Foxm1 transcription factor is required for maintenance of pluripotency of P19 embryonal carcinoma cells

Zhongqiu Xie, Guixiang Tan, Miao Ding, Difei Dong, Tuanhui Chen, Xiangxian Meng, Xiaoqin Huang and Yongjun Tan*

State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Biology, Hunan University, Changsha, Hunan 410082, China

ABSTRACT

Transcription factor Foxm1 plays a critical role during embryonic development and its expression is repressed during retinoic acid (RA)-induced differentiation of pluripotent P19 embryonal carcinoma cells at the early stage, correlated with downregulation of expression of pluripotency markers. To study whether Foxm1 participates in the maintenance of pluripotency of stem cells, we knock down Foxm1 expression in P19 cells and identify that Oct4 are regulated directly by Foxm1. Knockdown of Foxm1 also results in spontaneous differentiation of P19 cells to mesodermal derivatives, such as muscle and adipose tissues. Maintaining Foxm1 expression prevents the downregulation of pluripotency-related transcription factors such as Oct4 and Nanog during P19 cell differentiation. Furthermore, overexpression of FOXM1 alone in RA-differentiated P19 cells (4 days) or human newborn fibroblasts restarts the expression of pluripotent genes *Oct4*, *Nanog* and *Sox2*. Together, our results suggest a critical involvement of Foxm1 in maintenance of stem cell pluripotency.

INTRODUCTION

Pluripotent stem cells are undifferentiated cells that can give rise to several lineages of differentiated cell types (1). They are the founder cells for every organ, tissue and cell in the body. These cells are characterized by the ability to self-renew and maintain pluripotency, which allows them to fulfill their multiple functions, namely to provide enough cells during organogenesis, to control tissue homeostasis and, in addition, to ensure regeneration and repair. Among the well-established pluripotent cells, such as embryonic stem cells (ESCs) and embryonic germ cells (EGCs), embryonal carcinoma cells (ECCs) are derived from teratocarcinomas and have been well characterized as pluripotent cell lines that can be maintained as undifferentiated cells and induced under controlled conditions to differentiate *in vitro* to any cell type of all three germ layers (2), providing an attractive cell model system for studying pluripotent stem cells. The mouse P19 EC cell line was derived from a teratocarcinoma in C3H/He mice, produced by grafting an embryo at 7 days of gestation to testes of an adult male mouse (3). The cells contain a normal karyotype, predicting that the cells do not possess any gross genetic abnormalities. When injected into mouse blastocysts, P19 cells differentiate into a broad range of cell types in the resulting chimeras (4). P19 cells can differentiate *in vitro* into derivatives of all three germ layers depending on chemical treatment and growth conditions (5–7).

Understanding how pluripotency is regulated will have a large impact on developmental biology studies and regenerative medicine. Several transcription factors are required for maintenance of pluripotency, including Oct4 Sox2, and Nanog, and inactivation of these genes leads to loss of pluripotent stem cells and aberrant differentiation (8–11). Recently, overexpression of a cocktail of transcription factors (Oct4, Sox2, c-Myc and Klf4 or Oct4, Sox2, Lin-28 and Nanog) has resulted in the induction of pluripotency in somatic cells (12–14). These induced pluripotent stem cells (iPSCs) have all the properties of pluripotent cells, and the mechanism of this induction includes modification of epigenetics that mediate large-scale regulation of gene expression patterns (15). Growing evidence suggests that additional factors also contribute to the control of pluripotency (16,17) and identification of novel factors that are involved in maintenance of pluripotency is crucial and necessary for future application of pluripotent stem cell derivatives in regenerative

*To whom correspondence should be addressed. Tel/Fax: +86 731 88823211; Email: yjtan@hnu.cn; ytan1231@yahoo.com

medicine and for reprogramming of differentiated somatic cells.

Transcription factor Foxm1 belongs to the fork head/winged-helix family of transcription factors that play important roles in maintaining pluripotency (18,19), cellular proliferation and differentiation during embryonic development (20–23) and also play roles in cancer (24). Foxm1 is expressed in proliferating mammalian cells (25,26), where it regulates transcription of cell cycle genes critical for progression into DNA replication and mitosis(27–30). Foxm1 has been shown to be highly expressed in multipotent progenitor cells, such as proliferating neural stem cells in germinal regions of central nervous system (31,32) and progenitor cells of hair follicles (33), and to inhibit differentiation of the progenitors, implicating that Foxm1 plays in maintaining multipotent progenitor cells from divergent embryonic lineages. Although the functions of Foxm1 in pluripotent cells are not characterized, we have found in this study that pluripotent stem cells express Foxm1 and its expression is dramatically downregulated upon differentiation, suggesting that Foxm1 expression in pluripotent stem cells is functionally significant. Thus, Foxm1 appears to be a likely candidate for searching novel factors in controlling the pluripotency of stem cells.

In this study, we have investigated the possible role of Foxm1 in maintenance of pluripotency of P19 EC cells. We have shown that in response to retinoic acid (RA) treatment, P19 cells lose their pluripotency rapidly, evidenced by decreased expression of pluripotent stem cell markers, and Foxm1 expression is also repressed. The downregulation of Foxm1 protein levels happens before the decrease of Oct4 and Nanog upon differentiation. We have used an adenovirus-based vector to express Foxm1-specific siRNA to knock down Foxm1 expression in pluripotent P19 cells. The expression of Oct4 and Nanog are diminished by Foxm1 knockdown and the Oct4 promoter is regulated directly by Foxm1. Knockdown of Foxm1 in P19 cells also results in spontaneous differentiation of P19 cells to mesodermal derivatives, such as muscle and adipose tissues. We have used an adenovirus vector to maintain Foxm1 expression, which results in the prevention of Oct4 and Nanog downregulation during P19 cell differentiation. In differentiated cells, such as RA-differentiated P19 cells (4 days) or human newborn fibroblasts, overexpression of FOXM1 alone restarts the expression of *Oct4*, *Nanog* and *Sox2*. Together, our results suggest a critical involvement of Foxm1 in maintenance of stem cell pluripotency.

MATERIALS AND METHODS
Cell culture and RA-induced neural differentiation

The P19 EC cell line and human newborn fibroblasts was purchased from ATCC. Adenovirus-purification 293A cell line was purchased from Invitrogen. P19 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 7.5% calf serum, 2.5% fetal bovine serum (Gibco) and 0.5% penicillin streptomycin (Gibco) at $37^\circ C$ in 5% CO_2. 293A and human newborn fibroblasts were maintained in DMEM containing 10% fetal bovine serum. For RA-induced differentiation, P19 cell aggregates were formed by placing 3×10^6 cells in a 100 mm bacteriological dish (Petri dish) (Falcon) with addition of 5×10^{-7} M all-*trans*-RA (Sigma) for 4 days. Subsequently, the aggregates were replated on tissue culture dishes (Corning) for further differentiation.

