77.01	77.83	69.28	60.65
	S	17.24	12.35	22.45	23.44
	G2/M	5.75	9.82	8.27	15.91
N:3	G0/G1	81.13	73.64	65.22	59.37
	S	9.92	17.82	24.19	23.92
	G2/M	8.95	8.54	10.59	16.71
Mean	G0/G1	80.42	76.54	68.29	59.23
	S	12.60	14.75	23.60	24.23
	G2/M	6.98	8.71	8.11	16.54

E-Ras

		Cell cycle distribution(%)			
Trials	Phase	0hr	6hr	12hr	24hr
N:1	G0/G1	84.92	60.07	44.05	42.56
	S	7.11	24.16	51.37	39.76
	G2/M	7.97	15.77	4.58	17.68
N:2	G0/G1	82.31	58.99	43.11	29.50
	S	8.84	26.02	53.28	50.46
	G2/M	8.85	14.99	3.61	20.04
N:3	G0/G1	81.17	59.09	41.04	27.12
	S	10.81	25.99	56.43	47.96
	G2/M	8.02	14.92	2.53	24.92
Mean	G0/G1	82.80	59.38	42.73	33.06
	S	8.92	25.39	53.70	46.06
	G2/M	8.28	15.23	3.57	20.88

Fig. 3 (*continued*).

30 min at room temperature, the cells were incubated with primary antibodies overnight at 4 °C and cells were incubated for 1 h at RT with secondary antibodies. The following primary antibodies were used for immunofluorescence: goat anti-E-Ras (1:200; Santa Cruz), mouse anti-Oct4 (1:200; Santa Cruz), mouse anti-Nanog (1:200; Abcam), mouse anti-SSEA1 (1:200; Abcam), mouse anti-*glial fibrillary acidic protein*(GFAP) (1:200; Abcam), goat anti-α-fetoprotein(AFP) (1:200; Santa Cruz) and mouse anti-α-*smooth muscle actin*(α-SMA) (1:200; Santa Cruz). Secondary antibodies included Alexa Fluor 488 conjugated goat anti-mouse IgG (1:200; Invitrogen), Alexa Fluor 555-conjugated goat anti-mouse IgG (1:200; Invitrogen) and Alexa Fluor 555-conjugated donkey anti-goat IgG (1:500; Invitrogen). Nuclei are stained with 4′, 6′-Diamidino-2-phenylindole dihydrochloride (DAPI; Sigma Aldrich). Confocal microscopy images were obtained using a Zeiss LSM 710 (Carl Zeiss).

2.5. WST-1 cell proliferation assay

For the WST-1 assay, each cell was serum-starved for 24 h and seeded into 96-well plates (5×10^3 per well). 10 μl WST-1 [4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate] reagent (Roche) was added per well for 4 h at 37 °C in 5% CO_2. The 96-well plates were analyzed optically at 450 and 650 nm.

2.6. Cell counts-based proliferation assay

The cells were plated onto 6 well at 5×10^4 cells per well. After 48 h, cells were harvested and cell number was counted by a hemocytometer under inverted microscope. Cell numbers were expressed as mean from one representative experiment out of three.

2.7. Flow cytometry analyses

The cell cycle was analyzed by measuring the amount of propidium iodide (PI)-labeled DNA in ethanol-fixed NIH-3T3 cells and E-Ras overexpressed NIH-3T3 cells. To synchronize cell cycle, each cells were starved of serum for 48 h. After that, they were treated with 10% serum media to enter ino G1 and S phase for 0, 6, 12 and 24 h. Each cells were harvested and fixed with cold 90% ethanol in FACS tubes at 4 °C for 30 min. Then, cells were stained with propidium iodide (PI; Sigma Aldrich) containing 0.5 mg/ml RNase (Sigma Aldrich) for 30 min at room temperature. DNA content (2N-G0/G1 and 4N-G2/M) was then measured by flow cytometry analysis. Flow cytometry was performed on a Caliber FACS machine (Becton Dickinson). The percentages of cells in different phases of the cell cycle were analyzed by software (CellFit™). All experiments were performed in triplicate to assess consistency of response.

2.8. Protein preparation and Western blot analyses

Nuclear protein and cytosolic protein of each cell were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Thermo). 25 μg of lysed proteins was separated by dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred from gel onto polyvinyl difluoride (PVDF) membrane. The membrane was blocked for 1 h with blocking solution (5% skim milk) and incubated overnight at 4 °C with primary antibodies: anti-E-Ras (Santa Cruz; 1:2000), anti-cyclin D (cell signaling; 1:1000), anti-cyclin E (cell signaling; 1:1000), anti-SP1 (Millipore; 1:1000), anti-pSP1 (Assay Biotech; 1:1000), anti-ERK (cell signaling; 1:1000), anti-pERK (cell signaling; 1:1000), anti-p38 (cell signaling; 1:1000), anti-pp38 (cell signaling; 1:1000), anti-JNK (cell signaling; 1:1000), anti-pJNK (cell signaling; 1:1000), and anti-α tubulin (Santa Cruz; 1:5000). The membrane was incubated for 1 h at RT with individual secondary antibodies: anti-goat IgG HRP (Santa Cruz; 1:10,000), anti-rabbit IgG HRP (Santa Cruz; 1:10,000), and anti-mouse IgG HRP (Santa Cruz; 1:10,000). Detection was done using an ECL kit (GE healthcare).

2.9. Luciferase assay

NIH-3T3 cells and E-Ras overexpressed NIH-3T3 cells were transfected with pGL3-cyclin D promoter luciferase reporter vector and Renilla vector using Lipofectamine 2000 (Invitrogen). After 6 h, the cells were harvested for firefly luciferase activities with Dual Luciferase Reporter Assay Kit (Promega) according to the manufacturer's instructions. For pGL3-cyclin E promoter activity, pGL3-cyclin E promoter luciferase reporter vector was used instead of pGL3-cyclin D promoter luciferase reporter vector. Luciferase reporter plasmids of mouse cyclin D promoter and cyclin E promoter were charitably provided by Dr. Johan Auwerx (CNRS/INSERM/University Louis Pasteur). The Sp1 point mutants of the pGL3-cyclin D and E promoter were generated using QC lightning Multi site-directed mutagenesis kit (Stratagene) and performed according to the manufacturer's instructions. Details of PCR primers are list in the Supplemental Table S2.

2.10. Alkaline phosphatase staining

ALP staining was performed by a standard protocol. In brief, the reprogrammed transgenic MEFs were rinsed with PBS, fixed in 100% methanol, rinsed with PBS again and then overlaid with BCIP/NBT substrate (5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium; Dako), followed by incubation at room temperature for 1 h in the dark. The plates were scanned using an office scanner.

