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Stem cell-specific expression of Dax1 is conferred by STAT3 and Oct3/4 in embryonic stem cells

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ABSTRACT

Embryonic stem (ES) cells are pluripotent cells derived from inner cell mass of blastocysts. An orphan nuclear receptor, Dax1, is specifically expressed in undifferentiated ES cells and plays an important role in their self-renewal. The regulatory mechanism of *Dax1* expression in ES cells, however, remains unknown. In this study, we found that STAT3 and Oct3/4, essential transcription factors for ES cell self-renewal, are involved in the regulation of *Dax1* expression. Suppression of either STAT3 or Oct3/4 resulted in down-regulation of *Dax1*. Reporter assay identified putative binding sites for these factors in the promoter/enhancer region of the *Dax1* gene. Chromatin immunoprecipitation analysis suggested the *in vivo* association of STAT3 and Oct3/4 with the putative sites. Furthermore, gel shift assay indicated that these transcription factors directly bind to their putative binding sites. These results suggest that STAT3 and Oct3/4 control the expression of *Dax1* to maintain the self-renewal of ES cells.

Mouse embryonic stem (ES) cells are derived from inner cell mass (ICM) of blastocysts, and maintain pluripotency to differentiate into all types of somatic and germ cells [1,2]. In addition, mouse ES cells can self-renew to produce two identical stem-cell daughters upon cell division in the presence of leukemia inhibitory factor (LIF). It has been shown that four transcription factors—STAT3, Nanog, Oct3/4, and Sox2—play an important role in the self-renewal of mouse ES cells [reviewed in Ref. 3]. STAT3 is a downstream molecule of LIF, and expression of its dominant-negative mutant induces differentiation of ES cells [4]. Artificial activation of STAT3 using STAT3ER, a fusion protein consisting of STAT3 and the ligand-binding domain of estrogen receptor, can maintain ES cell self-renewal even in the absence of LIF [5]. These observations indicate that STAT3 activation is essential and sufficient for the maintenance of self-renewal. Nanog is a homeobox transcription factor, whose overexpression can bypass the requirement of LIF for self-renewal [6,7]. Since it was recently demonstrated that this homeobox transcription factor is dispensable for ES self-renewal [8], Nanog seems to be a self-renewal promoting factor. Oct3/4 is a member of the POU transcription factor family. Targeted disruption of this gene results in loss of the pluripotent ICM, and conditional repression of *Oct3/4* in ES cells leads to differentiation into trophoectoderm, indicating that Oct3/4 is a central player of the self-renewal in ES cells [9,10]. The function of Oct3/4 is synergistically activated with Sox2, a member of high mobility group (HMG) transcriptional factor, in expression of various self-renewal-specific genes [reviewed in Ref. 3].

Dax1 (also known as Nr0b1 and Ahch) belongs to the nuclear hormone receptor superfamily. Dax1 has been shown to play an important role in the establishment and maintenance of steroid producing tissues such as testis and adrenal cortex [reviewed in Ref. 11]. The N-terminal domain of Dax1 contains three LXXLL motifs involved in protein–protein interaction. In the C-terminus, Dax1 carries a ligand-binding domain that shares a homology with other nuclear receptors including steroidogenic factor 1 (SF1). The role of Dax1 in steroidogenesis is well established, whereas the failure to generate *Dax1* knockout mice has suggested that Dax1 may play a certain role at earlier stage than steroidogenesis during embryo development [12]. In fact, *Dax1* is expressed in ICM as well as in ES cells [13]. Suppression of *Dax1* expression either by knockdown or inducible knockout method induced ES cell differentiation [14]. Additionally, Dax1 binds with Nanog and shares multiple targets with Oct3/4 and Nanog [15,16]. These findings suggest that Dax1 is another key player in the maintenance of ES cell self-renewal.

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Despite its importance in ES cells, the mechanism how the expression of *Dax1* is maintained in ES cells remains unknown. In the present study, by combination of reporter, chromatin immunoprecipitation, and electrophoretic mobility shift assays, we demonstrated that expression of *Dax1* is under the control of STAT3 and Oct3/4 in self-renewing ES cells.