Adenovirus purification, infection and siRNA treatment

The adenovirus-based vector AdFoxm1siRNA that expresses Foxm1-specific siRNA was constructed with Invitrogen Block-it Adenoviral RNAi Expression System (Cat. No K4941-00 and V492-20), following the manufacturer's instructions. A siRNA sequence 5′-GGA CCA CTT CCC TTA CTT T-3 from mouse Foxm1 cDNA was used to design a double-strand DNA that was recombinated into the adenovirus vector. The constructions of human FOXM1-expression adenovirus AdFOXM1 and control virus AdLacZ or AdGFP were described previously (34). The large-scale adenovirus purification and viral infections were carried out as previously described (35). For siRNA treatment, mOct4 siRNA (sc-36124) and control siRNA (sc-37007) were purchased from Santa Cruz. The siRNA transfection was performed according to the manufacturer's instructions.

Isolation of RNA, reverse transcription polymerase chain reaction and quantitative real-time PCR

Total RNA isolated from cell cultures were routinely used for reverse transcription polymerase chain reaction (RT-PCR) or quantitative real-time PCR (qPCR). Total RNA was prepared by using RNAprep Pure Cell/Bacteria Kits (Tiangen Biotech, China), following the manufacturer's instructions. For RT-PCR, the cDNAs were synthesized using RevertAid™ First Strand cDNA Synthesis Kits (Fermentas) with total RNA as templates. PCR amplification was performed with Taq DNA polymerase (Promega) with following sense (S) and antisense (AS) primers, annealing temperature (T_a) and number of PCR cycles (N): mFoxm1-S, 5′-CAC TTG GAT TGA GGA CCA CTT-3′ and mFoxm1-AS, 5′-GTC GTT TCT GCT GTG ATT CC-3′ (T_a: 57.5°C N: 35); mOct4-S, 5′-CAC TTT GGC ACC CCA GGC TA-3′ and mOct4-AS, 5′-GCC TTG GCT CAC AGC ATC CC-3′ (T_a: 58°C, N: 30); mSox2-S, 5′-TGA CCA GCT CGC AGA CCT AC-3′ and mSox2-AS 5′-GGA GGA AGA GGT AAC CAC GG -3′ (T_a: 55°C, N: 35); mFgf4-S, 5′-CAC GCG GCA CGC AGA ATT GG-3′ and mFgf4-AS, 5′-ATG CTC ACC ACG CCT CGC TG-3′ (T_a: 60°C, N: 30); mCripto-S, 5′-CAT GGC ACC TGG CTG CCC AA-3′ and mCripto-AS, 5′-GGC AGG CGC CAG CTA GCA TA-3′ (T_a: 60°C, N: 30); mEras -S, 5′-TGG GCG TCT TTG CTC TTG-3′ and mEras -AS 5′-TCG GGT CTT CTT GCT TGA TT-3′ (T_a: 62°C, N: 35); mUtf1-S, 5′-CCG TCG CTA CAA GTT CCT CA-3′ and mUtf1-AS, 5′-GCA GCA ACG CGG TAT TCA-3′ (T_a: 58.5°C, N: 30); mEsg1-S, 5′-TGG TGA CCC TCG TGA CCC GT-3′ and mEsg1-AS, 5′-ACA TGG CCT GGC TCA CCT GC-3′ (T_a: 63°C, N: 30); mRex1-S,

5′-GGG TGC AAG AAG AAG CTG AG-3′ and mRex1-AS, 5′-GCG TGG GTT AGG ATG TGA AT-3′ (T_a: 57.5°C, N: 30); mCyclophilin-S, 5′-GGC AAA TGC TGG ACC AAA CAC-3′ and mCyclophilin-AS, 5′-TTC CTG GAC CCA AAA CGC TC-3′ (T_a: 57.5°C, N: 22); hFOXM1-S, 5′-GGA GGA AAT GCC ACA CTT AGC G-3′ and hFOXM1-AS, 5′-TAG GAC TTC TTG GGT CTT GGG GTG-3′ (T_a: 55.7°C, N: 35); hOct4-S, 5′-AAG CGA TCA AGC AGC GAC TAT-3′ and hOct4-AS, 5′-GGA AAG GGA CCG AGG AGTA CA-3′ (T_a: 60°C, N: 30); hNanog-S, 5′-CAA AGG CAA ACA ACC CAC TT-3′ and hNanog-AS, 5′-TCT GCT GGA GGC TGA GGT AT-3′ (T_a: 60°C, N: 30); hSox2-S, 5′-ACC TAC AGC ATG TCC TAC TC-3′ and hSox2-AS, 5′-CAT GCT GTT TCT TAC TCT CCT C-3′ (T_a: 60°C, N: 32); and hCyclophilin-S, 5′-GCA GAC AAG GTC CCA AAG ACA G-3′ and hCyclophilin-AS, 5′-CAC CCT GAC ACA TAA ACC CTG G-3′ (T_a: 55.7°C, N: 22). For qPCR, two samples were collected at each time point. Each sample was analyzed in triplicate with cyclophline as the inner control by real-time PCR with SYBR Green Supermix (Bio-Rad, USA). Amplification data were collected by Mastercycler ep realplex and analyzed by the realplex2.0 software (Eppendorf).

Western blot analysis and flow cytometry

To measure protein levels, cell lysates were resolved by denaturing gel electrophoresis before electrotransfer to Protran nitrocellulose membrane. The membrane was subjected to western blot analysis with antibodies against proteins of interest as described previously (36). The signals from the primary antibody were amplified by horse radish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (Bio-Rad, Hercules, CA, USA), and detected with Enhanced Chemiluminescence Plus (ECL-plus, Amersham Pharmacia Biotech, Piscataway, NJ, USA). The following antibodies and dilutions were used for western blotting: rabbit anti-Foxm1 (1:500; abcam ab47808), rabbit anti-Oct4 (1:500; Chemicon AB3209), rabbit anti-Nanog (1:2500; Chemicon AB9220), rabbit anti-Sox2 (1:1000; abcam ab59776), rabbit anti-Nestin (1:2500; Chemicon AB5922), mouse anti-Tubulin βIII (1:1000; Chemicon MAB1637) and mouse anti-β-Actin (1:10000; Sigma AC-15). For flow cytometry, cells at the certain time point were dissociated with 0.025% trypsin and 10^7 cells were incubated with 10 μl of antibody against protein of interest in 100 μl buffer (0.5% BSA, 2 mM EDTA, 1X PBS) for 10 min at 4°C. The cells were washed by adding 2 ml buffer and centrifuged 10 min ($300\,g$) and resuspended in 500 μl buffer. Samples were analyzed for flow cytometry on FACSCalibur (BD Biosciences) with SSEA-1-PE antibody (R&D Systems FAB2155P).

Teratoma formation

Cells (1.5×10^6 cells/mouse) were injected subcutaneously into the dorsal flank of 6-week-old male nude mice. Three weeks after the injection, teratomas that had formed were fixed overnight in 4% PFA and embedded in paraffin. Sections were stained with hematoxylin and eosin dyes.

Alkaline phosphatase staining

Cells were fixed with 50% acetone and 50% methanol at room temperature for 2 min and stained using an alkaline phosphatase (ALP) staining kit (Vector Laboratories Burlingame) according to a standard protocol.

Chromatin immunoprecipitation assays and cotransfection assays

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (35). For immunoprecipitation, 2 μl of rabbit anti-FOXM1 serum (27) or control serum was used. The ChIP DNA sample or 5% total input was used in PCR reaction with the following primers: mOct4 promoter −3155 bp forward: 5′-GCC TTG GAC CTT TGT TCT TAT CAC-3′ and mOct4 promoter −2985 bp backward: 5′-TCT GTC TCG GAG TTC TGT CTG GAG-3′ (T_a: 56°C, N: 35); and mOct4 promoter −1335 bp forward: 5′-GGA GCA GAC AGA CAA ACA CCA TC-3′ and mOct4 promoter −1148 bp backward: 5′-TGG CGG AAA GAC ACT AAG GAG AC-3′ (T_a: 62.5°C, N: 35).

