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Oct3/4 directly regulates expression of E2F3a in mouse embryonic stem cells

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ABSTRACT

Embryonic stem (ES) cells, derived from the inner cell mass of blastocysts, have a characteristic cell cycle with truncated G1 and G2 phases. Recent findings that suppression of Oct3/4 expression results in a reduced proliferation rate of ES cells suggest the involvement of Oct3/4 in the regulation of ES cell growth, although the underlying molecular mechanism remains unclear. In the present study, we identified *E2F3a* as a direct target gene of Oct3/4 in ES cells. Oct3/4 directly bound to the promoter region of the *E2F3a* gene and positively regulated expression of E2F3a in mouse ES cells. Suppression of E2F3a activity by E2F6 overexpression led to the reduced proliferation in ES cells, which was relieved by co-expression of E2F3a. Furthermore, cell growth retardation caused by loss of Oct3/4 was rescued by E2F3a expression. These results suggest that Oct3/4 upregulates E2F3a expression to promote ES cell growth.

1. Introduction

Embryonic stem (ES) cells are established from the inner cell mass of blastocysts. In the presence of leukemia inhibitory factor (LIF), mouse ES cells can repeatedly self-renew and maintain their pluripotency, the ability to differentiate into all types of somatic and germ cells. Extensive studies revealed that several transcription factors, including STAT3, Oct3/4, Sox2, and Nanog, play important roles in the self-renewal of mouse ES cells [1]. For example, using dominant-negative and conditionally-active mutants, activation of STAT3, a well-known transcription factor downstream of LIF, was shown to be essential and sufficient for the maintenance of self-renewal [2,3]. Nanog was identified as a homeobox transcription factor whose overexpression can bypass the requirement of LIF for self-renewal [4,5]. Although dispensable for self-renewal maintenance, Nanog plays a crucial role in creating the pluripotent ground state in ES cells [6,7].

Oct3/4, which is encoded by the *pou5f1* gene, belongs to the POU family of transcription factors and regulates the expression of pluripotency-associated genes in cooperation with Sox2. Targeted disruption of the *pou5f1* gene in mice results in loss of the pluripotent inner cell mass [8] and conditional repression of this gene in ES cells leads to their differentiation into trophectoderm [9], indicating that Oct3/4 is a central player in the maintenance of ES cell self-renewal. Furthermore, the finding that Oct3/4 is one of four factors required for the production of induced pluripotent stem cells suggests that Oct3/4 is also important for the acquisition of pluripotency [10].

ES cells have a characteristic cell cycle with truncated G1 and G2 phases [11]. In most cells, retinoblastoma (Rb) protein binds to E2F and inhibits its transcriptional activity, which results in suppression of growth. When Rb is phosphorylated by the Cyclin D-Cdk4 complex, phosphorylated Rb dissociates from the E2F-Rb complex and E2F is thereby activated. Activation of E2F results in induction of Cyclin E expression, and the Cyclin E-Cdk2 complex phosphorylates several target molecules, including Rb, to drive cells into S phase. On the other hand, mouse ES cells display high Cdk2 activity that lacks normal cell cycle periodicity [12]. As a result, the Cyclin E-Cdk2 complex continuously phosphorylates Rb to release E2F, and E2F target genes are constitutively expressed throughout the cell cycle, leading to unusually rapid proliferation without gap phases.

The mammalian E2F family of transcription factors consists of eight known genes encoding nine E2F proteins, with the *E2F3* locus encoding two distinct isoforms, E2F3a and 3b, which are transcribed from distinct promoters [13–16]. Of the eight E2Fs, E2F1, E2F2, and

Abbreviations: cDNA, complementary DNA; ChIP, chromatin immunoprecipitation; ES, embryonic stem; LIF, leukemia inhibitory factor; Rb, retinoblastoma.