2.11. In vitro differentiation of iPS cells

The 4 factors derived iPSC and combination of 4 factors and E-Ras derived iPSC from MEF were harvested by trypsinization and plated on the ultra low cluster plates (Costar, corning NY 14831) and incubated for five days in the medium without LIF. After incubation, aggregated cells were transferred to gelatin-coated cell culture dishes and incubated another five days. Cells were fixed and incubated with anti-*glial fibrillary acidic protein* (GFAP; Ectoderm marker), anti-α-fetoprotein (AFP; Endoderm marker) and anti-α-smooth muscle actin (SMA; Mesoderm marker) along with 4′-6-diamidino-2-phenylindole (Sigma).

3. Results

3.1. E-Ras is differentially expressed among mouse ES cell-lines

Previously we have demonstrated that iPS cells are derived successfully by treating fibroblast with protein extracts from mES cells of C57 strain but not E14 mES cell lines (Cho et al. 2010). The result of proteomic analysis between two mES cells shows difference in E-Ras protein level (Jin et al. 2011). We examined the expression level of mRNA and proteins in C57 and E14 mES cells to confirm the validity of the previous proteomic data (Fig. 1A, B and C). Real-time RT-PCR showed that the mRNA expression level of E-Ras in C57 mES cells was almost 2.5-fold higher than that in

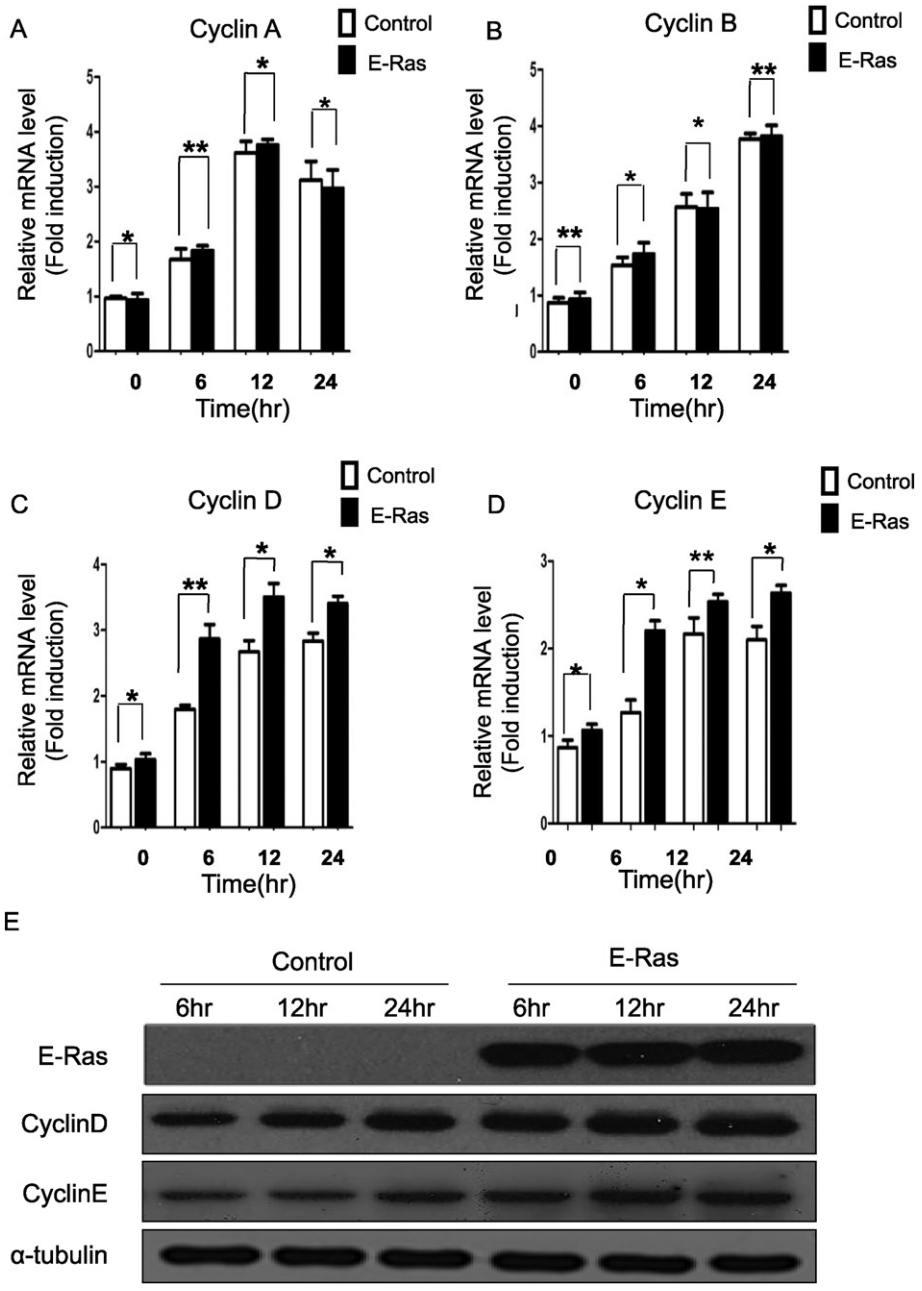


Fig. 4. E-Ras promotes cell cycle via up-regulation of cyclins D and E. (A)(B)(C)(D)The expression level of cyclins A, B, D and E were assayed by real-time PCR at 0, 6, 12, and 24 h after serum stimulation in the serum-starved E-Ras over-expressing cells and controls. The induction of cyclins D and E mRNA expression was earlier and greater in E-Ras over-expressing cells than in control cells. The induction of cyclins A and B was not affected by E-Ras over-expression. *$p < 0.005$; **$p < 0.001$. (E) Changes in the expression of cyclins. D, E proteins were analyzed by Western blotting at 6, 12, and 24 h after serum stimulation in the serum-starved E-Ras over-expressing cells and control ones. Proteins of cyclins D and E were higher in E-Ras over-expressing cell than in control ones.

E14 mES cells (Fig. 1A). The protein expression of E-Ras was 4.5-fold higher in C57 mES cells than in E14 mES cells (Fig. 1B). The immunoreactivity for E-Ras was also higher in C57 mES cells than that in E14 mES cells (Fig. 1C and D). Taken together, the expression level of E-Ras was highly distinguishable among well-established mouse ES cell-lines.