Materials and Methods

Cell culture and antibodies. ES cell lines A3-1, EB5, 2TS22C, and ZHBTc4 were cultured on gelatin-coated dishes with LIF-supplemented medium as described previously [5,10,17,18]. STAT3ER-expressing A3-1 cells were cultured in the medium containing 1 μM 4-hydroxytamoxifen (Sigma, St Louis, MO). HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Antibodies against Dax1 (sc-13064), STAT3 (sc-482) and Oct3/4 (sc-9081) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-tubulin antibody (DM-1A) was purchased from ICN Biomedicals (Irvine, CA).

Plasmid construction. Mammalian expression vector pCAGIP-STAT3ER was constructed by inserting the coding region of STAT3ER [5] into the XhoI site of pCAG-IP[19]. Construction of pCAG-wtSTAT3-IP and pCAG-dnSTAT3-IP was described before [20]. Promoter and enhancer regions of mouse *Dax1* gene were PCR-amplified from genomic DNA of ZHBTc4 cells using Pfx DNA polymerase (Invitrogen, Carlsbad, CA). To construct pDax1-luc(−4474/+4811), a promoter element (−4474/+26) and an enhancer element (+780/+4811) were subcloned into the XhoI and SfiI sites and the BamHI and SalI sites of pGL4.10 (Promega, Madison, WI), respectively. From pDax1-luc(−4474/+4811), pDax1-luc(−572/+4811), pDax1-luc(−572/+2436), and pDax1-luc(−572/+1463) were constructed by PCR. Oct3/4 mutation (ACACGCAT to ACACTAAT) and/or Sox2 mutation (TACAATG to TATGGGG) were introduced into pDax1-luc(−572/+2436) by PCR using Pfx DNA polymerase to obtain pDax1-luc(−572/+2436)(OmS), pDax1-luc(−572/+2436)(OSm) and pDax1-luc(−572/+2436)(OmSm). pDax1-luc(−5105/+26) was constructed by inserting a promoter region (−5105/+26) into the SmaI and XhoI sites of pGL2-basic (Promega). pDax1-luc(−2042/+26) and pDax1-luc(−740/+26) were constructed from pDax1-luc(−5105/+26). To obtain pDax1-luc(−740/+26)(STATm), a mutation (TTCTAGGGA to GTATCGATG) was introduced into the putative STAT3 site in pDax1-luc(−740/+26) using KOD DNA polymerase (Toyobo, Osaka, Japan).

Transfection. Plasmids were introduced into cultured cells either by lipofection with LipofectAMINE 2000 (Invitrogen), or calcium phosphate-mediated transfection. Synthesized siRNAs for *Oct3/4* (5′-AGCCUUAAGAACAUGUGUA-3′) and *MAP2* (5′-CAGGGCACCUA UUCAGAUAUU-3′), purchased from Xeragon (Germantown, MD), were transfected with TransMessenger Transfection Reagent (Qiagen, Hilden, Germany).

Northern blot and RT-PCR analyses. Northern blot analysis was carried out as described in [21]. Briefly, total RNA (10 μg) was separated by electrophoresis and transferred to a nylon membrane. The membrane was hybridized with ^{32}P-labeled probe of full-length *Dax1*, *Rex1*, or *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* cDNA. After washing, radiolabeled bands on the membrane were visualized with a BAS-2000 image analyzer (Fuji Film, Tokyo, Japan). For RT-PCR analysis, total RNA was converted to cDNA by Superscript III reverse transcriptase (Invitrogen) or RevertTraAce (Toyobo) with oligo(dT)$_{12-18}$ primers. Quantitative RT-PCR and microarray analysis were performed as described in [17]. The expression level of each gene in ES cells was normalized relative to that of *GAPDH*. Primers used in RT-PCR analysis are listed in Table S1.