For cotransfection assays, the mouse Oct4 promoter regions were PCR amplified from mouse genomic DNA with the following primers: mOct4 −1331 Mlu1: 5′-CGA CGC GTC AGA GCA TGG TGT AGG AGC A-3′ or mOct4-1249 Mlu1: 5′-CGA CGC GTT AAG CAC CAG GCC AGT AAT G-3′, and mOct4+29 BglII: 5′-GGA AGA TCT GTG GAA AGA CGG CTC ACC TA-3′, and cloned into the corresponding Mlu1 and BglII sites of the pGL3 basic Luciferase vector (Promega). The mouse Oct4 promoter −3550 to −2817 region was PCR amplified from mouse genomic DNA with the following primers: mOCT4 −3550 Mlu1: 5′-CGA CGC GTC TAA CAC GAG TGA TTT CCC TGC TC-3′ and mOct4-2817 Mlu1 backward: 5′-CGA CGC GTG GTG TCT GAA GTA CTT ACG-3′, and cloned into the corresponding Mlu1 site of pGL3-Oct4-1361-bp-promoter. The site-directed mutagenesis was performed by GenScript. P19 cells were transfected with 200 ng of either CMV-FOXM1 cDNA or CMV empty expression vectors and 1500 ng of the Luciferase reporter constructs containing different mouse Oct4 promoter regions using Lipofecter 2000 (Invitrogen). Protein extracts were prepared from transfected P19 cells at 48 h following DNA transfection and the Dual-Luciferase Assay System (Promega) was used to measure Luciferase enzyme activity following the manufacturer's instructions.

Statistical analysis

We used Microsoft Excel Program to calculate SD and statistically significant differences between samples using the Student's t-test. The asterisks in each graph indicate statistically significant changes with P-values calculated by Student's t-test: *$P < 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$. P-values < 0.05 were considered statistically significant.

RESULTS

To confirm that P19 EC cells are pluripotent stem cells, we tested whether the cells were capable of forming teratomas upon subcutaneous inoculation into nude mice. The teratomas formed by P19 cells contained tissues of all three germ layers including neural rosette (ectoderm), cuboidal epithelium (endoderm) and muscle (mesoderm) (Supplementary Figure S1A), revealing that P19 cells possess the pluripotency. P19 cells were known to differentiate to neural cells by RA treatment in vitro when aggregated to form embryoid bodies (EBs) (5,7). We confirmed that RA-induced P19 cell neural differentiation displayed at least three phases: the first phase in which P19 cells lost their pluripotency, marked by decrease of alkaline phosphatase activity (Supplementary Figure S1B) and disappearance of the expression of the pluripotency marker Nanog at RA induction (Supplementary Figure S1C); the second phase in which certain percentage of P19 cells committed to the neural differentiation and neural stem-like cells appeared, evidenced by elevated expression of neural stem cell marker Nestin that peaked at Day 3 after RA treatment (Supplementary Figure S1C) and increased cell populations positive with neural stem cell-surface marker Prominin-1 (homolog of human CD133) (Supplementary Figure S1D); and the terminal differentiation phase (6–7 days following RA treatment) in which differentiated neuronal cell populations appeared, evidenced by elevated expression of neuron-specific marker NeuroD1 and Tubulin βIII (Supplementary Figure S1C and S1E-K).

Foxm1 is repressed at early time points during differentiation and knockdown of Foxm1 results in loss of pluripotency of P19 cells

Transcription factor Foxm1 highly expressed in P19 pluripotent cells and its expression decreased dramatically between Day 1 to Day 2 following RA treatment (Figure 1A and B), suggesting the reduction of Foxm1 was one of the early events during differentiation of the pluripotent cells and Foxm1 might participate in the maintenance of P19 cell pluripotency. The reduction of Foxm1 expression post RA treatment was correlated with the decreased expression of many of the known pluripotency-related genes, such as Oct4, Nanog, Sox2, Fgf4, Cripto and Utf1 (Figure 1A and B and Supplementary Figure S2). Interestingly, the downregulation of Foxm1 occurred before the decrease of Oct4 expression during differentiation, implicating that Foxm1 might be one of the upstream controlling factors of Oct4 expression in the maintenance of stem cell pluripotency. This idea was further confirmed by Foxm1 knockdown experiments, in which the infection of AdFoxm1siRNA resulted in the decreased activity of alkaline phosphatase in P19 cells (Figure 1C). Control adenovirus infections determined that almost 100% of the cells were infected with the viral dosage at 10 plaque-forming units (pfu)/cell (35) and the infection of AdFoxm1siRNA could knockdown the Foxm1 mRNA expression around 95% (Figure 1D, qPCR). The decreased expression of Foxm1 resulted in

downregulation of levels of cell cycle-related genes such as Cyclin D1, Skp2, Cdc25B and Cdk1 and consequently the decrease of proliferation rate in P19 cells (Supplementary Figure 3S) as expected because Foxm1 was found to stimulate transcription of cell cycle genes critical for progression into DNA replication and mitosis (27–30). On the other hand, knockdown of Foxm1 does not affect survival of P19 cells (Supplementary Figure S3 and Figure 3C). The inhibition of Foxm1 expression by AdFoxm1siRNA also abolished the expression of Oct4 and Nanog in P19 cells (Figure 1D and E), implicating that the levels of Oct4 and Nanog in pluripotent stem cells rely on the Foxm1 expression. Taken together, our data reveal that Foxm1 highly expressed in the undifferentiated P19 pluripotent cells and was downregulated by the differentiation process. Importantly, inhibition of its expression caused the loss of pluripotency of P19 cells, implying its role in maintaining the pluripotency of stem cells.

Knockdown of Foxm1 resulted in spontaneous mesodermal differentiation of P19 cells