3.2. E-Ras over-expression promotes a cell proliferation and cell cycle

To assess the effects of E-Ras on cell proliferation, we manufactured E-Ras over-expressed NIH-3T3 cells by infection with E-Ras retroviral vector. We confirmed the high level of E-Ras expression in the transduced cells (Fig. 2A). After gene transduction, E-Ras over-expressed cells grew faster than control cells did under the same growth condition. Cell numbers were greater in E-Ras transduced group than control cells after sub-culturing equal numbers. During 6 days culture, E-Ras over-expressed cells grew faster than control ones (Fig. 2B). On the sixth day, the growth rate of E-Ras over-expressed cells was twice as fast as that of control ones (E-Ras over-expressed cells, 16×10^5; control cells, 8×10^5) (Fig. 2C). In the WST-1 assay, E-Ras over-expressed cells were 30% more proliferative than control cells (Fig. 2D). These

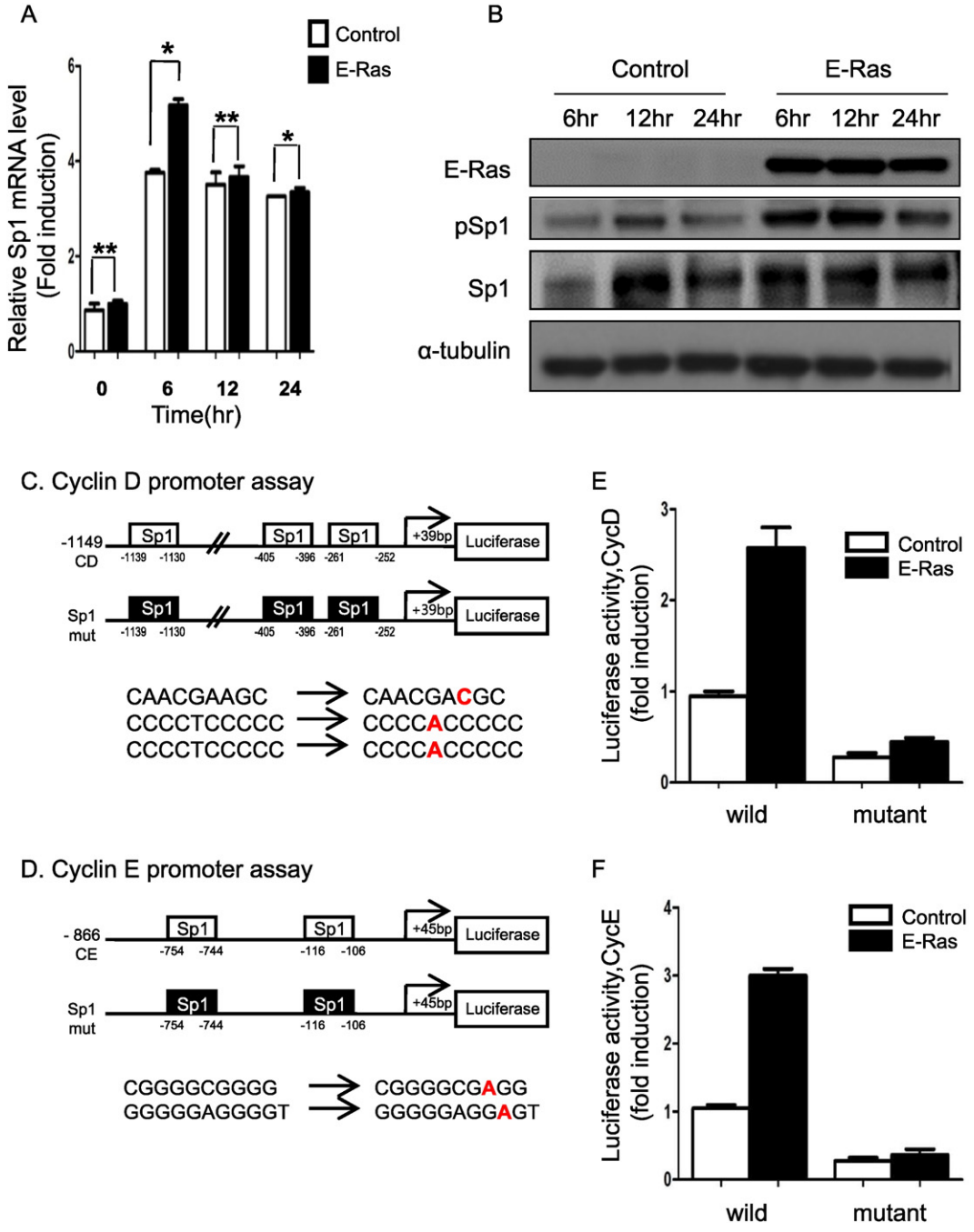


Fig. 5. E-Ras enhances cyclins D and E promoter activity through Sp1. (A) The mRNA level of Sp1 was analyzed by real-time PCR at 0, 6, 12, and 24 h after serum stimulation in the serum-starved E-Ras over-expressing cells and control ones. The induction of Sp1 mRNA expression was greater in E-Ras over-expressing cells than in control ones. *$p < 0.005$; **$p < 0.001$. (B) Changes in the expression of total Sp1 and phosphorylated Sp1 were analyzed by Western blotting at 6, 12, and 24 h after serum stimulation in E-Ras over-expressing cells and control. The induction of phosphorylated Sp1 as well as total Sp1 amount was greater in E-Ras over-expressing cells than in control ones. (C–F) Promoter assay of cyclins D and E genes. (C, D) Schematic presentation of pGL3 basic luciferase reporter vector and Sp1 deletion mutants of cyclins D or E. (E, F) The activation of cyclin D and cyclin E promoter was higher in E-Ras over-expressing cells than in control ones. Deletion mutants of the Sp1 reduced promoter activity both in E-Ras over-expressing cells and control ones. Abbreviations: Sp1, specificity protein 1, mut, mutant.

results verified that E-Ras promotes cell growth. To further understand how E-Ras stimulates cell proliferation, we analyzed cell cycle through measuring DNA contents of E-Ras over-expressed cells and control cells by propidium iodide (PI) flow cytometric assay (Fig. 3A, B and C). To synchronize cell cycle, each cell was starved of serum for 48 h and forced to enter into G1 and S phase by applying 10% serum media. E-Ras over-expressed cells entered S phase between 6 and 12 h after serum-stimulation, which was earlier than control cells. These results indicated that constitutive over-expression of E-Ras in NIH 3T3 cells advanced cell cycle more quickly.