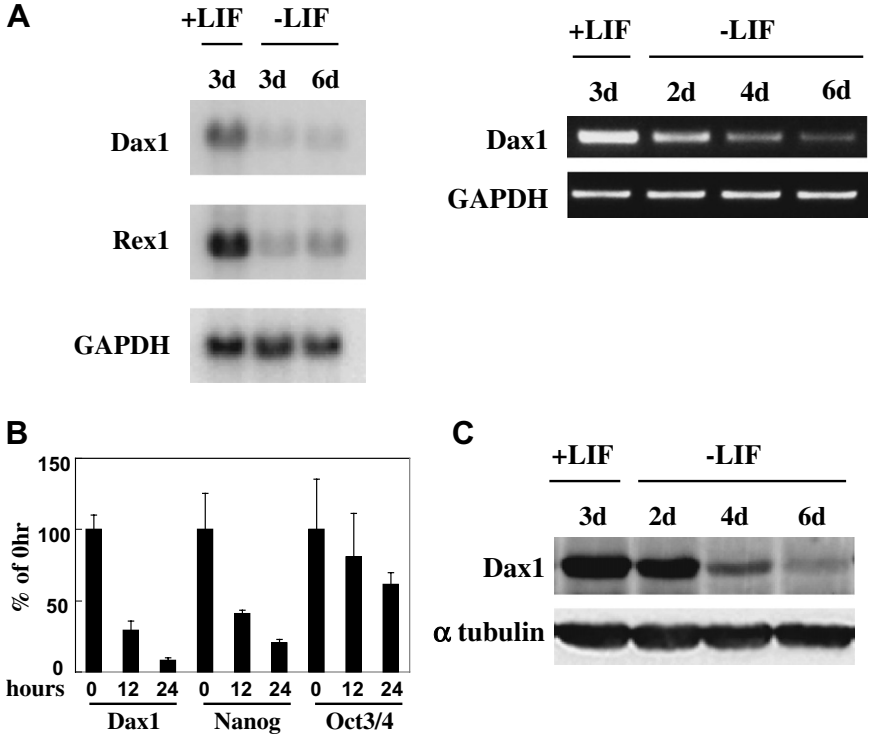

Fig. 1. Dax1 is expressed specifically in self-renewing ES cells. (A) Reduction of *Dax1* mRNA by LIF withdrawal. After culture with (+) or without (−) LIF for the indicated periods, A3-1 cells were subjected to Northern blot analysis (left panels) or RT-PCR analysis (right panels). *Rex1* and *GAPDH* were used as a self-renewal marker and an internal control, respectively. (B) Decrease of *Dax1* mRNA upon LIF withdrawal. After culturing in the absence of LIF for the indicated periods, EB5 cells were subjected to quantitative RT-PCR analysis. (C) Down-regulation of Dax1 protein upon LIF removal. After culture in the presence (+) or absence (−) of LIF for the indicated periods, A3-1 cells were subjected to Western blot analysis using antibodies against Dax1 and α-tubulin (internal control). Data shown are representative of multiple experiments.

Chromatin immunoprecipitation (ChIP) assay and electrophoretic mobility shift assay (EMSA). ChIP assay and EMSA were performed as described previously [21]. In ChIP analysis, after cross-linking with 1% formaldehyde, sonicated chromatin was immunoprecipitated with either anti-STAT3 or anti-Oct3/4 antibody. The precipitated DNA was amplified by PCR using Ampliqon III (Funakoshi, Tokyo, Japan) with primers listed in Table S1. In EMSA, nuclear extract or recombinant protein was incubated with ^{32}P-labeled DNA probe in the presence or absence of 100-fold molar excess of cold competitors. Samples were separated on a 4% native polyacrylamide gel, and radiolabeled bands were visualized with BAS-2000. Oligonucleotides used were listed in Table S2.

Results and discussion

Previously, it was demonstrated that *Dax1* mRNA is highly expressed in self-renewing ES cells [13]. It was also reported recently that *Dax1* mRNA is enriched in an ICM-like subpopulation of ES cells [22]. Therefore, we first confirmed the self-renewal-specific expression of Dax1 both in mRNA and protein levels. Northern blot analysis, as well as RT-PCR analysis, showed that the expression of *Dax1* mRNA was strong in ES cells cultured in the presence of LIF and reduced upon differentiation induced by removal of LIF (Fig. 1A and B). In agreement with this, Dax1 protein was specifically expressed in self-renewing ES cells and down-regulated upon LIF withdrawal (Fig. 1C). Furthermore, the down-regulation of *Dax1* mRNA was detected as early as 12 h after LIF withdrawal (Fig. 1B), suggesting that the *Dax1* down-regulation is not due to ES differentiation, but to suppression of the LIF-signaling pathway. These results indicate that the expression of *Dax1* is restricted to self-renewing ES cells and depends on LIF stimulation.

