Biochemical and Biophysical Research Communications 431 (2013) 444–449

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Reprogramming of mouse fibroblasts into induced pluripotent stem cells with Nanog

Jai-Hee Moon [a,1], Wonjin Yun [a,1], Jihyun Kim [a], Solji Hyeon [a], Phil Jun Kang [a], Gyuman Park [a], Aeree Kim [b], Sejong Oh [c], Kwang Youn Whang [d], Dong-Wook Kim [e], Byung Sun Yoon [a], Seungkwon You [a,*]

[a] Laboratory of Cell Function Regulation, Division of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul, Republic of Korea
[b] Department of Pathology, College of Medicine, Korea University Guro Hospital, Seoul, Republic of Korea
[c] Division of Animal Science, Chonnam National University, Gwangju 500-757, Republic of Korea
[d] Division of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul, Republic of Korea
[e] Department of Physiology, Center for Cell Therapy, Yonsei University College of Medicine, Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 8 December 2012
Available online 16 January 2013

Keywords:
iPSCs
Nanog
Bmi1
Sonic hedgehog

ABSTRACT

Oct4–Sox2–Nanog transcriptional networks are critical for the maintenance of embryonic stem (ES) cell self-renewal and induction of pluripotency. However, in transcription factor-induced reprogramming of somatic cells into induced pluripotent stem cells (iPSCs), Nanog is initially dispensable and Oct4 remains the sole factor that could not be substituted/omitted. Here, we show that mouse fibroblasts could be reprogrammed into iPSCs by Nanog and Bmi1, which replaces Sox2, Klf4, and c-Myc, in the absence of Oct4. Furthermore, we show that in the presence of shh agonists (oxysterol and purmophamine), which replaces the function of Bmi1, a single transcription factor, Nanog is sufficient to reprogram mouse fibroblasts into iPSCs. Nanog-induced iPSCs resemble mESCs in terms of morphology, global gene expression profiles, epigenetic status and pluripotency both *in vitro* and *in vivo*. These findings support that Nanog can replace the Oct4 for the somatic cell reprogramming and underlie the mechanisms of Nanog in reprogramming process.

1. Introduction

Transcription factors are involved in the regulation of both embryonic stem cell (ESC) pluripotency and somatic cell reprogramming [2]. Indeed, the successful reprogramming of adult somatic cells into ES-like cells indicates that factors expressed in the latter may confer differentiation competency to the former [7]. In an elegant screen of 24 gene candidates selected for their link to ESC pluripotency, Yamanaka's group identified four factors (Oct4, Sox2, Klf4, and c-Myc) that were sufficient to reprogram mouse fibroblasts into induced pluripotent stem cells (iPSCs). Surprisingly, however, Nanog was not among this canonical quartet of transcription factors [8]. Moreover, the addition of Nanog to this quartet has not heretofore been shown to increase reprogramming efficiency. In fact, overexpression of Nanog in neural stem cells (NSCs) did not cause any obvious phenotypic changes, in contrast to overexpression of Oct4 [3]. On the other hand, NSCs with increased Nanog expression generated more hybrid colonies with ESC-like characteristics than did wild-type NSCs. Furthermore, Nanog is expressed either weakly or not at all in incompletely reprogrammed cells that fail to properly activate the endogenous pluripotency transcriptional circuitry, and selection or screening for activation of endogenous Nanog expression facilitates the isolation of fully reprogrammed iPSCs [8]. In addition, Nanog in combination with Oct4 and Sox2 facilitates molecular reprogramming of somatic cells [10], supporting its role in the generation of iPSCs.

This study examined the contribution of Nanog to somatic cell reprogramming in mice. The combination of Bmi1 and Oct4 [5] with Nanog (hereafter termed BON) was first introduced into mouse embryonic fibroblasts (MEFs). Overexpression of BON compared with the vector control (BO) increased the number of ESC-like colonies (Fig. 1A). Thus, Nanog apparently improved the reprogramming efficiency of MEFs.