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E2F3 are considered "transactivators" that activate transcription of target genes for DNA replication and G1/S transition. Overexpression of these E2Fs is sufficient to drive cells into S phase [13,17,18] and loss of these transcriptional activators results in a block in proliferation [19]. Although *E2F1*$^{-/-}$*E2F2*$^{-/-}$ mice are viable and develop to adulthood, *E2F1*$^{-/-}$*E2F3*$^{-/-}$ and *E2F2*$^{-/-}$*E2F3*$^{-/-}$ mice die early in embryogenesis owing to growth retardation, indicating that among the three activators, E2F3 is particularly important for cellular proliferation. Of the two E2F3 isoforms, inactivation of E2F3a results in a low-penetrance proliferation defect *in vitro*, whereas loss of E2F3b shows no effect [13,20].

Recently, it was reported that knockdown or knockout of Oct3/4 reduces the proliferation rate in ES cells [21,22]. These findings suggest that Oct3/4 is involved in the regulation of ES cell growth. However, it remains unclear how Oct3/4 regulates ES cell growth. In the present study, we identified *E2F3a* as a direct target gene of Oct3/4 in ES cells, and found that Oct3/4 regulates E2F3a expression to promote ES cell growth.

2. Materials and methods

2.1. Cell culture, transfection, and the luciferase assay

The mouse ES cell lines E14 (E14tg2a), which was obtained from American Type Culture Collection, and ZHBTc4 [9], a kind gift from Dr. H. Niwa (RIKEN CDB, Japan), were cultured on gelatin-coated dishes in LIF-supplemented medium as described previously [23], except that the culture medium contained 150 μM 1-thioglycerol (Sigma) instead of β-mercaptoethanol. HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (BioWest, France). Plasmids were introduced into cultured cells using LipofectAMINE2000 (Invitrogen, Carlsbad, CA). Cell extracts for the luciferase assay were prepared 2 days after transfection, and luciferase activity was measured with a dual-luciferase assay kit (Promega, Madison, WI) using an AB-2200 luminometer (ATTO, Tokyo, Japan).

2.2. Plasmid construction

The coding sequences of *E2F3a* and *E2F6* were amplified by PCR from complementary DNA (cDNA) of E14 ES cells using the primers listed in Supplementary Table S1. pCAGIP-myc-E2F3a and pCAGIP-myc-E2F6 were constructed by inserting myc-tagged E2F3a- and E2F6-coding fragments into the mammalian expression vector pCAG-IP [23], respectively. pCAGIH3-myc-E2F3a was produced by inserting a myc-tagged E2F3a-coding fragment into pCAG-IH3 [24].

An upstream region of the *E2F3a* gene (−501/−1 region) was amplified by PCR using the primers listed in Supplementary Table S1 and cloned into the *Acc*65I and *Xho*I sites of pGL4.10 (Promega) to obtain pGL4.10-E2F3a(-501). Mutations (ATGCAAAT to CCCACGTC) were introduced into the Oct3/4-binding element by PCR using the primers listed in Supplementary Table S1. The PCR products were then cloned into pGL4.10 to obtain pGL4.10-E2F3a(-501)mutOct.

2.3. RT-PCR analysis

Total RNA was isolated from ES cells with Sepasol-RNA I Super G reagent (Nakalai Tesque, Kyoto, Japan) and converted to cDNA using ReverTraAce (Toyobo, Osaka, Japan) with oligo(dT)$_{12-18}$ primers (Nippon EGT, Toyama, Japan). Gene expression was examined by PCR using specific primers (Supplementary Table S2). Glycerol-3-phosphate dehydrogenase was used as an internal control.

2.4. Biotinylated DNA pulldown assay

A biotinylated DNA pulldown assay was performed as described previously [25] using the oligonucleotides listed in Supplementary Table S3. Briefly, the biotin-labeled, double-stranded oligonucleotide was mixed with extracts of HEK293 cells transfected with pCAGIP-myc-Oct3/4 [26] and incubated with streptavidin-agarose (Novagen, Darmstadt, Germany) with or without a 100-fold excess of non-labeled competitor oligonucleotide. The beads were washed three times, and bound proteins were subjected to Western blot analysis using an anti-Myc antibody (sc-40; Santa Cruz Biotechnology, Santa Cruz, CA).