Since LIF stimulation directly leads to activation of STAT3 in ES cells, we examined the effect of a dominant-negative mutant of STAT3 on *Dax1* expression. *Dax1* mRNA was detected in ES cells transfected with wild-type STAT3, as well as control ES cells, whereas its expression level was reduced by transfection with the dominant-negative mutant of STAT3 (Fig. 2A). We also examined the expression of *Dax1* in STAT3ER-expressing ES cells, where it was strongly detected when cells were cultured in the presence of 4-hydroxytamoxifen (4HT), an activator of STAT3ER, but decreased after removal of 4HT (Fig. 2A). These results suggest the possibility that Dax1 is a downstream target of STAT3 in ES cells.

To investigate the relationship between Dax1 and Oct3/4, we examined the effect of *Oct3/4* knockdown on *Dax1* expression. ES cells were transfected with *Oct3/4* siRNA and the expression level of *Dax1* mRNA was determined. RT-PCR analysis revealed that the expression levels of both *Oct3/4* and *Dax1* were reduced by *Oct3/4* knockdown (Fig. 2B). To confirm this, we used ZHBTc4 cells, in which tetracycline (Tet) stimulates down-regulation of *Oct3/4* expression [10]. ZHBTc4 cells were treated with or without Tet, and the expression of *Dax1* and *Oct3/4* was determined (Fig. 2B). As reported previously [10], Tet stimulation completely repressed the expression of *Oct3/4*, which was recovered by removal of Tet. Similarly, *Dax1* expression was dramatically decreased by Tet stimulation, and restored after Tet removal. The decrease of *Dax1* was