Oct4 and Nanog are key regulators that are essential for the formation and/or maintenance of the inner cell mass (ICM) during mouse preimplantation development and for the self-renewal of pluripotent ESCs [4]. Therefore, the most likely mechanism for the Nanog-mediated maintenance of self-renewal and the undifferentiated state of ESCs is through the modulation of Oct4 and/or Sox2 levels. In support of this hypothesis, half of the genes bound by Oct4 are bound by Sox2 and more than 90% of the gene promoter regions bound by Oct4 and Sox2 are also bound by Nanog [1]. These results prompted our next investigation into

* Corresponding author. Fax: +82 2 3290 3507.
E-mail address: bioseung@korea.ac.kr (S. You).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.12.149

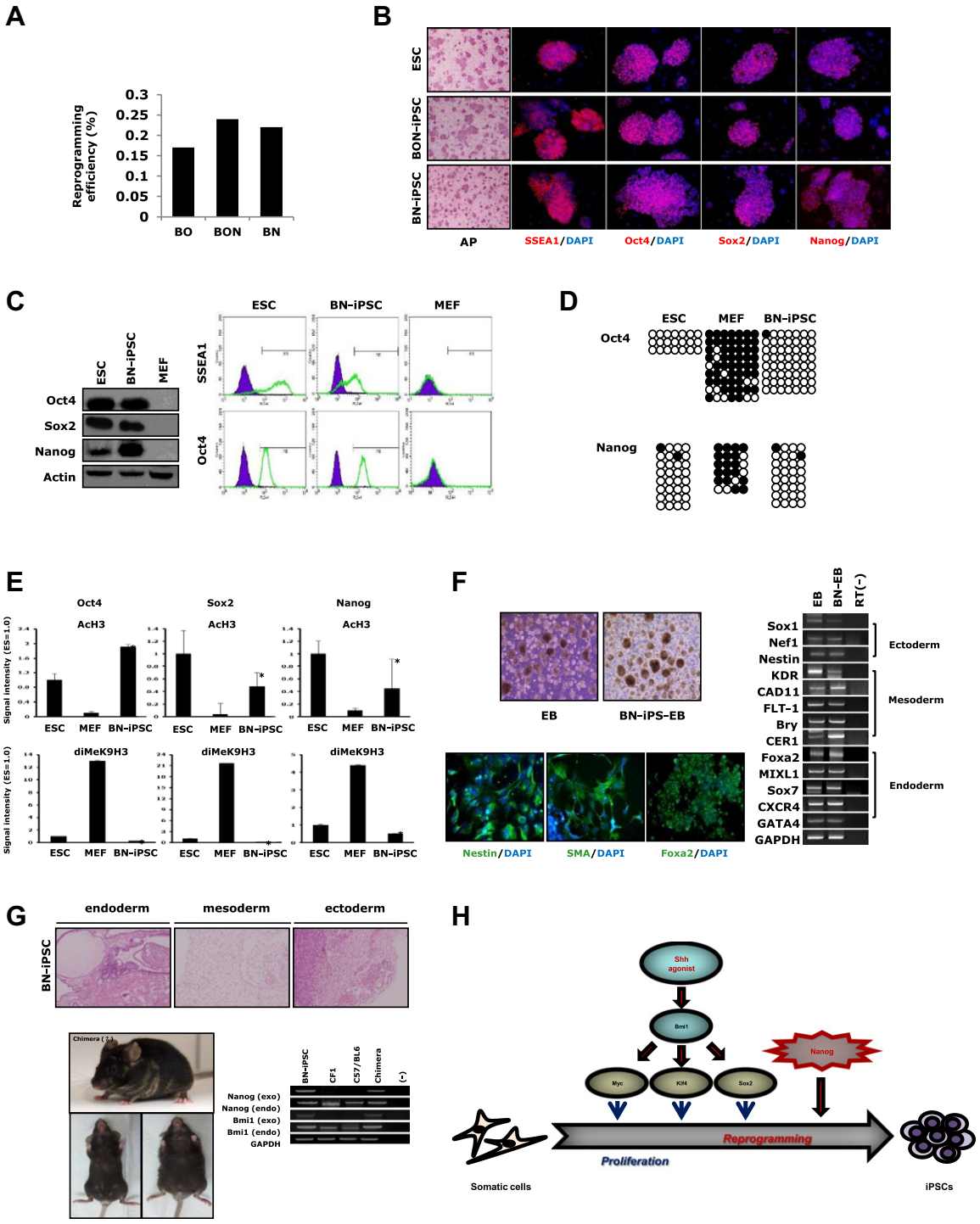

Fig. 1. Characterization of iPSCs generated from MEFs by retroviral transduction with Nanog. (A) Reprogramming efficiencies of transcription factor combinations for the induction of iPSCs (B, Bmi1; O, Oct4; N, Nanog). The reprogramming efficiency of each combination was quantified by determining the percentage of Nanog-positive colonies. (B) Characterization of mESCs (top), BON-iPSCs (middle), and BN-iPSCs (bottom) by alkaline phosphatase (AP) staining and immunofluorescence staining for the pluripotency markers SSEA1, Oct4, Sox2, and Nanog. Scale bars, 200 μm. (C) Western blotting (left) and FACS (right) analyses of mESC marker genes. (D) DNA methylation analysis of the promoter regions of the Oct4 and Nanog genes. Each row of circles for a given amplicon represents the methylation status of CpG dinucleotides for the corresponding region in one bacterial clone. Open and filled circles indicate unmethylated and methylated CpG dinucleotides, respectively, in the Oct4 and Nanog promoter regions for mESCs, parental MEFs, and BN-iPSCs (E) Dimethylation (lysine 9 of histone H3; diMeK9H3) and acetylation (histone H3; AcH3) status of the histone subunits of the Oct4, Sox2, and Nanog promoter regions. Data were analyzed by ChIP in mESCs, MEFs, and BN-iPSCs, and then quantified by qRT-PCR. Each value for MEFs and BN-iPSCs is the mean ± the SD and is shown relative to the corresponding value for mESCs ($n = 3$). $^{*}P < 0.05$ compared with MEFs. (F) *In vitro* differentiation of BN-iPSCs. RT-PCR (right panel) and immunostainimg (left panel) analyses of BN-iPSC-derived cells of all three germ layers are shown after EB differentiation. Scale