2.5. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed using the OneDay ChIP Kit (Diagenode, Philadelphia, PA) according to the manufacturer's protocol. Briefly, ES cells overexpressing Oct3/4 were fixed with 1% formaldehyde and genomic DNA was sheared. The sheared DNA was then immunoprecipitated with normal rabbit IgG (sc-2027; Santa Cruz Biotechnology) or an anti-Oct3/4 antibody (sc-9081; Santa Cruz Biotechnology). To detect precipitated genomic DNA, PCR analysis was performed using the primers 5′-AGT AGG ACT TTG CCC CAC CT-3′ and 5′-TGA AGG GAT ACG GTT TAC GC-3′.

2.6. WST-1 assay

After 2 or 3 days of culture in 96-well microplates, the cell proliferation reagent WST-1 (Roche, Mannheim, Germany) was added to each well and the number of viable cells was estimated by measuring absorbance at 450 nm with Sunrise Remote R (TESCAN, Männedorf, Switzerland).

3. Results

3.1. Oct3/4 regulates E2F3a expression in ES cells

First, to confirm the previous finding that suppression of Oct3/4 results in a reduced proliferation rate in ES cells [21,22], we examined the effect of Oct3/4 suppression on the growth of ES cells using ZHBTc4 cells, in which expression of Oct3/4 is suppressed by treatment with tetracycline [9]. The growth rate of ZHBTc4 cells was reduced in the presence of tetracycline (*i.e.*, by the absence of Oct3/4), as determined by the WST-1 cell proliferation colorimetric assay (Fig. 1A), suggesting that Oct3/4 regulates ES cell growth.

E2F activators (E2F1, E2F2, E2F3a, and E2F3b) play a central role in the regulation of proliferation [13]; therefore, we next examined whether the expression levels of E2F activators are regulated by Oct3/4. ZHBTc4 cells were cultured in the presence or absence of tetracycline, and the expression levels of E2F activators were compared (Fig. 1B). mRNA expression levels of E2F2 and E2F3a were markedly reduced by tetracycline treatment, while that of E2F1 was only slightly reduced and that of E2F3b was increased, suggesting that Oct3/4 positively regulates E2F2 and E2F3a at the transcriptional level.

3.2. The promoter region of E2F3a contains a putative Oct3/4-binding site

Oct3/4 recognizes and binds to 5′-ATGCAAAT-3′ [27]. When we searched for a putative Oct3/4-binding site(s) in the *E2F2* and *E2F3a* genes, we discovered one putative Oct3/4-binding site in the 0.5-kb upstream region of the *E2F3a* gene and named this site 'E2F3a-Oct' (Fig. 2A). The identification of this site raised the possibility that *E2F3a* is a direct target gene of Oct3/4; therefore, we first examined

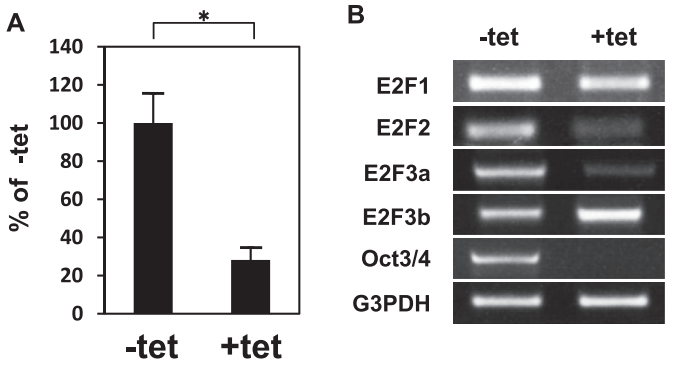

Fig. 1. Oct3/4 regulates E2F3a expression in ES cells. (A) Suppression of ES cell growth by Oct3/4 knockout. ZHBTc4 cells were cultured in the presence of LIF with (+tet) or without (−tet) tetracycline for 3 days, and were then subjected to the WST-1 assay. Bars and error bars represent the means and standard deviations, respectively ($n = 3$). *, $P < 0.05$. (B) Expression levels of E2F1, E2F2, E2F3a, and E2F3b in the presence or absence of Oct3/4. ZHBTc4 cells were cultured in the presence of LIF with tetracycline for 1 day (+tet) or without tetracycline for 2 days (−tet). The expression levels of E2F1, E2F2, E2F3a, E2F3b, and Oct3/4 were examined by RT-PCR analysis. Glycerol-3-phosphate dehydrogenase (G3PDH) was used as an internal control. All results are representative of three independent experiments.

if the 0.5-kb promoter region of the *E2F3a* gene shows any promoter activity. As expected, this region showed a strong promoter activity in ES cells (Fig. 2B). Furthermore, the promoter activity was decreased by the presence of tetracycline in ZHBTc4 cells (Fig. 2C), suggesting that this 0.5-kb region is regulated by Oct3/4.