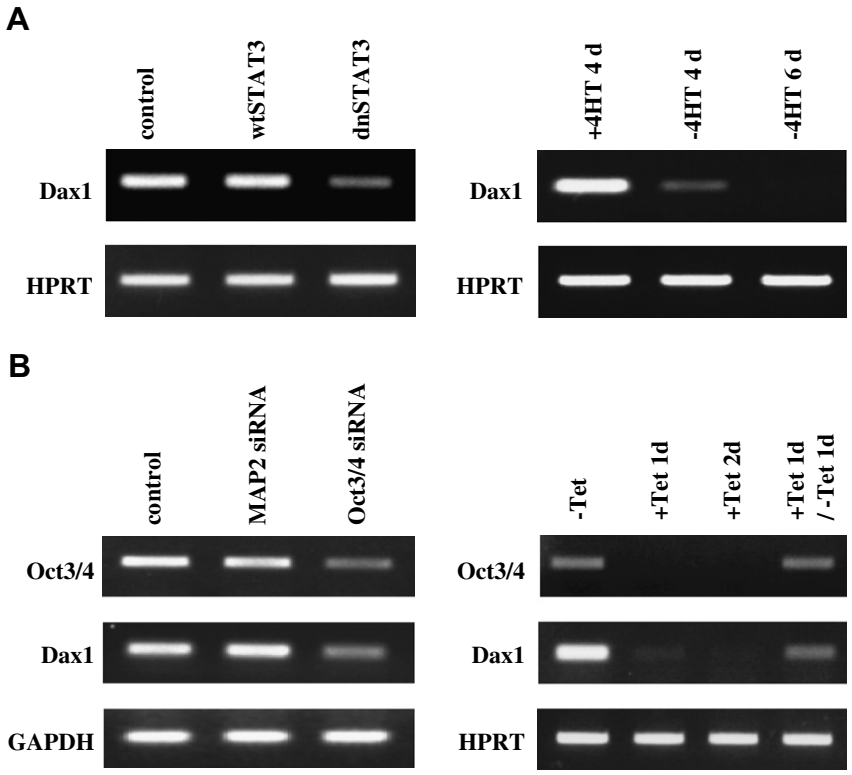

Fig. 2. Regulation of *Dax1* by STAT3 and Oct3/4. (A) Suppression of STAT3 leads to reduction in *Dax1* expression. After transfection with pCAGIP (control), pCAG-wtSTAT3-IP (wtSTAT3), or pCAG-dnSTAT3-IP (dnSTAT3), A3-1 cells were re-seeded and selected with 1 μg/ml puromycin for another three days and subjected to RT-PCR analysis (left panels). STAT3ER-expressing A3-1 cells were cultured with (+) or without (−) 4-hydroxytamoxifen (4HT) in the absence of LIF for four or six days and subjected to RT-PCR analysis (right panels). *Hypoxanthine-guanine phosphoribosyltransferase (HPRT)* was used as an internal control. (B) Repression of *Oct3/4* expression induces down-regulation of *Dax1*. After transfection with synthesized *Oct3/4* siRNA, A3-1 cells were cultured for two days and subjected to RT-PCR analysis (left panels). *MAP2* siRNA was used as a negative control. ZHBTc4 cells were cultured in the presence of LIF with (+) or without (−) tetracycline (Tet) for the indicated period (right panels). To restore the expression of *Oct3/4*, the culture medium of Tet-treated cells was changed to a Tet-free medium, and cells were cultured for one more day (+Tet1d/−Tet1d). The expression levels of *Oct3/4* and *Dax1* were examined by RT-PCR analysis. All results are representative of three separate experiments.