To test whether knockdown of Foxm1 promoted spontaneous differentiation in P19 cells, we analyzed the expression levels of differentiation markers in AdFoxm1siRNA-infected P19 cells, including Map2 (ectoderm), Brachyury (mesoderm) and Gata4 (endoderm). Without obvious effects on the expression of Map2 and Brachyury (data not shown), knockdown of Foxm1 in P19 cells resulted in a rapid increase of Gata4 expression (Figure 2A). Gata4 was known to be one of the major transcription factors that stimulated cardiogenesis during development (37–39) even though it was frequently used as a marker for endodermal derivatives during differentiation of pluripotent stem cells (40). Flow cytometry was performed to measure the expression of SSEA-1, which highly expresses in mouse pluripotent stem cells, in P19 cells and AdFoxm1siRNA-infected P19 cells (3d). We used mouse embryonic fibroblasts as negative control cells and mouse ES cells as positive control cells. AdFoxm1siRNA infection resulted in significant decrease of SSEA-1 in P19 cells (Figure 2B). We performed the teratoma formation in vivo with AdFoxm1siRNA-infected P19 cells and found that P19 cells with decreased levels of Foxm1 produced significantly smaller teratomas (Figure 2C). Compared with the control P19 cell-formed teratomas, which expressed all three germ layer differentiation markers, teratomas formed with AdFoxm1siRNA-infected P19 cells only expressed mesodermal marker Brachyury (Figure 2D). Moreover, the expression of cardiac muscle-specific Actc1 was detected in AdFoxm1siRNA-infected teratomas, implicating that inhibition of Foxm1 limited the multipotentials of P19 cell differentiation and caused P19 cell spontaneous differentiation to cardiomyocytes in vivo. This possibility was further confirmed by hematoxylin and eosin-stained sections from teratomas of AdFoxm1siRNA-infected P19 cells, in which only mesodermal derivatives such as muscle and adipose connective tissues were observed (Figure 2E).
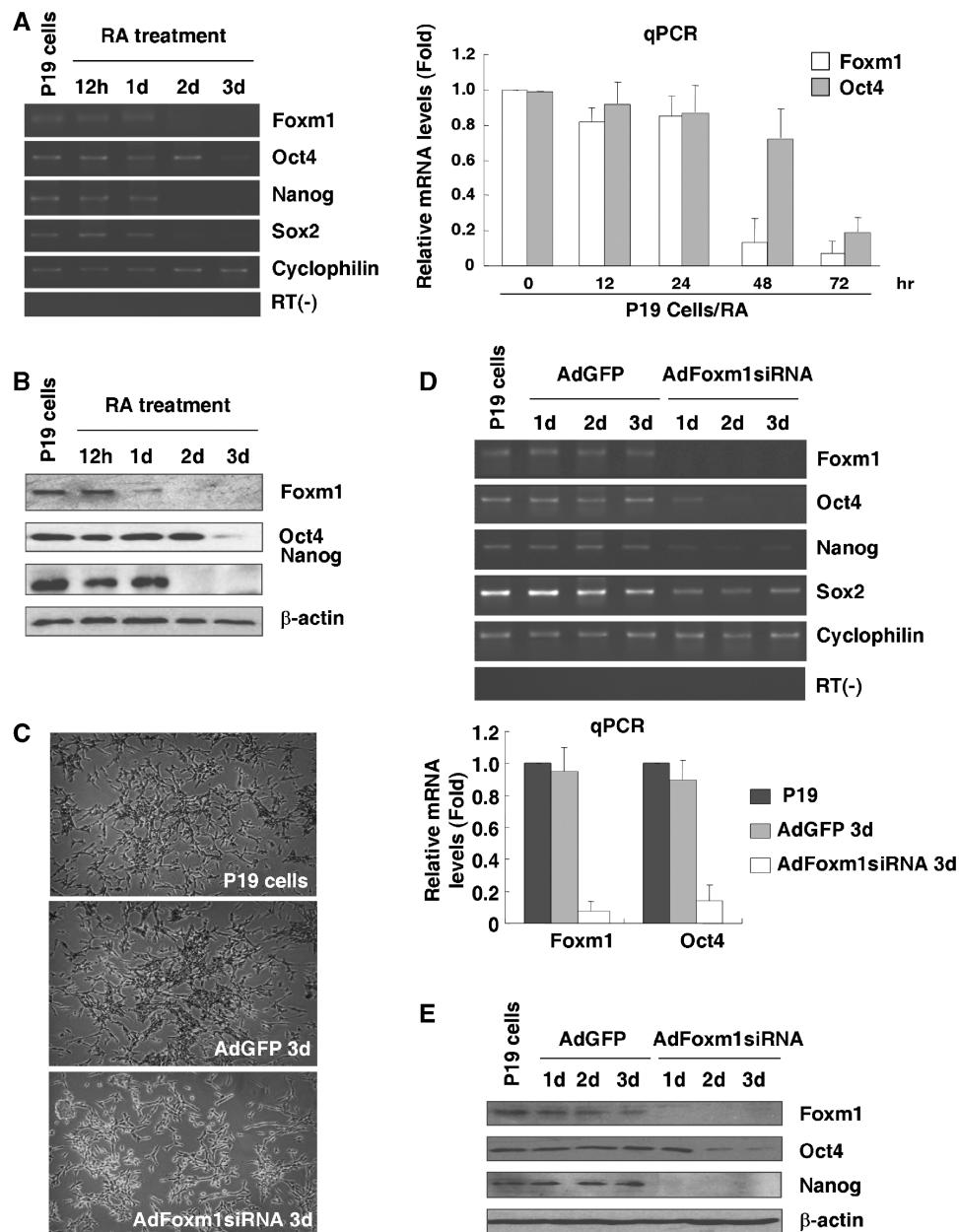
Figure 1. The expression of Foxm1 was repressed during RA-induced P19 cell differentiation and knockdown of Foxm1 decreased alkaline phosphatase activity and the expression of pluripotency genes. (**A** and **B**) Gene expression analysis of Foxm1, Oct4, Nanog and Sox2 during RA-induced differentiation of P19 cells by RT-PCR or quantitative real-time PCR (qPCR) (A) or western blotting (B). Cyclophilin or β-actin was used as the loading control. (**C**) P19 cells, P19 cells infected with AdGFP control virus or AdFoxm1siRNA virus (10 pfu/cell, 3 days) were stained for alkaline phosphatase. (**D** and **E**) Knockdown of Foxm1 decreased the expression of pluripotency markers in P19 cells. P19 cells were infected with AdGFP or AdFoxm1siRNA (10 pfu/cell) and gene expression analysis of Foxm1, Oct4, Nanog and Sox2 was performed by RT-PCR or qPCR (D) or western blotting (E).