bars, 200 μm. (G) *In vivo* differentiation of BN-iPSCs. BN-iPSC-derived teratomas differentiated into cells of all three germ layers (upper panel); a BN-iPSC-derived chimeric mouse and germline transmission of BN-iPSCs are also shown (lower panel). PCR was performed on genomic DNA to detect exogenous and endogenous transgene integration in BN-iPSC-derived chimeric mice. Scale bars, 200 μm. (H) Schematic diagram summarizing the present data.

whether Nanog could replace Oct4 and Sox2 during the reprogramming process.

2. Materials and methods

2.1. Cell culture and conditions

The mESCs and iPSCs were sub-cultured on mitomycin C-treated mouse (CF1 strain) feeder cell layers and maintained in mESC medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 0.1 mM β-mercaptoethanol, 1% penicillin–streptomycin, and 1000 units/ml leukemia inhibitory factor (LIF; Millipore). The cells were dispersed every 3–5 days with 0.05% trypsin–EDTA and passaged by seeding at a density of 2×10^5 cells/well in a 6-well plate. The MEFs and TTFs were cultured in DMEM containing 10% FBS [5]. All mouse experiments were approved by the Animal Care Committee at the College of Life Science and Biotechnology, Korea University, and were performed in accordance with government and institutional guidelines and regulations.

To induce reprogramming of MEFs into BN-iPSCs, MEFs (2×10^5) were infected with retroviral supernatants containing Bmi1 and Nanog and then cultured in mESC medium. Two days after the initial transduction, cells were sub-cultured on mitomycin C-treated mouse (CF1 strain) feeder layers and maintained in mESC medium. To induce reprogramming into OxyN-, PN-iPSCs, and TTF-derived OxyN-iPSCs, fibroblasts were cultured in NSC medium [5] containing either purmorphamine (1 μM; Calbiochem) or 25-hydroxycholesterol (oxysterol; 0.1 μM, 0.5 μM; Sigma Chemical Co.) for 1 day [5]. Next, retroviral supernatants containing Nanog were added to the cells along with purmorphamine or oxysterol in NSC medium containing 6 μg/ml polybrene. A third round of transduction was performed in the presence of Nanog, and the cells were incubated for another 2 days. Two days after the final transduction, the cells were transferred and grown on mitomycin C-treated mouse feeder layers in ESC medium. To quantify the efficiency of iPSC generation, the number of Nanog-positive colonies was counted and expressed as a percentage of the total number of infected cells.