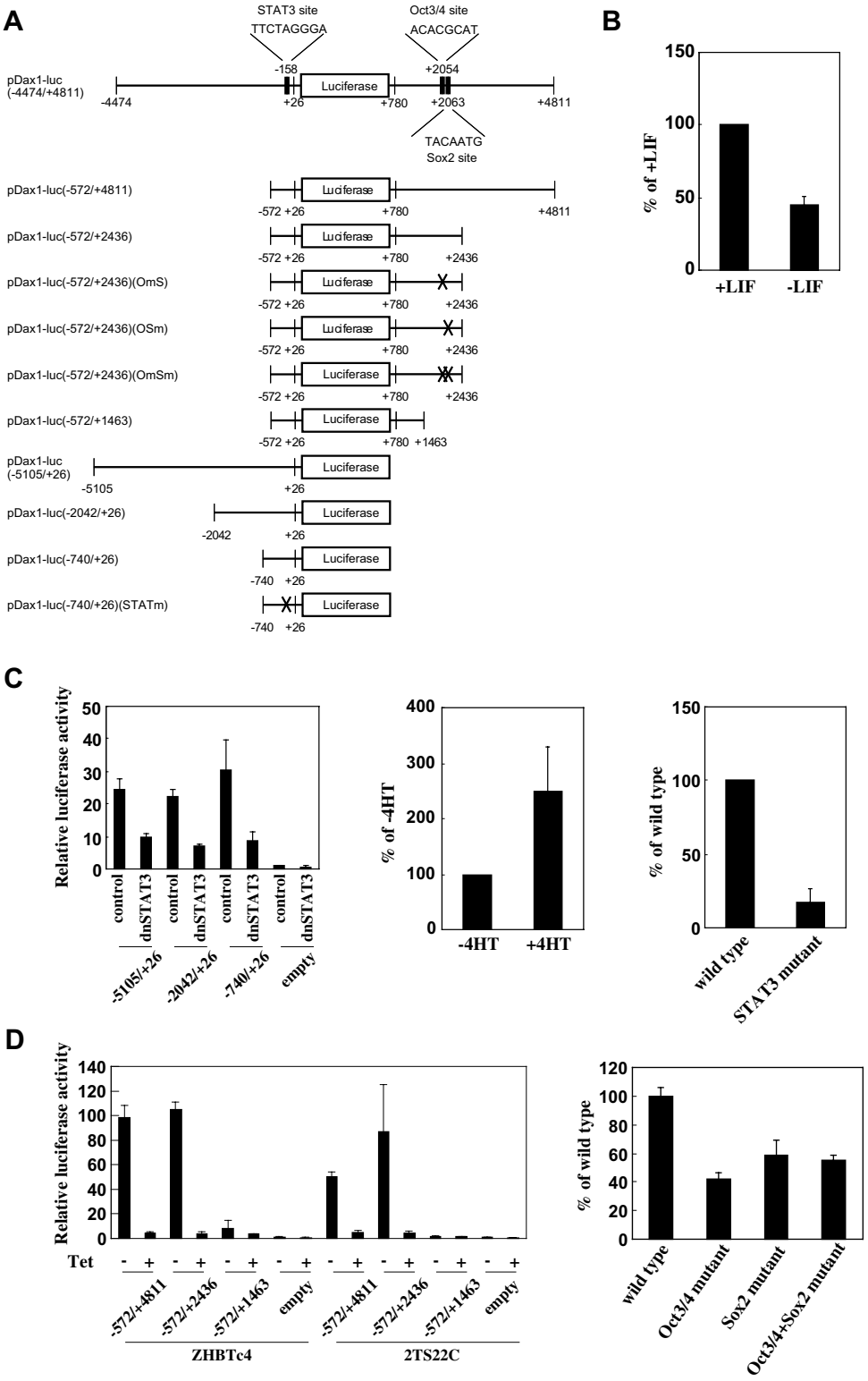

Fig. 3. Identification of putative binding sites for STAT3 and Oct3/4. (A) Schematic view of pDax1-luc(−4474/+4811), which carries the promoter and enhancer regions of the *Dax1* gene, and its mutants. Putative binding sites for STAT3, Oct3/4 and Sox2 are indicated. (B) LIF stimulates the promoter/enhancer activity of the *Dax1* gene. One day after transfection with pDax1-luc(−4474/+4811), A3-1 cells were split into two dishes and cultured for two days in the presence or absence of LIF. Bars represent the means and standard deviations ($n = 3$). (C) Identification of putative STAT3 site in the promoter region of *Dax1*. A3-1 cells were co-transfected with the indicated reporter plasmid and pCAGIP (control) or pCAG-dnSTAT3-IP (dnSTAT3) (left panel). A3-1 cells were co-transfected with pDax1-luc(−740/+26) and pCAGIP-STAT3ER, and cultured in the presence or absence of 4HT (middle panel). A3-1 cells were transfected with pDax1-luc(−740/+26) (wild type) or pDax1-luc(−740/+26)(STATm) (STAT3 mutant) (right panel). In each experiment, transfected cells were cultured for two days. The bars represent the means and standard deviations ($n = 3$). (D) Identification of putative Oct3/4 site in the enhancer region of *Dax1*. ZHBTc4 and 2TS22C cells were transfected with the indicated plasmid and cultured in the presence or absence of Tet for one day (left panel). ES cells were transfected with pDax1-luc(−572/+2436)(wild type), pDax1-luc(−572/+2436)(OmS)(Oct3/4 mutant), pDax1-luc(−572/+2436)(OSm)(Sox2 mutant), or pDax1-luc(-572/+2436)(OmSm)(Oct3/4+Sox2 mutant) and cultured for one day (right panel). The bars represent the means and standard deviations of triplicates. Similar results were obtained in multiple independent experiments.

observed 12 h after Tet addition (Fig. S1). These findings suggest the possibility that Oct3/4 regulates *Dax1* expression in ES cells.