Foxm1 binds to and stimulates Oct4 promoter

To test whether Foxm1 was one of the regulators of Oct4 transcription, we scanned $-5\,\mathrm{kb}$ promoter region of mouse Oct4 gene with the Foxm1 DNA binding consensus sequence, and found multiple tandem Foxm1 putative binding sites at the regions of -3434 to $-3415\,\mathrm{bp}$, -2895 to $-2880\,\mathrm{bp}$ and -1328 to $-1313\,\mathrm{bp}$ in the Oct4 promoter (Figure 3A). We used ChIP assays to determine the Oct4 promoter regions that mediate Foxm1 binding to endogenous Oct4 promoter at physiological conditions. The chromatin of P19 cells or RA-induced (3d) P19 cells was cross-linked, sonicated to DNA fragments of 500–1000 nt in length, and then immunoprecipitated (IP) with either rabbit Foxm1 antiserum or rabbit serum (control). The amount of promoter DNA associated with the IP chromatin was quantitated by RT-PCR with primers specific to Oct4 promoter region $-3155\,\mathrm{bp}$ to $-2985\,\mathrm{bp}$ or $-1335\,\mathrm{bp}$ to $-1148\,\mathrm{bp}$. Compared to the RA treated
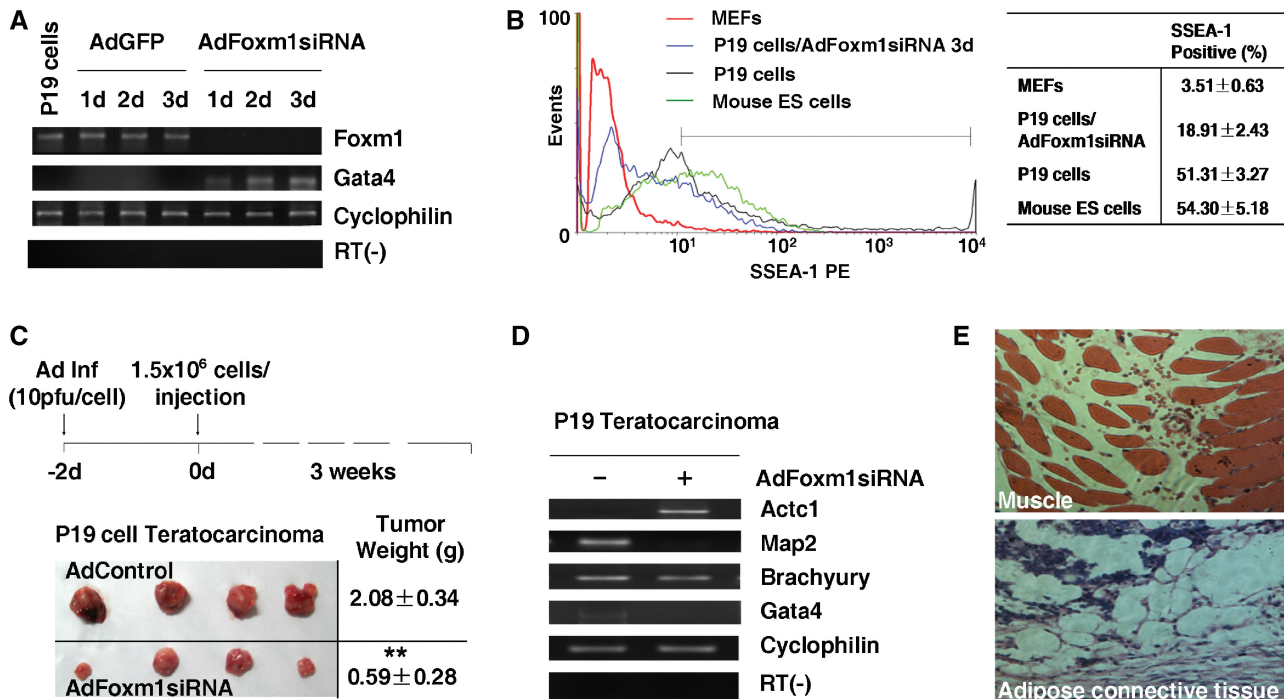
Figure 2. Knockdown of Foxm1 resulted in spontaneous mesodermal differentiation of P19 cells. (**A**) Knockdown of Foxm1 increased the expression of differentiation marker Gata4 in P19 cells. P19 cells were infected with AdGFP or AdFoxm1siRNA (10 pfu/cell) and gene expression analysis of Foxm1 and Gata4 was performed by RT-PCR. (**B**) Knockdown of Foxm1 resulted in significant decrease of SSEA-1 in P19 cells. Flow cytometry was used to measure cell populations of SSEA-1 in P19 cells and AdFoxm1siRNA-infected P19 cells (3d). MEFs were used as negative control cells and mouse ES cells were used as positive control cells. (**C**) Teratoma formation was abrogated in P19 cells with decreased levels of Foxm1. Teratomas collected from the nude mice injected with Adcontrol or AdFoxm1siRNA-infected P19 cells are shown. (**D**) Teratomas formed with AdFoxm1siRNA-infected P19 cells expressed mesodermal marker Brachyury and cardiac muscle-specific Actc1. The total RNAs isolated from teratomas of P19 cells or AdFoxm1siRNA-infected P19 cells were analyzed for selected genes by RT-PCR. (**E**) Knockdown of Foxm1 in P19 cells resulted in differentiation of mesodermal derivatives in teratoma formation. Hematoxylin and eosin-stained sections from teratomas of AdFoxm1siRNA-infected P19 cells after 3 weeks were shown.

samples that showed no Foxm1 specific binding, P19 pluripotent cell samples showed obvious binding activities of Foxm1 on the Oct4 promoter region around -3 kb but not the -1.3 kb region (Figure 3B). These results confirmed that Foxm1 bound directly to endogenous Oct4 promoter at -3 kb upstream region in pluripotent stem cells. To test whether Foxm1 activates Oct4 promoter, luciferase reporter plasmids with different length of mouse Oct4 promoter regions were constructed and transfected into P19 cells with the CMV-Foxm1 expression vector or a CMV empty expression vector. Luciferase enzyme activity was analyzed following transfection. Cotransfection of Foxm1 expression vector caused a significant increase in -3 kb Oct4 promoter activity (Figure 3C). The activities of Oct4 promoters with different length were inhibited at different levels during RA-induced P19 cell differentiation (Figure 3D). Furthermore, only the activities of the Oct4 promoter that binds Foxm1 were affected significantly by Foxm1 depletion (Figure 3E). To further prove that Oct4 is a *bona fide* target for Foxm1, we generated point mutations on the Foxm1 putative binding site A and B, and found that the luciferase activities of mutated promoters were decreased significantly compared with that of the intact promoter when cotransfected with the Foxm1 expression plasmid (Figure 3F). Together, these results demonstrated

that the Oct4 gene was one of the direct transcriptional targets of Foxm1 in pluripotent stem cells.

Maintained expression of FOXM1 prevented the downregulation of pluripotency marker expression during P19 cell differentiation