2.2. RT-PCR and qRT-PCR analyses

For RT-PCR and qRT-PCR analyses, cDNA was amplified in triplicate with a reverse-transcription-negative blank of each sample and a no-template blank that served as the negative control [5]. Amplification curves and gene expression levels were normalized to those for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was used as an internal standard. The primers used for RT-PCR and qRT-PCR are listed in Supplementary Table S1.

2.3. Protein expression analysis

Protein expression was analyzed by Western blotting, FACS, and immunocytochemistry as described previously [5] with minor modifications for immunocytochemistry. Cells were then fixed, permeabilized, and incubated with primary antibodies (Supplementary Table S2). Alkaline phosphatase (AP) staining was performed by using the ESC Characterization Kit (Millipore) according to the manufacturer's instructions.

2.4. Genomic DNA methylation analysis

Isolation and sodium bisulfite conversion of genomic DNA isolated from MEFs, TTFs, ESCs, and iPSCs was performed by using the Genomic DNA Purification Kit (Promega) and the EpiTect Bisulfite Kit (Qiagen) [5]. The primers used for promoter fragment PCR amplification are listed in Supplementary Table S1.

2.5. Chromatin immunoprecipitation analysis coupled to qRT-PCR detection

Chromatin immunoprecipitation (ChIP) was performed on MEFs, TTFs, ESCs, and iPSCs by using anti-acetyl H3 and anti-dimethyl K9 H3 antibodies and the EZ ChIP Kit (Millipore), according to the manufacturer's instructions. The PCR primers used for qRT-PCR are listed in Supplementary Table S1.

2.6. In vitro and in vivo differentiation

For *in vitro* differentiation of iPSCs, single cells were obtained by trypsinization and cultured in suspension with EB formation medium (Millipore) without LIF for 7 days. Spontaneous differentiation was evaluated by immunostaining for representative lineage-specific markers with the indicated antibodies at various time points (5–7 days). For *in vivo* differentiation of iPSCs, cells (1×10^6 cells/mouse) were injected under the kidney capsule into the dorsal flank of Balb/c nude mice [5]. Tissue sections (5–6 μm thick) were cut and processed for hematoxylin-eosin staining.

For chimera formation, 4–5-weeks-old female mice (C57BL/6 N) were generated, as previously described [5]. Chimerism was ascertained by the appearance of an albino coat color (derived from the iPSCs) in black host pups. High-contribution chimeras were mated with C57BL/6 J mice to test for germline transmission. Genotyping was performed with genomic DNA isolated from iPSCs and the tails of chimeric and germline-transmitted offspring.

2.7. Statistical analysis

Data were analyzed by using analysis of variance (ANOVA) with the general linear model procedures of the Statistical Analysis System (SAS, 9.13 software package). Data were expressed as the means ± the SD, and $P < 0.05$ was considered statistically significant.

3. Results and discussion

When Bmi1 and/or Nanog were introduced into MEFs in the absence of Oct4 and Sox2, ESC-like colonies were successfully generated by the transduction of Bmi1 and Nanog together (BN), but not by Bmi1 or Nanog alone (data not shown). The estimated reprogramming efficiency of MEFs into BO-, BON-, and BN-iPSCs was 0.17%, 0.24%, and 0.22%, respectively (Fig. 1A). Established BON- and BN-iPSCs were similar to mouse embryonic stem cells (mESCs) in terms of their morphology and expression of ESC-specific markers under mESC culture conditions (Fig. 1B). Western blotting and fluorescence-activated cell sorting (FACS) analyses confirmed the protein expression of Oct4, Sox2, and Nanog in BN-iPSCs (Fig. 1C). The epigenetic status of BN-iPSCs in regard to the Oct4 and Nanog promoter regions was more similar to that of mESCs compared with the parental MEFs (Fig. 1D and E) consistent with the epigenetic remodeling that occurs during reprogramming.

BN-iPSCs exhibited the capacity to differentiate into cells of all three germ layers, as assessed by embryoid body (EB) and teratoma formation (Fig. 1F and G). Furthermore, BN-iPSCs were able to produce germline-competent adult chimeric mice (Fig. 1G), demonstrating that Nanog in combination with Bmi1 is sufficient to replace Oct4 and to reprogram mouse fibroblasts into iPSCs.