To determine if Dax1 is a direct target of STAT3 and Oct3/4, we first performed a reporter assay to identify their binding sites. Both a 4.5-kb (−4512/+26) of 5'-flanking and a 4.0-kb (+780/+4811) of 3′-flanking regions of the *Dax1* gene were cloned, and inserted into a reporter plasmid carrying *luciferase* gene (Fig. 3A). When this reporter plasmid was introduced into ES cells, the promoter activity was increased by the presence of LIF (Fig. 3B), suggesting that the cloned region contains the regulatory region for *Dax1* expression. When we examined the effect of the dominant-negative mutant of STAT3 on the promoter activity of a region (−5105/+26), the mutant strongly inhibited the promoter activity (Fig. 3C), suggesting the existence of a STAT3 binding site within this region. Deletion analysis revealed that the putative STAT3 site is located within the region −740/+26. To confirm this, we examined the effect of artificial activation of the STAT3 signaling pathway using STAT3ER system on the promoter activity of this region. As expected, the activation of STAT3 increased the promoter activity. When the consensus-binding motif recognized by STAT3 was searched for in this region, one putative STAT3 site (5′-TTCTAGGGA-3′) was found, at −158. We introduced a mutation into this site and found that the mutation resulted in the decreased promoter activity. These results suggest that STAT3 binds to this putative site. Similarly, when we introduced the reporter plasmid carrying the region (−572/+26 plus +780/+4811) into ZHBTc4 cells, suppression of *Oct3/4* resulted in reduced promoter/enhancer activity (Fig. 3D). Deletion analysis identified the putative Oct3/4 binding site within the region +1463/+2436. By searching here for the consensus-binding motif of Oct3/4, one putative site (5′-ACACGCAT-3′) was found at +2054. Mutation at this site decreased the promoter/enhancer activity, suggesting that Oct3/4 binds to this site. We also noticed a putative Sox2 binding site (5′-TACAATG-3′) lying adjacent to the putative Oct3/4 site. Since Oct3/4 often cooperates with Sox2 to promote promoter/enhancer activity of the target genes, the same reporter plasmids were introduced to 2TS22C cells, in which tetracycline stimulation down-regulates *Sox2* [17]. As in the case of Oct3/4, promoter/enhancer activity was reduced by either suppression of *Sox2* expression or mutation at the putative Sox2 binding site. These results suggest that Oct3/4 and Sox2 bind to these sites.

To examine the interaction of STAT3 and Oct3/4 with their putative binding sites in intact cells, we performed ChIP assay (Fig. 4A). In STAT3ER-expressing ES cells, when stimulated with 4HT, a DNA fragment encompassing from −187 to +87 and containing the putative STAT3 site was precipitated with anti-STAT3 antibody, while this co-precipitation was not observed in non-stimulated cells. The observed co-precipitation was specific for this region, since the precipitation of a putative Oct3/4 site-containing DNA fragment (+2014 to +2356) was not observed with anti-STAT3 antibody. Similarly, anti-Oct3/4 antibody specifically precipitated the fragment containing the Oct3/4 site, but not the STAT3 site. These results suggest *in vivo* association of STAT3 and Oct3/4 with the promoter/enhancer region of *Dax1* through their own putative binding sites and that there is no interaction between STAT3 and Oct3/4. We also carried out electrophoretic mobility shift assay (Fig. 4B). When nuclear extracts prepared from STAT3ER-expressing HEK293 cells were stimulated with 4HT and incubated with the STAT3 probe (an oligonucleotide containing the putative STAT3 binding site), we observed the mobility shift of the STAT3 probe,