In order to determine the effects of maintained Foxm1 expression during differentiation of P19 cells, we used an adenovirus vector AdFOXM1 (34) constructed with human FOXM1 cDNA to prevent the downregulation of Foxm1 protein post RA treatment. AdFOXM1 infection resulted in efficient expression of exogenous human FOXM1, with no effects on the expression of endogenous Foxm1 in P19 cells (Figure 4A). Then, P19 cells infected with AdFOXM1 were induced to differentiation according to the standard protocol one day after viral infection. Maintained expression of FOXM1 prevented the decrease of mRNA levels of pluripotency genes such as Oct4, Nanog, Sox2 and endogenous Foxm1 that were downregulated by differentiation in P19 cells or control AdGFP-infected P19 cells (Figure 4B). The analysis of Oct4 protein levels confirmed the prevention of Oct4 protein decrease during P19 cell differentiation by AdFOXM1 infection (Figure 4C). Next, we studied the effects of re-expression of FOXM1 in differentiated P19 cells. P19 cell EBs were treated with RA for 4 days till the
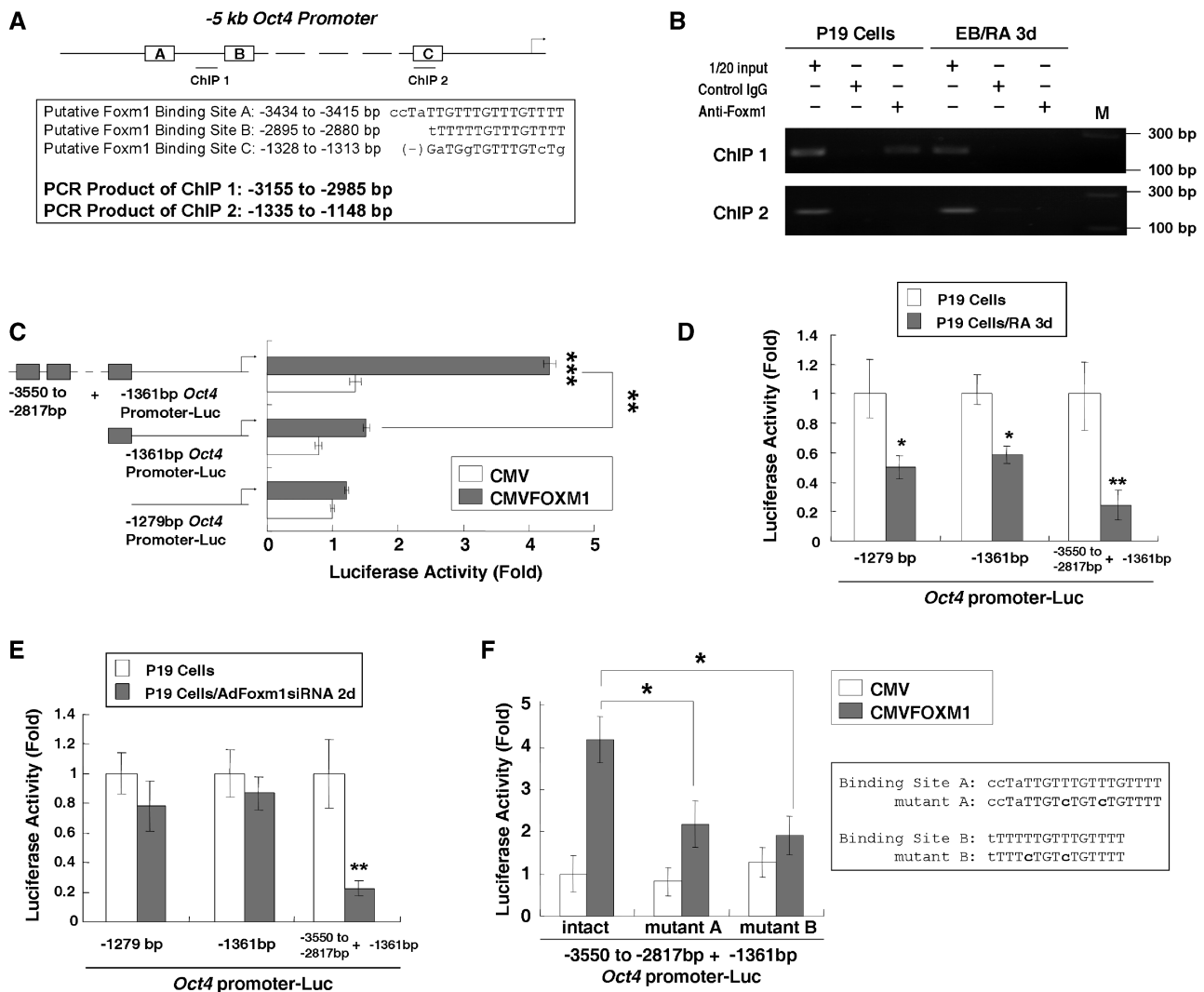
Figure 3. Foxm1 binds to and stimulates Oct4 promoter. (**A**) The predicted positions of putative Foxm1 binding sites in −5 kb mouse Oct4 promoter by gene sequence analysis and the positions of primers designed for ChIP assays. (**B**) FoxA1 bound to endogenous Oct4 promoter. ChIP assays were used to show direct binding of Foxm1 to endogenous Oct4 promoter regions. The chromatin of P19 cells or RA-induced (3d) P19 cells was cross-linked, sonicated, and immunoprecipitated (IP) with either Foxm1 antiserum or rabbit serum (control) and the amount of promoter DNA associated with the IP chromatin was quantitated by RT–PCR with primers specific to different Oct4 promoter regions. The predicted size of the PCR product was 170 bp (ChIP1) or 187 bp (ChIP2). (**C**) The −3-kb region of Oct4 promoter mediated the transcription activity of Foxm1. The Oct4 promoters with different length were constructed into luciferase reporter plasmid. The different reporter plasmid (1.5 μg) and loading control pRL-CMV luciferase reporter plasmid (20 ng) were transfected into P19 cells with the CMV-FOXM1 expression vector (200 ng) or a CMV empty expression vector (200 ng). Protein lysates were prepared at 24 h following transfection, and used to measure dual Luciferase enzyme activity. (**D**) The activities of Oct4 promoters with different length were inhibited at different degree during RA-induced P19 cell differentiation. The different reporter plasmid (1.5 μg) was transfected into P19 cells, followed by RA-induced differentiation. Three days later, the luciferase activities were measured. (**E**) Only the Oct4 promoter that binds Foxm1 was inhibited by Foxm1 depletion. The different reporter plasmid (1.5 μg) was transfected into P19 cells, followed by AdFoxm1siRNA infection. Two days later, the luciferase activities were measured. (**F**) Point mutations on Foxm1 binding sites of the −3 kb Oct4 promoter inhibited Foxm1-mediated transcription activity. Intact or mutated −3 kb Oct4 promoter-luciferase reporter plasmids (1.5 μg) were transfected into P19 cells with the CMV-FOXM1 expression vector (200 ng). The luciferase activities were measured at 24 h following transfection. The asterisks indicate statistically significant changes: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

cells lost the pluripotency and consequently the expression of Oct4, Nanog and Sox2. The differentiated cells were infected with AdFOXM1 or control AdGFP and the expression analysis of selected genes were performed. We found that the expression of exogenous FOXM1 in the differentiated P19 cells (RA/EB 4 day) started the expression of Oct4, Nanog, Sox2 and endogenous Foxm1 (Figure 4D). The analysis of Oct4 and Nanog protein