Importantly, RT-PCR and quantitative real-time PCR (qRT-PCR) analyses confirmed the reactivation and expression of endogenous

Oct4, Sox2, Nanog, c-Myc, Klf4, E-Ras, and Rex1 in the BN-iPSCs (Supplementary Fig. S1A). The expression of both Nanog and Bmi1 transgenes was efficiently silenced in the reprogrammed cells (Supplementary Fig. S1B).

We previously reported that the activation of sonic hedgehog (shh) signaling (by shh, oxysterol, or purmorphamine) compensates for the effects of Bmi1 and, together with Oct4, reprograms MEFs into iPSCs [5]. Therefore, we tested whether MEFs could be reprogrammed into iPSCs by the combination of Nanog and oxysterol, or Nanog and purmorphamine. Treatment of MEFs with either compound activated the shh pathway (data not shown) and enhanced the reprogramming of MEFs into iPSCs by forced expression of Nanog. The resulting iPSCs, obtained by one-factor (1F) combinations of Nanog and oxysterol or Nanog and purmorphamine, are hereafter termed OxyN- and PN-iPSCs. OxyN- and PN-iPSCs were next analyzed by the same tests as described above for BN-iPSCs. Like BN-iPSCs, OxyN- and PN-iPSCs were very similar to mESCs in all properties examined (Fig 2 and Supplementary Fig. S2).

We next investigated whether adult tail-tip fibroblasts (TTF) could be reprogrammed into iPSCs by using the same combination of oxysterol and Nanog that was used to reprogram MEFs. As a result, iPSC colonies were successfully generated, and two iPSC lines were established from the Nanog-induced adult fibroblasts (hereafter termed TTF-derived OxyN-iPSCs). The TTF-derived OxyN-iPSCs were also similar to mESCs (Fig. 3), demonstrating that mouse fibroblasts can be reprogrammed into pluripotent stem cells by Nanog in combination with Shh agonists.

Our study therefore showed that Nanog can enhance the reprogramming efficiency of mouse fibroblasts into iPSCs by forced expression of Bmi1 and Oct4. In addition, Nanog alone can replace the function of Oct4 and reprogram mouse fibroblasts into iPSCs in the presence of small molecules. OxyN-, PN-, and BN-iPSCs all resembled mESCs at the molecular level and with respect to their differentiation potential (see schematic diagram, Fig. 1H). These re-