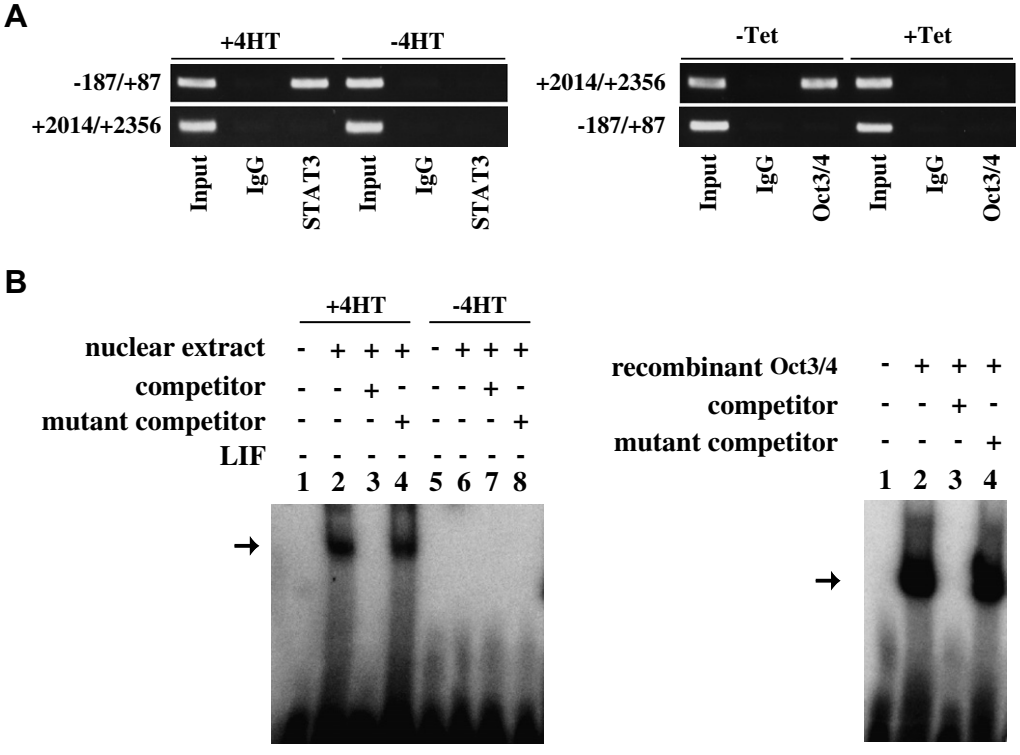

Fig. 4. STAT3 and Oct3/4 bind to promoter and enhancer regions of the *Dax1* gene. (A) *In vivo* association of STAT3 and Oct3/4 with the promoter/enhancer region of *Dax1*. Association of STAT3 with the promoter region of *Dax1*(−187/+87) (left panel). STAT3ER-expressing A3-1 cells were cultured in LIF-free medium with or without 4HT for four days. Association of Oct3/4 with the enhancer region of *Dax1*(+2014/+2356) (right panel). ZHBTc4 cells were cultured with or without Tet for four days. In both experiments, cells were subjected to ChIP assay with control antibody (IgG), anti-STAT3 antibody (STAT3) or anti-Oct3/4 antibody (Oct3/4). Results are representatives of three independent experiments. (B) STAT3 and Oct3/4 directly bind to their putative binding sequences. Nuclear extracts from STAT3ER-expressing HEK293 were stimulated with 4HT to increase the DNA binding activity of STAT3ER, and incubated with ^{32}P-labeled STAT3 probe (left panel). Recombinant MBP-Oct-3/4-His6 was incubated with ^{32}P-labeled Oct-3/4 probe (right panel). In both experiments, non-specific binding was determined using a 100-fold molar excess of unlabeled competitor DNA and mutant competitor DNA. Arrows indicate specific complex positions. Results are representative of three separate experiments.

suggesting that STAT3 binds with the putative binding site. Similarly, incubation of recombinant Oct3/4 with the Oct3/4 probe resulted in the retarded mobility of the probe. In both cases, the mobility shifts were inhibited by unlabeled competitor, but not by mutant competitor, indicating the sequence-specific interaction between transcription factors and oligonucleotides. Taken together, these data indicate that Dax1 is a direct target molecule of STAT3 and Oct3/4.

In conclusion, we demonstrated here that STAT3 and Oct3/4 directly bind to the promoter and enhancer regions of the *Dax1* gene and positively regulate *Dax1* expression. In addition, our data suggest that Oct3/4 cooperates with Sox2 for *Dax1* expression, which corresponds well with the previous observation that knockout of *Sox2* in ES cells induces the down-regulation of *Dax1* [17]. In contrast, it is unlikely that Nanog, another important player for ES self-renewal, regulates *Dax1* expression, since knockout of this gene in ES cells gives no effect on the expression level of *Dax1* [8]. Previous studies reported that *Dax1* promoter contains multiple binding sites for SF1, through which SF1 stimulates the transcription of *Dax1* [23–25]. We, however, found by RT-PCR and microarray analyses that the expression level of *SF1* is very low in ES cells (data not shown). Indeed, Northern blot analysis could not detect *SF-1* expression in ES cells [26]. Furthermore, in contrast to *Oct3/4*-null mice, *SF1*-null mice survive *in utero* and die within the first week of birth [27]. It is therefore unlikely that *Dax1* expression is controlled by SF1 in ES cells. Interestingly, it has been reported that LRH1 maintains *Oct3/4* expression through an SF1 response element in ES cells [28]. It is then possible that, in addition to STAT3 and Oct3/4, LRH1 is involved in regulating *Dax1* expression in self-renewing ES cells through binding to the SF1 sites.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.04.154.

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