To determine if the E2F3a-Oct site is involved in the promoter activity of the 0.5-kb region, we introduced mutations into this site (Fig. 2A). Promoter activity was reduced when E2F3a-Oct was mutated, suggesting that Oct3/4 regulates E2F3a expression through this site (Fig. 2B).

3.3. Oct3/4 directly binds to E2F3a-Oct in vitro and in vivo

To determine if Oct3/4 can bind to the E2F3a-Oct site *in vitro*, we performed a pulldown assay using a biotinylated oligonucleotide named E2F3a-05, which contains the E2F3a-Oct site. Oct3/4 was precipitated by biotinylated E2F3a-05 oligonucleotides (Fig. 3A). Furthermore, when the pulldown assay was performed in the presence of a 100-fold excess of non-labeled E2F3a-05 oligonucleotide, the precipitation of Oct3/4 was inhibited, while mutant E2F3a-05 oligonucleotide showed no effect on the precipitation. These results suggest that Oct3/4 binds to the E2F3a-Oct site of E2F3a-05 oligonucleotides.

To examine whether Oct3/4 binds to the E2F3a-Oct site *in vivo*, we performed a ChIP assay (Fig. 3B). When Oct3/4-overexpressing ES cells were subjected to ChIP analysis, a DNA fragment containing the E2F3a-Oct site was precipitated with an anti-Oct3/4 antibody but not with a control antibody, suggesting the association of Oct3/4 with the E2F3a-Oct site in intact ES cells. Taken together, these results suggest that *E2F3a* is a direct target of Oct3/4 in ES cells.

3.4. E2F3a restores the reduced proliferation rate upon Oct3/4 suppression

To evaluate the role of E2F3a in the proliferation of ES cells, we suppressed E2F3a activity and examined the effect on ES cell growth. Owing to functional redundancy among E2F activators, it was expected that suppression of E2F3a would be compensated by other E2F activators. We therefore utilized E2F6, which inhibits all E2F activators including E2F3a [28]. When overexpressed in ES cells, E2F6 reduced the growth rate of ES cells (Fig. 4A and