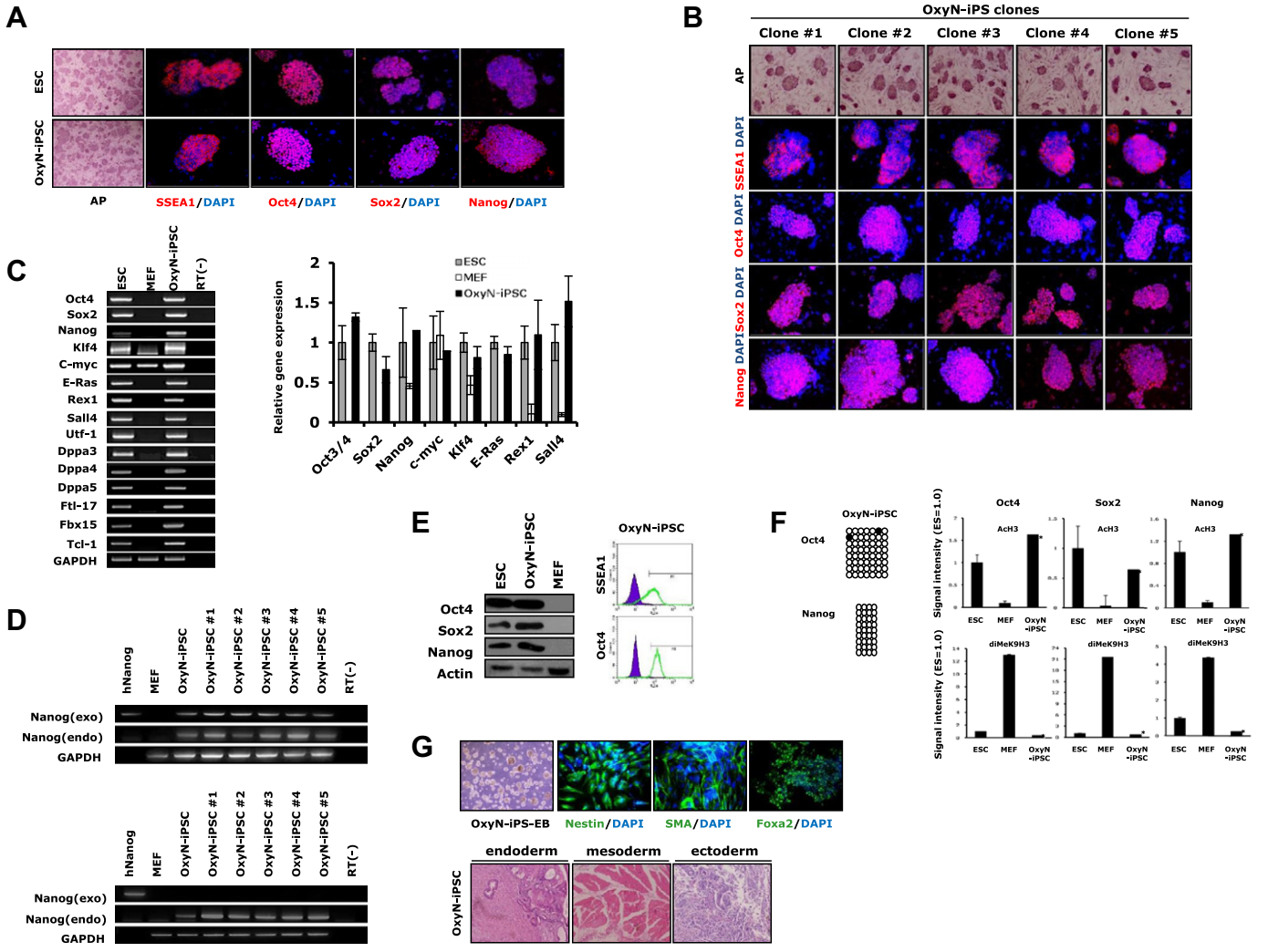

Fig. 2. Generation and characterization of OxyN-iPSCs. (A) and (B) AP staining and immunofluorescence detection of mESC marker genes. Scale bars, 200 μm. (C) RT-PCR and qRT-PCR analyses of mESC marker genes in OxyN-iPSCs. Each value is the mean ± the SD ($n = 3$). (D) PCR analysis of genomic DNA was performed to detect integration of exogenous Nanog in MEFs and OxyN-iPSCs (upper panel). hNanog was used as the positive control by using expression plasmid DNA. RT-PCR was performed to detect expression of exogenous Nanog transcripts in MEFs and OxyN-iPSCs (lower panel). (E) Western blotting and FACS analyses of ESC marker genes. (F) DNA methylation analysis of the Oct4 and Nanog gene promoter regions was performed by using bisulfite genomic sequencing (left panel). Open and closed circles indicate unmethylated and methylated CpG dinucleotides, respectively. Dimethylation (lysine 9 of histone H3; diMeK9H3) and acetylation (histone H3; AcH3) status of the histone subunits of the Oct4, Sox2, and Nanog promoter regions (right panel). Data were analyzed in mESCs, MEFs, and OxyN-iPSCs by ChIP and then quantified by qRT-PCR. Each value for MEFs and OxyN-iPSCs is the mean ± the SD and is shown relative to the corresponding value for mESCs ($n = 3$). $^{*}P < 0.05$ compared with MEFs. (G) *In vitro* and *in vivo* differentiation of OxyN-iPSCs as assessed by EB and teratoma formation, respectively. Immunofluorescence staining of EBs showed cell types that were positively stained for differentiation markers (upper panel). Hematoxylin–eosin staining of teratomas revealed cells of all three germ layers (lower panel). Scale bar, 200 μm.