levels further confirmed the results (Figure 4E). Flow cytometry was performed to measure the SSEA-1 expression in the differentiated P19 cells infected with AdFOXM1 or AdGFP. The differentiated P19 cells (RA 4d) were used as negative control cells. We found that the AdFOXM1 infection in differentiated P19 cells resulted in significant increase of SSEA-1 (Figure 4F). The staining of alkaline phosphatase was performed to show that
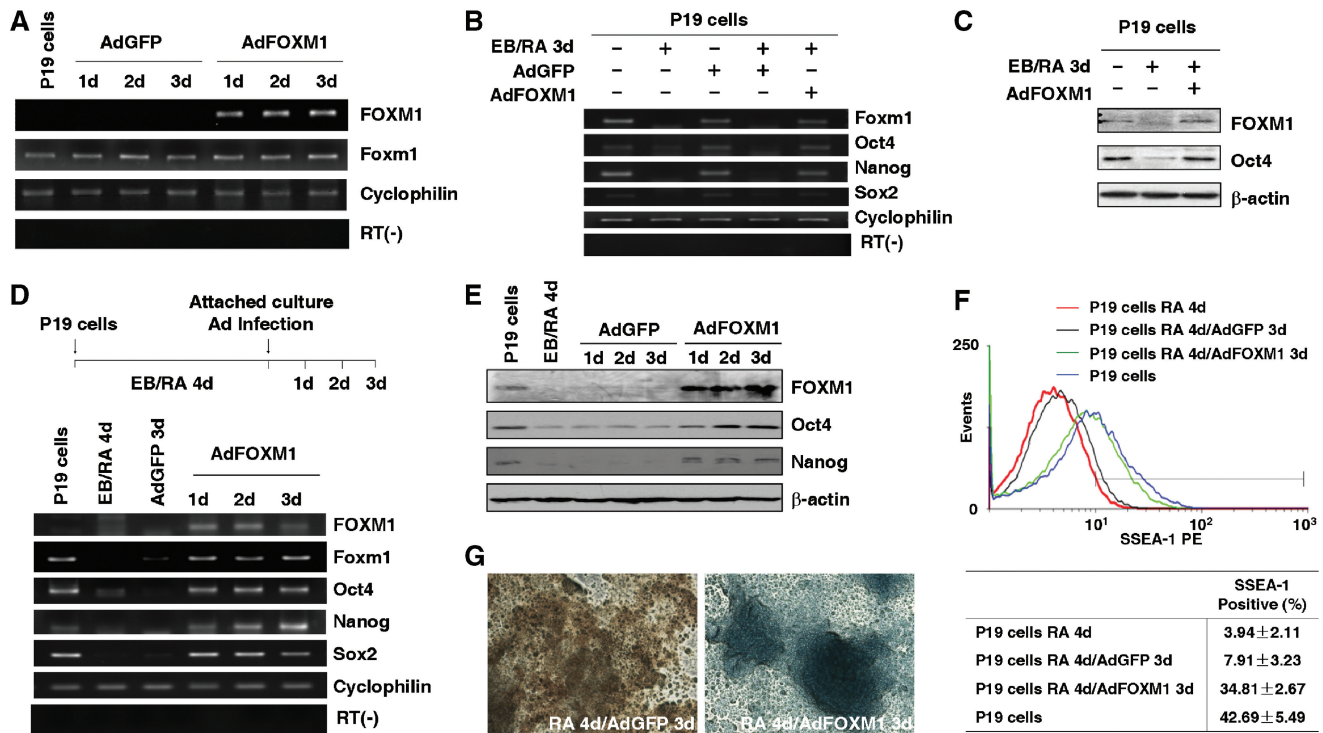
Figure 4. Maintained expression of FOXM1 prevented the downregulation of pluripotency marker expression during P19 cell differentiation. (**A**) Overexpression of FOXM1 in P19 cells by AdFOXM1 infection. P19 cells were infected with AdGFP or AdFOXM1 (10 pfu/cell) and mRNA levels of exogenous FOXM1, endogenous Foxm1, and loading control Cyclophilin were measured by RT-PCR. (**B** and **C**) P19 cells infected with AdGFP or AdFOXM1 were induced to differentiation according to the standard protocol one day after viral infection. Gene expression analysis of selected pluripotency genes was performed by RT–PCR (**B**) or western blotting (**C**). (**D** and **E**) The overexpression of FOXM1 activated expression of Foxm1, Oct4, Nanog and Sox2 in the differentiated P19 cells. P19 cells were differentiated by RA treatment and 4 days later the cells were infected with AdGFP or AdFOXM1. The expression analysis of selected genes was performed by RT-PCR (**D**) or western blotting (**E**). (**F**) The overexpression of FOXM1 activated expression of of SSEA-1 in differentiated P19 cells. Flow cytometry was used to measure cell populations of SSEA-1 in P19 cells (RA 4d), P19 cells (RA 4d)/AdGFP (3d), P19 cells (RA 4d)/AdFOXM1 (3d) and P19 cells. P19 cells (RA 4d) were used as negative control cells and P19 cells were used as positive control cells. (**G**) P19 cells (RA 4d)/AdGFP (3d) and P19 cells (RA 4d)/AdFOXM1 (3d) were stained for alkaline phosphatase.

FOXM1 overexpression also resulted in higher activities of alkaline phosphatase in the differentiated P19 cells infected with AdFOXM1 (Figure 4G). Therefore, these data indicated a potential of Foxm1 to induce the expression of pluripotency-related genes in differentiated cells.

The overexpression of FOXM1 activated the expression of pluripotency-related genes in human newborn fibroblasts

We used human newborn fibroblasts to test whether overexpression of FOXM1 is able to induce the expression of pluripotency-related genes in well differentiated somatic cells. Human newborn fibroblasts were infected three times by AdFOXM1 (100 pfu/cell) at 4 days interval between each infection. Control AdLacZ infections determined that almost 100% of the cells were infected with the viral dosage at 100 pfu/cell (Supplementary Figure S4). We noticed that the multiple AdFOXM1 infections caused dramatic morphology changes of human newborn fibroblasts, which formed cell aggregates and colonies afterwards (Figure 5A). Moreover, we found that the colonies formed by AdFOXM1-infected human newborn fibroblasts possessed high levels of alkaline phosphatase activity (Figure 5B), suggesting that these colonies started to have some characteristics similar to pluripotent

stem cells. Next, the human newborn fibroblasts infected with AdFOXM1 were analyzed for pluripotency markers by RT-PCR. We found that the overexpression of FOXM1 activated expression of OCT4, NANOG and SOX2 in human newborn fibroblasts (Figure 5C). Based on the findings that mouse and human somatic cells can be induced to acquire pluripotency by overexpression of specific transcription factors such as OCT4 and NANOG, the data of the ectopic expression of FOXM1 in human newborn fibroblasts suggested that FOXM1 might be one of the candidates for reprogramming differentiated somatic cells to iPS cells. Future work will be focused on testing this possibility.