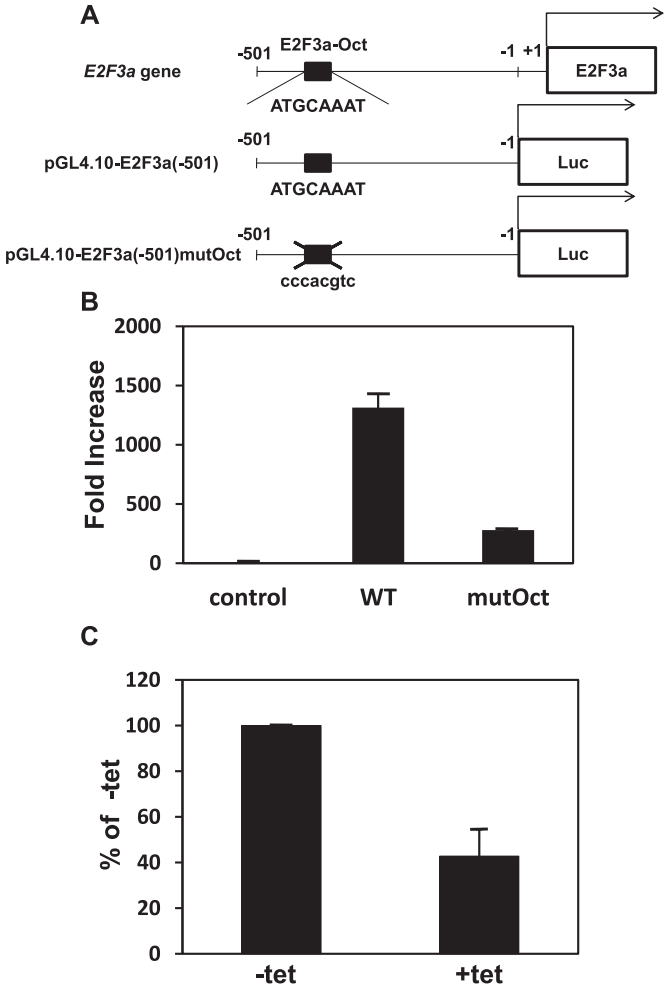

Fig. 2. Oct3/4 regulates the promoter region of the *E2F3a* gene. (A) Schematic views of pGL4.10-E2F3a(-501), which carries the promoter region of the *E2F3a* gene, and its mutants. A putative binding site for Oct3/4 is indicated. (B) Promoter activity is reduced when E2F3a-Oct is mutated. ES cells were transfected with pGL4.10 (control), pGL4.10-E2F3a(-501) (WT), or pGL4.10-E2F3a(-501)mutOct (mutOct), cultured for 2 days, and subjected to the luciferase assay. (C) The promoter activity of *E2F3a* is reduced upon loss of Oct3/4. ZHBTc4 cells were transfected with pGL4.10-E2F3a(-501) and cultured in the presence (+tet) or absence (−tet) of tetracycline for 2 days. In both experiments, bars and error bars represent the means and standard deviations, respectively ($n = 3$).

Supplementary Fig. S1A). Importantly, this E2F6-mediated reduction in growth was alleviated by co-expression of E2F3a. These results suggest the involvement of E2F3a in the regulation of ES cell growth.

Lastly, we examined the role of E2F3a in Oct3/4-regulated ES cell growth. For this, we examined if E2F3a recovers the retardation of cell growth induced by loss of Oct3/4 in ZHBTc4 cells. When we overexpressed E2F3a in ZHBTc4 cells, the reduced proliferative capacity upon tetracycline treatment (*i.e.*, Oct3/4 suppression) was restored (Fig. 4B and Supplementary Fig. S1B). Taken together, these results suggest that E2F3a regulates proliferation downstream of Oct3/4 in ES cells.

4. Discussion

We demonstrated that Oct3/4 directly bound to the promoter region of the *E2F3a* gene and that Oct3/4 positively regulated expression of E2F3a in mouse ES cells. Furthermore, ES cell growth

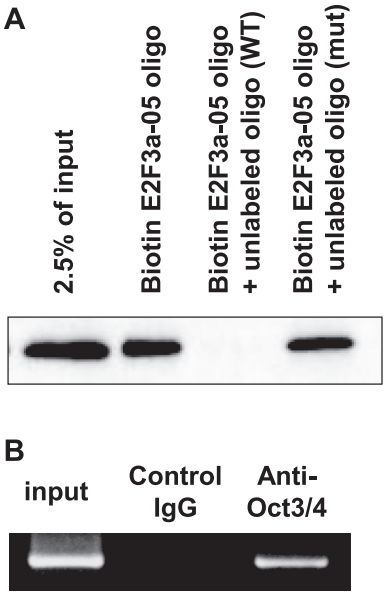

Fig. 3. Oct3/4 directly binds to the E2F3a-Oct site. (A) Oct3/4 binds to the E2F3a-Oct site *in vitro*. The biotin-labeled E2F3a-05 oligonucleotide was incubated with extracts of HEK293 cells transfected with myc-Oct3/4, with or without a 100-fold excess of non-labeled wild-type (WT) or mutated (mut) E2F3a-05 oligonucleotide. The precipitates, as well as cell extracts, were analyzed by Western blotting with an anti-c-Myc antibody. (B) Oct3/4 binds to the E2F3a-Oct site *in vivo*. A chromatin immunoprecipitation assay was performed using control IgG and an anti-Oct3/4 antibody. The precipitates were examined by PCR using specific primers described in Materials and methods. All results are representative of three independent experiments.

was retarded by loss of Oct3/4 and E2F3a expression restored this retardation. These results suggest that Oct3/4 directly regulates expression of E2F3a to promote ES cell growth.