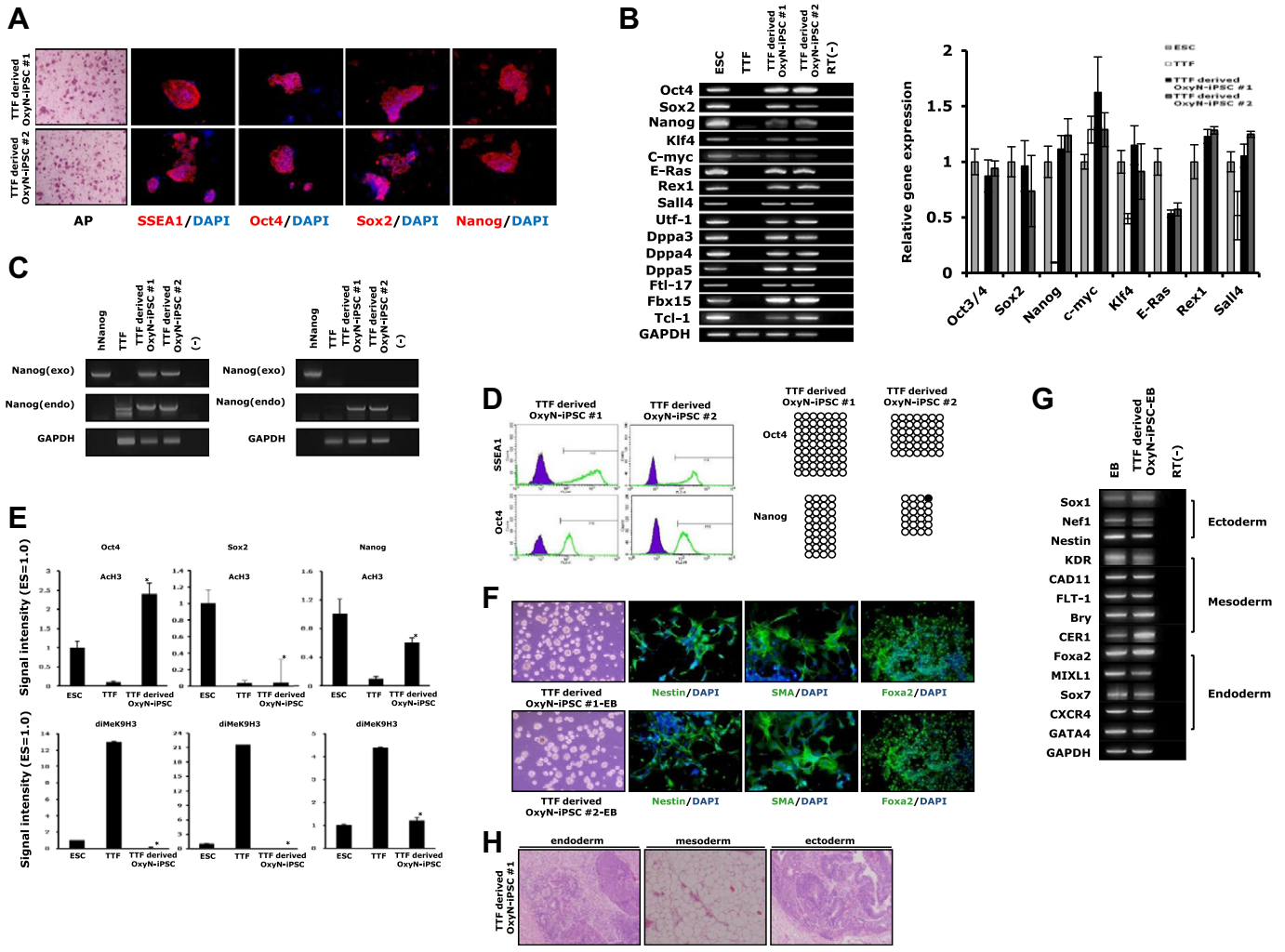

Fig. 3. Generation and characterization of TTF-derived OxyN-iPSCs. (A) OxyN-iPSCs generated from TTFs were stained for AP. Scale bars, 200 μm. Immunofluorescence staining of pluripotency and surface markers in TTF-derived OxyN-iPSCs. Nuclei were stained with DAPI (blue). Scale bars, 200 μm. (B) RT-PCR and qRT-PCR analyses for pluripotency markers in mESCs, TTFs, and TTF-derived OxyN-iPSCs. (C) PCR analysis of genomic DNA was performed to detect integration of exogenous Nanog in TTFs and TTF-derived OxyN-iPSCs (left). hNanog was used as the positive control by using expression plasmid DNA. RT-PCR was performed to detect expression of exogenous Nanog transcripts in TTFs and TTF-derived OxyN-iPSCs (right). (D) FACS analysis of mESC marker genes (left) and bisulfite sequencing analysis of Oct4 and Nanog promoter regions (right) in TTF-derived OxyN-iPSCs. Open circles represent unmethylated CpGs; closed circles represent methylated CpGs. (E) Dimethylation (lysine 9 of histone H3; diMeK9H3) and acetylation (histone H3; AcH3) status of the histone subunits of the Oct4, Sox2, and Nanog promoter regions. Data were analyzed by ChIP in mESCs, TTFs, and TTF-derived OxyN-iPSCs, and then quantified by qRT-PCR. Each value for TTFs and TTF-derived OxyN-iPSCs is the mean ± the SD and is shown relative to the corresponding value for mESCs ($n = 3$). $^{*}P < 0.05$ compared with TTFs. (F) Immunofluorescence staining of TTF-derived OxyN-iPSCs. Cells of all three germ layers were generated from the TTF-derived OxyN-iPSCs after EB differentiation. Nuclei were stained with DAPI (blue). Scale bars, 200 μm. (G) RT-PCR analysis of germ layer cells generated from TTF-derived OxyN-iPSCs after EB differentiation. Scale bars, 200 μm. (H) Teratoma formation at 8–10 weeks after transplantation of TTF-derived OxyN-iPSCs into Balb/c nude mice. Teratomas were sectioned and stained with hematoxylin-eosin. Histological sections are shown of identified cells representing all three germ layers. Scale bars, 200 μm.

sults are consistent with previous work demonstrating that Nanog is able to overcome multiple barriers to reprogramming [9], and that this effect is due to modulation of the pluripotency network co-regulated by Oct4, Nanog, and Sox2 [6].