DISCUSSION

Pluripotent stem cells are unique in their ability to remain self-renewing and pluripotency in vitro and these processes are tightly regulated by a network of transcription factors (41). Our results for the first time define a role for transcription factor Foxm1 in maintaining the pluripotency of stem cells. The loss of Foxm1 expression in pluripotent P19 cells results in the decreased activity of alkaline phosphatase and the diminished expression of
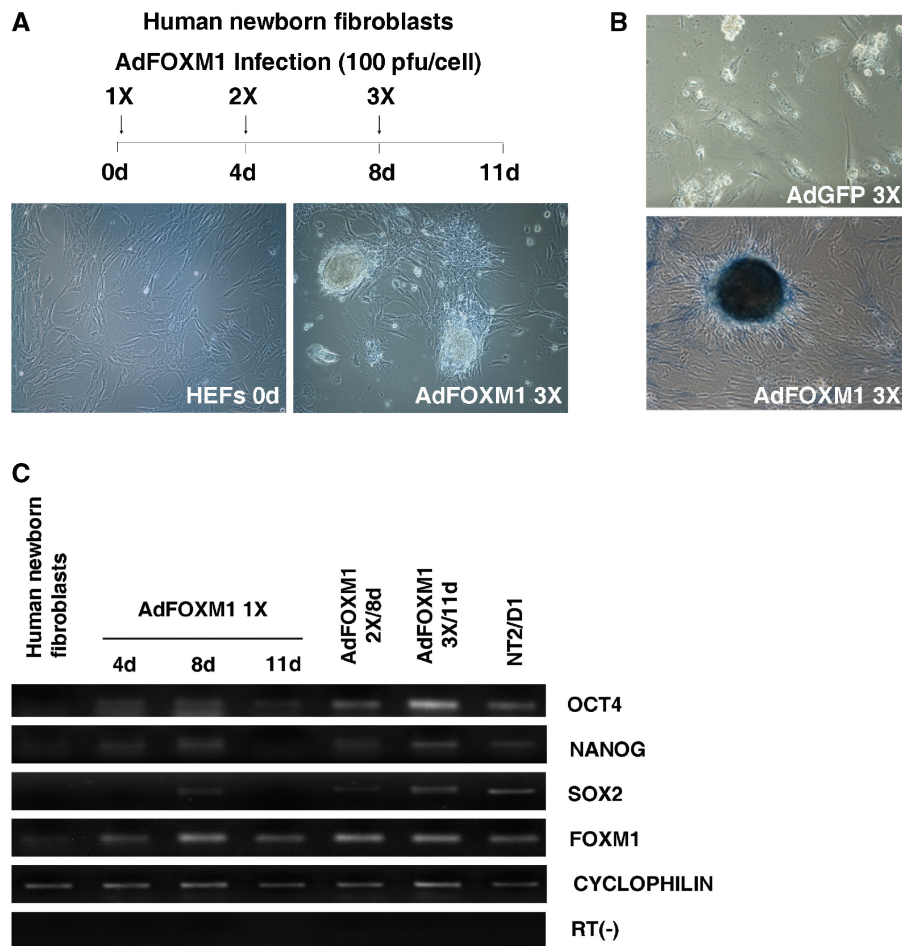
Figure 5. The overexpression of FOXM1 activated the expression of pluripotency-related genes in human newborn fibroblasts. (**A**) The morphology of FOXM1-overexpressed human newborn fibroblasts. Human newborn fibroblasts were infected three times by AdFOXM1 (100 pfu/cell) at 4 days interval between each infection. Pictures of human newborn fibroblasts or the cells post AdFOXM1 3X infections were taken at 200× magnification using a TE2000 microscope (Nikon). (**B**) FOXM1-overexpressed human newborn fibroblasts obtained high levels of alkaline phosphatase activity. The human newborn fibroblasts infected 3X with AdGFP or AdFOXM1 were stained for alkaline phosphatase. (**C**) The overexpression of FOXM1 activated expression of OCT4, NANOG and SOX2 in human newborn fibroblasts. The human newborn fibroblasts infected with AdFOXM1 were analyzed for selected genes by RT-PCR (C). The human EC cell line NT2/D1 was used as a positive control of human pluripotent stem cells.

pluripotency genes such as Oct4 and Nanog. Maintaining Foxm1 expression prevents the downregulation of Oct4 and Nanog during P19 cell differentiation. Furthermore, we also found that the stable expression of FOXM1 in P19 cells prevented the downregulation of Oct4 expression during RA-induced differentiation (Supplementary Figure S5). Moreover, overexpression of FOXM1 alone starts the expression of *Oct4*, *Nanog* and *Sox2* in RA-differentiated P19 cells or differentiated human newborn fibroblasts. It is well known that Oct4, Nanog and Sox2 form a regulatory feedback circuit to maintain pluripotency in human and mouse ESCs (42,43). This regulatory circuit, in which all three transcription factors regulate themselves as well as each other to form an interconnected autoregulation loop, has been identified to be essential to the ESC identity. Our results confirm that Foxm1 maintains Oct4 expression by directly binding to the Oct4 promoter, suggesting that Foxm1 is one of the upstream regulators of Oct4 and possesses a plausible position in the regulatory circuit of pluripotency.

This idea has been further confirmed by the experiments of the combinantion of Oct4 knockdown and FOXM1 overexpression in P19 cells. The Oct4 specific siRNA transfection (10 nM) that inhibits Oct4 expression resulted in the decreased levels of the alkaline phosphatase activity and the expression of Sox2 but not Nanog in P19 cells (Supplementary Figure S6). The AdFOXM1 infection to the Oct4 knockdown cells caused the elevation of alkaline phosphatase activity and the increased expression of Oct4 and Sox2 in these cells (Supplementary Figure S6), implicating that the overexpression of FOXM1 activates the transcription of Oct4 mRNAs which may compensate the siRNA-induced Oct4 mRNA degradation. Because Oct4 acts as a major regulator that activates target genes encoding pluripotency/self-renewal mechanisms and represses signaling pathways promoting differentiation (42–45), it is reasonable to believe that Foxm1 contributes to the hallmark characteristics of pluripotent stem cells through its regulation on Oct4 expression.

The expression of Foxm1 is required for the repression of pathways that promote differentiation because knockdown of Foxm1 results in activation of Gata4 expression and spontaneous differentiation of pluripotent P19 cells to mesodermal derivatives such as cardiac muscle especially. Although Gata4 is frequently used as a marker for endodermal derivatives during differentiation of pluripotent stem cells (46), Gata4 is also considered as a key regulator of cardiogenesis (39). Gata4 possesses the ability to specify endoderm with temporal and spatial specificity and to facilitate the generation of cardiomyocyte progenitors from associated mesoderm (40). It is known that the overexpression of Gata4 enhances cardiogenesis in P19 cells (47). It has been shown that a balance between levels of Nanog and Gata4/Gata6 is necessary for differentiation into primitive endoderm of the developing blastocyst (48). Nanog represses the expression of Gata6 and Gata4, and Gata4/Gata6 are upregulated in the absence of Nanog. It is interesting to notice that knockdown of Foxm1 in P19 cells results in the decreased expression of Nanog, which may explain that knockdown of Foxm1 results in activation of Gata4 expression in P19 cells. Whether the increased expression of Gata4 is directly regulated by the decrease of Foxm1 or through the reduced expression of other pluripotency factors, such as Nanog, need to be further investigated.

RA-initiated differentiation reduces the Foxm1 expression, which is correlated with the decreased expression of many of the known pluripotency-related genes. The model of RA-induced P19 cell differentiation, which mimics events that occur during early development, has been widely used for molecular analysis of *in vitro* differentiation (49–54). As an important molecule for controlling cell growth and differentiation in both embryo and adult, RA functions by binding to the ligand-inducible transcription factors (nuclear receptor proteins RARs and RXRs) that activate or repress the transcription of downstream target genes (55). RA-induced P19 cell differentiation is followed by the repression of pluripotency genes such as Oct4 (56). There are numerous publications suggesting that the repression of pluripotency genes during differentiation is regulated majorly by modifications of epigenetics, such as DNA methylation of specific promoter regions (57), histon modifications (58), and participation of microRNAs (59). For example, differentiation-related microRNA miR-124 is induced by RA to repress the expression of pluripotency-related Hes-1 during neuronal differentiation of P19 cells (60). Given the evidence that the reduction of Foxm1 expression is similar as that of many pluripotency genes post RA treatment, we imagine that the mechanisms of epigenetic modification are involved in the regulation of Foxm1 expression during RA-induced P19 cell differentiation. This hypothesis will be elucidated by future studies.

Direct reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) can be achieved by overexpression of transcription factors (Oct4, Sox2, c-Myc and Klf4 or Oct4, Sox2, Lin-28 and Nanog) (12–14). In this study, we demonstrate that expressions of Oct4, Nanog and Sox2 are upregulated by Foxm1 in differentiated P19 cells or human newborn fibroblasts. On the other hand, Foxm1 has been shown to activate the expression of c-Myc directly (61). Based on the findings in this study and published evidences that the increase of the cell division rate results in an accelerated kinetics of iPSC formation (62) and Foxm1 is a major stimulator of cell proliferation (27), we hypothesize that Foxm1 may be one of the candidates to help reprogramming somatic cells into iPSCs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online

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