3.3. E-Ras over-expression induces cyclins D and E expression through activation of Sp1

In order to understand the mechanism of E-Ras to facilitate cell cycle progression, we investigated the change of cell cycle related genes such as cyclins A, B, D, and E. Total RNA was isolated from E-Ras over-expressed cells and control ones that were prepared at 0, 6, 12, and 24 h after serum stimulation. mRNA level of cyclins A or B was induced after serum stimulation, which was not augmented by E-Ras. However, induction of mRNA level of cyclins D or E after serum stimulation was augmented by E-Ras over-expression (Fig. 4A, B, C and D). Western blotting result of cyclins D and E was analogous to real-time PCR result (Fig. 4E). Cyclins D and E are rate-limiting activators of the G1-to-S phase transition (Resnitzky et al. 1994). Therefore, rapid cell cycle by E-Ras might be dependent on the expression of cyclins D and E. To figure out the common transcription factor which can bind and activate the promoter of cyclins D and E, we used TRANSFAC database. From this analysis, Sp1 was selected since it was the only candidate that was predicted to be able to bind the promoter of both cyclins D and E genes. Next, we evaluated the expression of Sp1 by E-Ras. Real-time PCR and Western blot data demonstrated E-Ras increased not only the expression but also phosphorylation of Sp1 (Fig. 5A and B). To demonstrate whether E-Ras can activate the cyclins D and E promoter, we performed luciferase assay using full promoter sequence for both genes. Wild pGL3-cyclin D promoter vector was transfected into E-Ras over-expressed cells and control cells, and then luciferase activity was determined 6 h after transfection. As expected, E-Ras significantly activated cyclins D and E promoter. The promoter activity of cyclins D or E increased two or three times under E-Ras overexpression (Fig. 5E and F). Furthermore, to conduct an examination on the specific effect of Sp1 on cyclins D and E promoter, we constructed mutant luciferase vector which had mutation in Sp1 binding site on cyclins D and E promoter respectively (Fig. 5C and D). The red letter indicates sequence replacement. The mutation of Sp1 binding sites on cyclins D or E promoter obliterated promoter activity, which was not recovered even by E-Ras over-expression (Fig. 5F). Overall, these results suggest that E-Ras induced the expression and phosphorylation of Sp1. The activated Sp1 increased the expression of cyclins D and E through promoter activation of both genes.

3.4. The JNK signaling is stimulated by E-Ras and is associated with phosphorylation of Sp1

To investigate the mechanism how E-Ras phosphorylates Sp1, we examined Ras signaling pathway. PI3K and Raf are well known as representative downstream signaling proteins of Ras, but they are not related with regulating cell cycle via E-Ras activation (Takahashi et al. 2005; Moodie et al. 1993). Therefore, we paid attention to another signaling factor MEKK, the downstream messenger of Ras-PI3K or Ras–Raf pathway. There are ERK, p38 MAPK, and JNK in downstream of MEKK. The induction of phospho-ERK or phospho-p38 after serum stimulation was not affected by E-Ras overexpression (Fig. 6A). However, the induction of phospho-JNK after serum stimulation was augmented by E-Ras overexpression. The total ERK, p38, and JNK protein level had no difference regardless of E-Ras overexpression. These data demonstrated that JNK could be the link between E-Ras and Sp1, but not ERK or p38. To ascertain that phosphorylated JNK could activate Sp1, E-Ras over-expressed cells and control cells were exposed to pharmacologic JNK inhibitor SP600125 for 6 h (Fig. 6B). JNK inhibitor reduced not only phosphorylation of Sp1 but also expression of cyclins D and E, which was not recovered even by E-Ras overexpression (Fig. 6C). Moreover, to substantiate whether E-Ras-JNK regulates the cell cycle, we performed a FACS analysis at 0, 6, 12, and 24 h after serum stimulation using E-Ras overexpressing cells that were pretreated with PBS, Akt

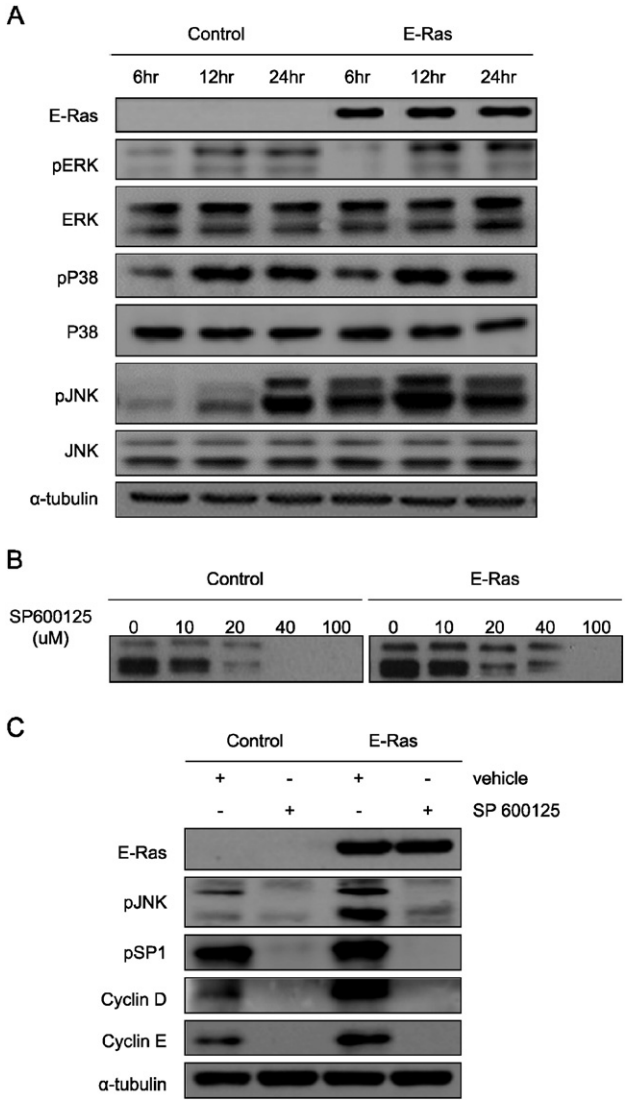
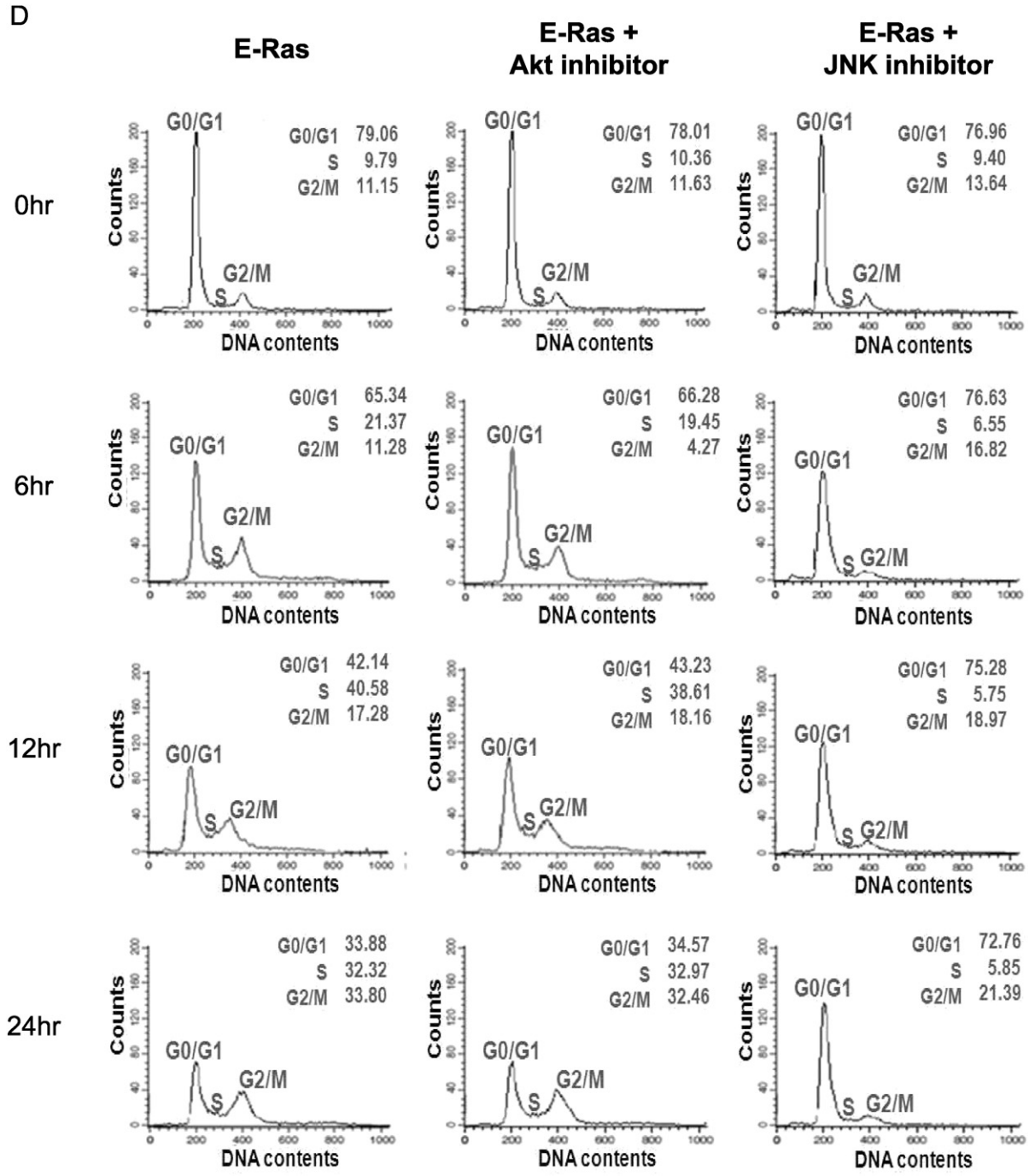