Although we showed that E2F3a is involved in the growth of ES cells, the underlying molecular mechanism remains unclear. E2F3 reportedly contributes to transcriptional regulation of the *cyclin E* gene [29]. Therefore, E2F3a may induce expression of cyclin E1 to drive cell cycle progression in ES cells. In support of this, expression of cyclin E1 in ES cells was decreased upon Oct3/4 knockout (data not shown).

In addition to E2F3a, the expression level of E2F2 was also reduced upon Oct3/4 knockout (Fig. 1B), suggesting that Oct3/4 positively regulates E2F2 expression. However, we do not know if this regulation is direct, since we could not find any putative Oct3/4-binding sites in the *E2F2* gene. Several reports suggested the involvement of E2F2 in cell growth regulation. For example, overexpression of E2F2 induces the proliferation of terminally differentiated cardiomyocytes [30]. The transcriptional activity of E2F2 promotes adult hepatocyte proliferation [31]. Therefore, it is possible that Oct3/4 regulates expression of not only E2F3a, but also of E2F2, to promote ES cell growth. In addition, Oct3/4 controls the expression of the Cdk inhibitor p21 [22]. p21 can bind to the G1 Cyclin/Cdk complex and thereby suppress cell growth; therefore, it is also possible that Oct3/4 promotes cell proliferation through the regulation of both E2F3a and p21 pathways.

Our present data, as well as previous reports [21,22], showed the involvement of Oct3/4 in ES cell growth. Oct3/4 is also involved in cancer cell growth. For example, Oct3/4 knockdown reduces the growth rate of human embryonal carcinoma cells [32]. Oct3/4 increases cyclin D1 expression in hepatocellular carcinoma cells [33]. Oct3/4 positively regulates Survivin expression to promote the proliferation of esophageal squamous cell carcinoma cells [34]. Similarly, E2Fs have been suggested to play important roles in cancer cell growth [16,35,36]. It is therefore possible that Oct3/4 controls E2Fs to regulate cancer cell growth.

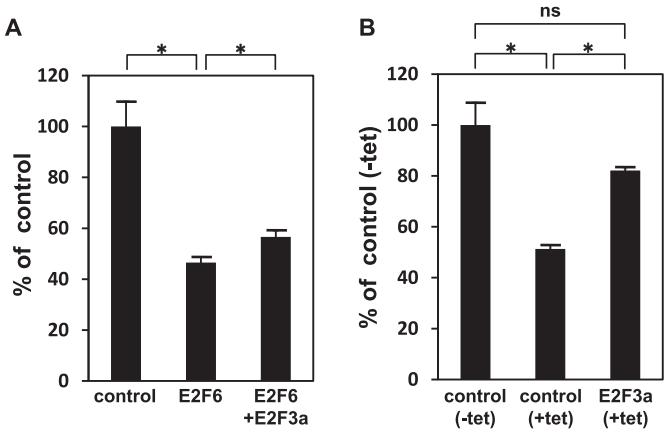

Fig. 4. E2F3a regulates ES cell growth downstream of Oct3/4. (A) Co-expression of E2F3a inhibits E2F6-mediated suppression of ES cell growth. Control ES cells, E2F6-overexpressing ES cells, and E2F6- and E2F3a-overexpressing ES cells were cultured in the presence of LIF for 3 days, and then subjected to the WST-1 assay. Bars and error bars represent the means and standard deviations, respectively ($n = 3$). *, $P < 0.05$. (B) E2F3a expression abolishes the negative effect of Oct3/4 suppression on cell proliferation. ZHBTc4 cells and E2F3a-overexpressing ZHBTc4 cells were cultured in the presence of LIF with (+tet) or without (−tet) tetracycline for 2 days, and were then subjected to WST-1 assay in triplicate. Bars and error bars represent the means and standard deviations, respectively. *, $P < 0.05$. ns, not significant. The results are representative of three independent experiments.

Conflict of interests

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.105.

Transparency document

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