Taken together, our findings represent an alternative mechanism for the reprogramming of somatic cells into iPSCs and suggest that Nanog is a key regulator of the reprogramming pathway. It is therefore important to understand how Nanog interacts with other reprogramming factors via the process of molecular communication. In this content, our study may help to unravel the role of Nanog during the induction of somatic cells into pluripotent cells.

Acknowledgments

This research was supported by a Grant from the Bio & Medical Technology Development Program of the National Research Foundation (NRF), funded by the Korean government (Ministry of Education, Science and Technology) (No. 2012-0006451), and by a Grant from the Korean Health Technology R&D Project, funded by the Ministry of Health and Welfare (No. A120392).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.149.

References

[1] L.A. Boyer, T.I. Lee, M.F. Cole, et al., Core transcriptional regulatory circuitry in human embryonic stem cells, Cell 122 (2005) 947–956.

[2] R. Jaenisch, R. Young, Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming, Cell 132 (4) (2008) 567–827.

[3] J.B. Kim, B. Greber, M.J. Arauzo-Bravo, et al., Direct reprogramming of human neural stem cells by OCT4, Nature 461 (2009) 643–649.

[4] K. Mitsui, Y. Tokuzawa, H. Itoh, et al., The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells, Cell 113 (2003) 631–642.
[5] J.H. Moon, J.S. Heo, J.S. Kim, et al., Reprogramming fibroblasts into induced pluripotent stem cells with Bmi1, Cell Res. 21 (2011) 1305–1315.
[6] G. Pan, J.A. Thomson, Nanog and transcriptional networks in embryonic stem cell pluripotency, Cell Res. 17 (2007) 42–49.
[7] M. Tada, Y. Takahama, K. Abe, et al., Nuclear reprogramming of somatic cells by *in vitro* hybridization with ES cells, Curr. Biol. 11 (2001) 1553–1558.
[8] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell 126 (2006) 663–676.
[9] T.W. Theunissen, A.L. van Oosten, G. Castelo-Branco, et al., Nanog overcomes reprogramming barriers and induces pluripotency in minimal conditions, Curr. Biol. 21 (2011) 65–71.
[10] J. Yu, M.A. Vodyanik, K. Smuga-Otto, et al., Induced pluripotent stem cell lines derived from human somatic cells, Science 318 (2007) 1917–1920.