Fig. 6. E-Ras increases the expression of cyclins D, and E through the activation of E-Ras–JNK pathway promotes the cell cycle through the JNK pathway. (A) Changes in the activation of MAPK proteins (ERK, p38, JNK) were analyzed by Western blot analysis at 6, 12, and 24 h after serum stimulation in the serum-starved E-Ras over-expressing cells and controls. All three MAPK activities were stimulated by serum, while total amount of MAPK proteins remained unchanged. Among them, induction of phosphorylated JNK was earlier and greater in E-Ras over-expressing cells than in control ones, whereas induction of ERK and p38 activities was not enhanced by E-Ras over-expression. (B) In order to inhibit JNK phosphorylation, both cells were treated with 50 μM SP600125 for 6 h. (C) Western blot analysis was used to determine phospho-Sp1, cyclin D, and cyclin E expression. As JNK phosphorylation was inhibited, the induction of phospho-Sp1, cyclin D, and cyclins E after serum-stimulation were prevented not only in controls but also in E-Ras overexpressing cells. Abbreviations: MAPK, mitogen-activated protein kinase; ERK, Extracellular signal-regulated kinase; p38, P38 mitogen-activated protein kinases; and JNK, c-Jun N-terminal kinases. (D) E-Ras overexpressing cells were incubated with working concentrations of the Akt inhibitor, JNK inhibitor, or DMSO as control. Each cell was treated with 10% serum media for 0, 6, 12, and 24 h after serum starvation for 48 h. Cell cycle was arrested only by JNK inhibition but not by Akt inhibition.



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inhibitor, or JNK inhibitor. Enhanced cell-cycle progression after serum stimulation by E-Ras was not affected by Akt inhibitor, but significantly retarded by JNK inhibitor (Fig. 6D). Taken together, our data indicate that E-Ras–JNK signaling pathway phosphorylates and activates Sp1, resulting in the expression of cyclins D and E, and cell cycle progression or proliferation.

3.5. E-Ras–JNK pathway enhances reprogramming by stimulating the cell cycle

Next, to investigate whether E-Ras–JNK pathway could affect reprogramming efficiency, we compared the efficiency of 4 factors-medicated iPS cell generation between presence and absence of E-Ras. After gene transduction, each group of cells was exposed in the presence or absence of JNK inhibitor. Cells were cultured under the standard condition for mouse iPS cell induction, and monitored daily for morphological changes (ESC-like colony formation). After 15 days of delivery of transgenes, we observed colonies positive for alkaline phosphatase (Fig. 7A). ES cell-specific genes including Oct4, Nanog, Rex1 and E-Ras were expressed in both colonies from 4 factor only and from 4 factor plus E-Ras (5 factors), while alpha smooth muscle actin was not expressed (Fig. 7B). Interestingly, addition of E-Ras on top of 4 factors significantly increased the number of colonies, which was totally blocked by JNK inhibitor (Fig. 7A and C). JNK inhibitor blocked not

only efficiency of reprogramming but also reprogramming itself. Thus, E-Ras is required for enhancing the efficiency of iPS cell generation and JNK might be a critical signaling messenger during reprogramming.

To investigate whether 4 factors and 5 factors derived iPS cells have the similar characteristics each other, the colonies were immunostained with the pluripotency markers including Oct4, Nanog and SSEA1. As shown in Fig. 7D and E, the stemness markers are expressed similarly in these two different colonies. Next, to determine the differentiation potential of these iPS cells, we used the EB-based spontaneous differentiation protocol. The iPS cells derived Embryoid bodies (EBs) were formed after 5 days of suspension and the EBs are attached on gelatin coated dish (Fig. 7F). After 5 days of attached culture, these differentiated cells from two different iPS colonies expressed specific markers of three germ layers, including GFAP for ectoderm, α-SMA for mesoderm and AFP for endoderm (Fig. 7G). From these results, we determined that the pluriptency of 5 factors (including E-Ras)-derived iPS cells were similar to those of 4 factors-derived iPS cells.

4. Discussion

In this study, we examined how E-Ras increased cell proliferation and stimulated the cell cycle as well as how it affected the efficiency of reprogramming of somatic cell. We demonstrated that E-Ras accelerated the cell cycle through the JNK pathway, leading to subsequent phosphorylation of Sp1 and increase of cyclins D and E. E-Ras improved the efficiency of 4 factors-mediated iPS cell generation, which was blocked by JNK inhibitor (highlight).

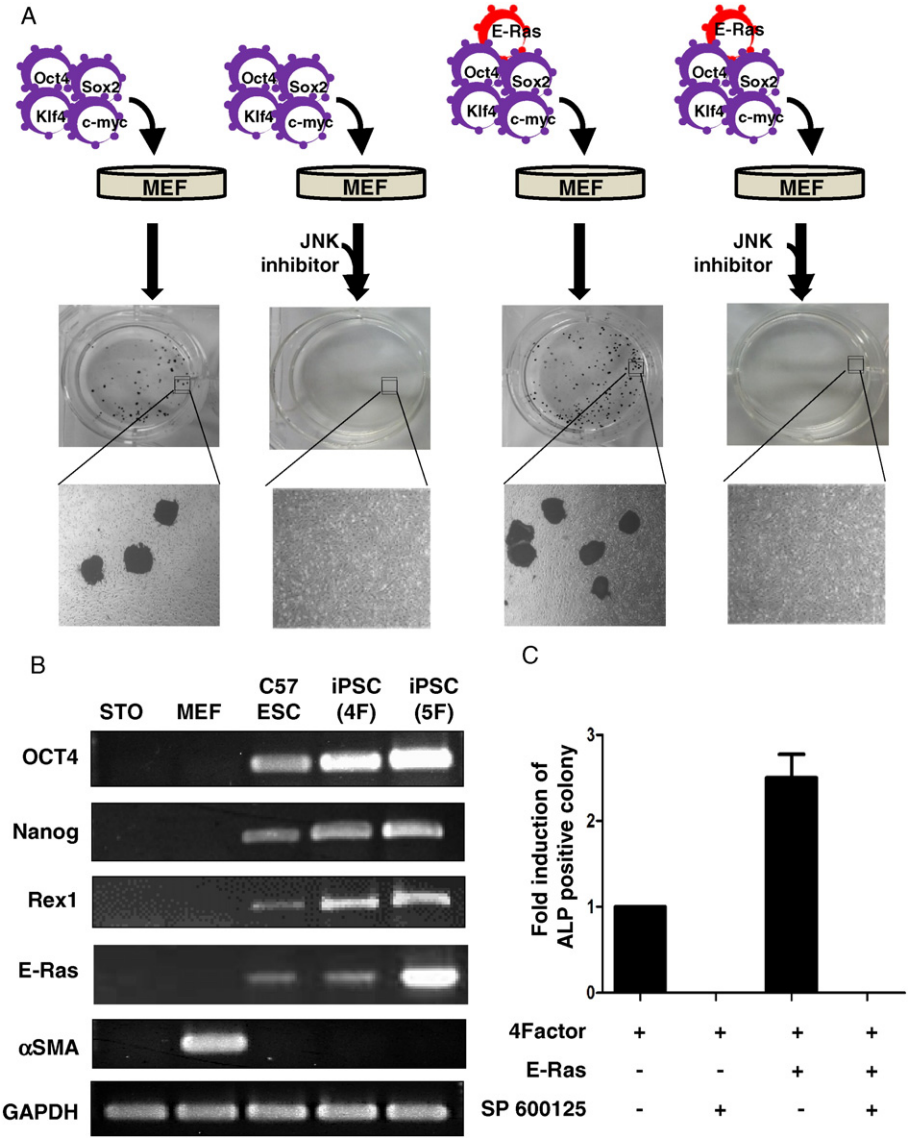
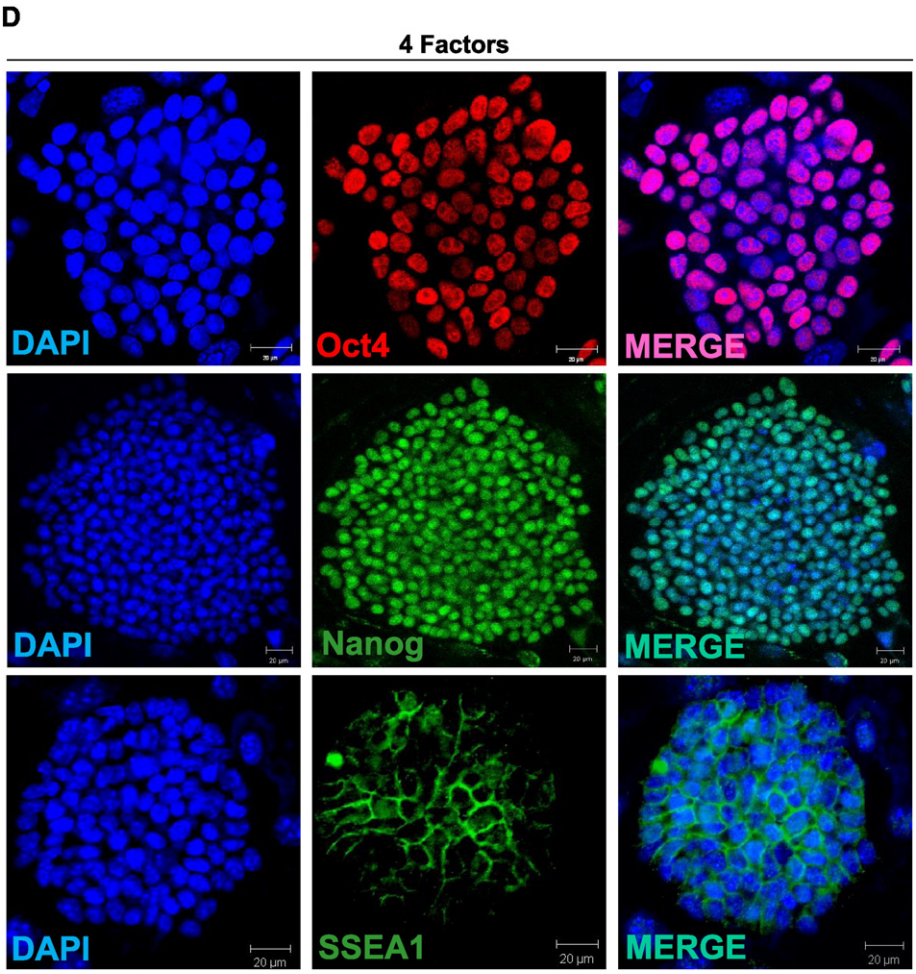


Fig. 7. E-Ras is required for efficient reprogramming through the JNK pathway. (A) MEFs were transduced with retroviruses containing four reprogramming factors (Oct4, Sox2, Klf4, and c-myc) or five factors (E-Ras combined with 4 factors). Cells were incubated with the JNK inhibitor or DMSO controls. A representative image of alkaline phosphatase (AP) staining was shown. (B) Expression of ES cell-specific genes including Oct4, Nanog, Rex1, E-Ras and differentiated gene (alpha smooth muscle actin) was analyzed in colonies from 4 factors only and from 4 factors plus E-Ras (5 factors) by RT-PCR. (C) The number of colonies or generation of iPS cells increased by E-Ras transduction, which was blocked by JNK inhibitor. (D)(E) iPSC generated from 4 factors with E-Ras and without E-Ras were stained with mouse anti-Oct4 (1:200; Santa Cruz), mouse anti-Nanog (1:200; Abcam) and mouse anti-SSEA1 (1:200; Abcam). Scale bars = 20 μm. (F) Images of EB formation (D5) (upper row) and spontaneous differentiation (D5 after attached) (lower row) were shown. Scale bars = 200 μm. (G) Immunostaining confirming in vitro differentiation into all three germ layers was shown. Images were stained with mouse anti-*glial fibrillary acidic protein* (GFAP) (1:200; Abcam), goat anti-α-fetoprotein (AFP) (1:200; Santa Cruz) and mouse anti-α-*smooth muscle actin* (SMA) (1:200; Santa Cruz). Scale bars = 20 μm.



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4.1. Is E-Ras a key molecule to regulate the efficiency of reprogramming?

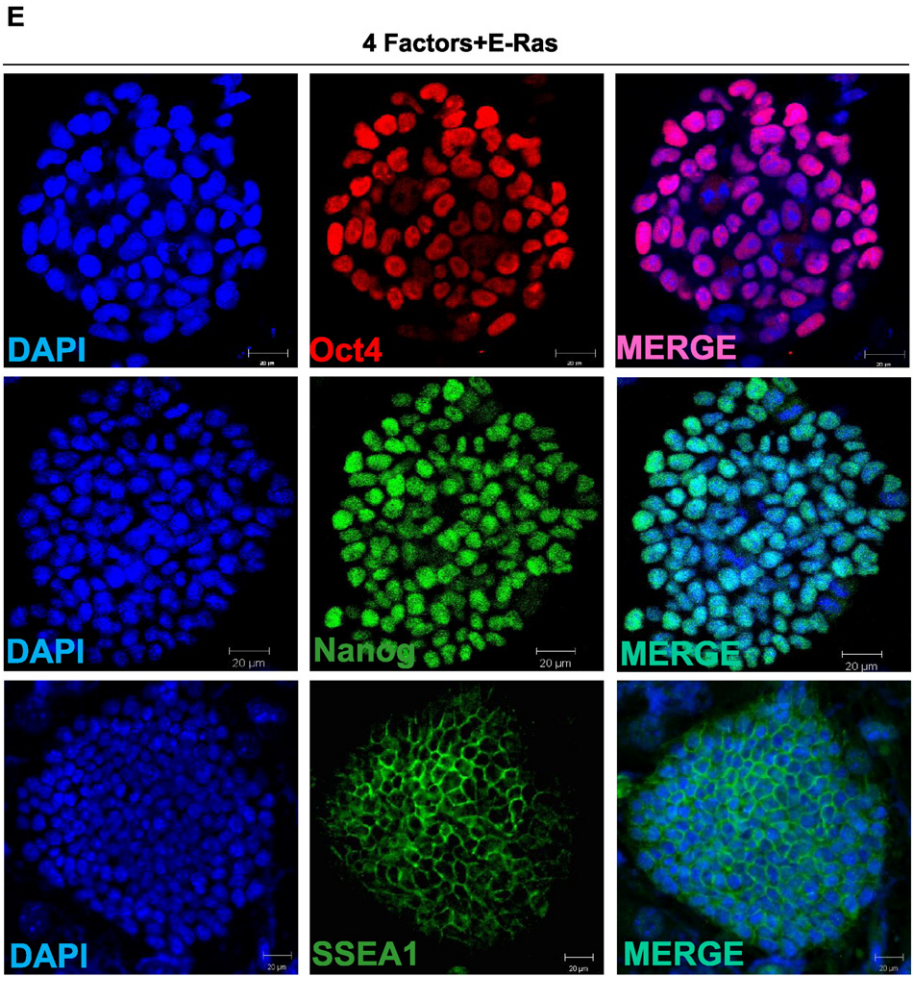
In our previous studies (Cho et al. 2010; Jin et al. 2011), E-Ras was suggested as a key molecule of reprogramming after proteomic analysis of two different mESC lines. It was the only molecule showing differential expression between two mESC lines; high in C57 mES cell line whose protein extracts could convert fibroblast to iPS cells, whereas low in E14 mES cells whose extracts could not induce reprogramming. In order to test this hypothesis, we made E-Ras construct and overexpressed it in somatic cells in the present study. To our expectation, E-Ras overexpression significantly enhanced the efficiency of 4 factors-medicated iPS cell generation from somatic cell. When E-Ras was combined with 4 factors, it improved the efficiency of iPS cell generation (Fig. 7). This result is corroborated by a recent study reporting the enhancing role of E-Ras in reprogramming (Yu et al. 2014). In this paper, Yu et al. demonstrated that E-Ras stimulated reprogramming of somatic cells through Akt and Foxo1 signaling axis. However, we found new downstream signaling pathway of E-Ras and it was JNK among several signaling molecules. Inhibition of JNK signaling even in the presence of E-Ras overexpression abolished iPS cell generation from somatic cell. This finding suggests that E-Ras is a key molecule to enhance reprogramming through JNK pathway.

4.2. Mechanism of E-Ras to regulate reprogramming: association between proliferation and reprogramming

E-Ras is not expressed in somatic cell or even in ES cell when they are differentiated. The most dramatic change after overexpression of E-Ras in somatic cell was cell-cycle progression and proliferation. One study suggested that normal proliferation of somatic cells is required for infection of Yamanaka factors and continuous rapid proliferation of somatic cells after viral infection is not help to induce the reprogramming (Xu et al. 2013). However, most researchers believe that a high proliferation rate required for establishment of human cell reprogramming and maintenance of embryonic stem cell identity. Because, many previous studies demonstrated that cell cycle arrest mediated through p21, p27 and INK4 is a barrier for cell reprogramming (Li et al. 2009; Hong et al. 2009; Ruiz et al. 2011) and the ectopic expression of CycD1, CycD2 and CycE increased reprogramming efficiency up to more than 2-fold compared to control (Ruiz et al. 2011). It has been proposed that in hES cells, lengthening of the average time spent in the G1, G2, and M phase increased propensity to differentiate (Ruiz et al. 2011; Lange and Calegari 2010). Collectively, fast cell cycle and high proliferation rate may prohibit differentiation potential and increase efficiency of somatic cell reprogramming. Since E-Ras increases cell proliferation and fast cell cycle through lengthening S phase and shortening G1, G2, it may influence efficiency of the reprogramming process.

4.3. Mechanism of E-Ras to regulate cell-cycle and cell proliferation

Other studies have noted that E-Ras affects cell proliferation, but its precise mechanism has not been understood. In this study we dissected the mechanism how E-Ras facilities cell cycle progression and proliferation. The most dramatic change after E-Ras expression in somatic cell



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was the increased transition from G1 to S phase, whose process is controlled by early cyclins, such as D and E. Interestingly, E-Ras did not increase later cyclins A and B which are expressed at later part of the cell cycle and regulate G2 to M transition (Fig. 4). This illustrates that E-Ras facilitates cell cycle mainly at the early phase. The question why E-Ras selectively increased cyclins D and E was solved by bioinformatics. We found that promoters of both cyclins D and E gene have Sp1 binding sites and confirmed that Sp1 was the common transcriptional activator for cyclins D and E by luciferase assay. Induction of cyclins D and E by E-Ras was obliterated by introduction of mutation at the sp1 binding sites of promoter. However, Sp1 binding element in the promoter of cyclins A and B is not reported. This signifies that Sp1 may link between E-Ras and G1-S cyclins, cyclins D and E. The amounts of phosphorylated Sp1 and total Sp1 in E-Ras over-expressed cells were increased more rapidly and dramatically compared to those of the control cells (Fig. 5). Interestingly, amount of total and phosphorylated form of Sp1 were decreased along with the phases of cell cycle, which corresponds to the established research data (Grinstein et al. 2002). Therefore, Sp1 could be a specific stimulator for regulating early cell cycle through cyclins D and E. Previous studies hypothesized that E-Ras activates only Akt pathway and increases cell proliferation, but it is not related with the cell cycle (Takahashi et al. 2003; Yu et al. 2014). In the present study, we investigated the missing link between E-Ras and Sp1 to regulate cell cycle. Since MEKK is one of major downstream messengers of Ras signaling pathway, we focused on MEKK family including ERK, p38, and JNK. JNK was the only kinase that was activated by stimulation of E-Ras. Pharmacological inhibitor of JNK attenuated the effects of E-Ras on cell cycle by down-regulating Sp1-cyclin D/E axis. In contrast, Akt inhibitor did not change the cell cycle in the present study (Fig. 6D), which was consistent with the other study (Takahashi et al. 2003). It signified that E-Ras augments the cell cycle progression through specific activation of JNK signaling. Thus the mechanism how E-Ras facilitates cell cycle is the sequential activation of JNK pathway, Sp1 transcription factor, increase of cyclins D and E, resulting in G1-S transition.

5. Conclusion

This study shows for the first time that E-Ras activates the JNK–Sp1 signaling pathway. Activated Sp1 binds to the cyclins D and E promoter and increases both gene and protein expression levels, resulting in an accelerated cell cycle and cell proliferation. Furthermore, we demonstrate that E-Ras promotes reprogramming of somatic cells by direct activation of JNK pathway.

Author contributions

No disclosure of potential conflicts of interest.

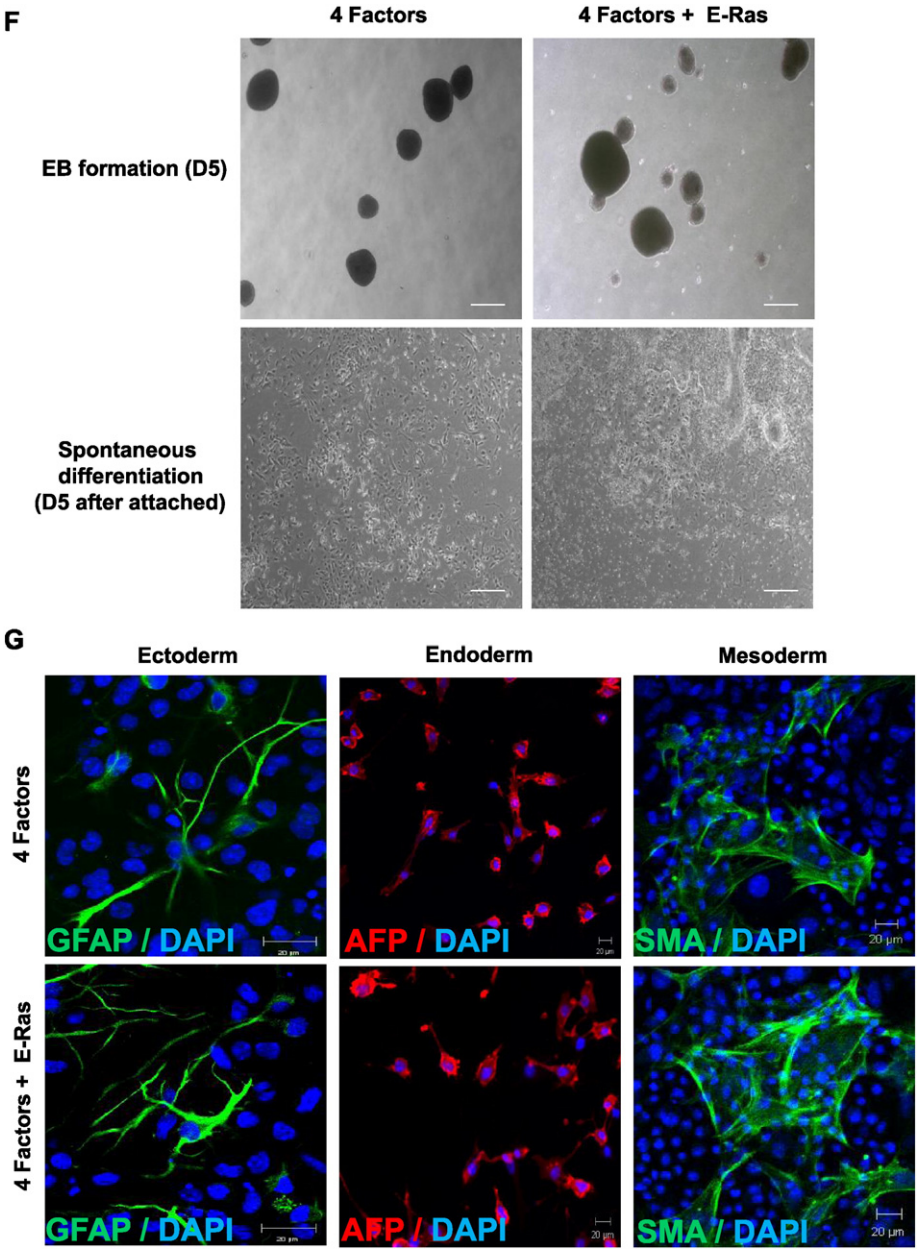

Fig. 7 (*continued*).

Acknowledgments

This study was supported by a grant from the Innovative Research Institute for Cell Therapy (A062260) and supported by the a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI14C1541and HI14C1277).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.09.004.

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