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Transcriptional regulation of OCT4 by the ETS transcription factor ESE-1 in NCCIT human embryonic carcinoma cells

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ABSTRACT

The epithelium-specific ETS transcription factor-1 (ESE-1) is physiologically important in the pathogenesis of various diseases. Recently, OCT4, a transcription factor involved in stem cell pluripotency, has been implicated in tumorigenesis. In this study, we invested the molecular mechanism by which ESE-1 regulates transcription of OCT4 in NCCIT human embryonic carcinoma cells. Real-time PCR analysis revealed that OCT4 levels were high in undifferentiated NCCIT cells but significantly decreased upon retinoic acid-mediated differentiation, concomitant with up-regulation of ESE-1 expression. OCT4 mRNA level rose following shRNA-mediated knockdown of ESE-1, but declined when ESE-1 was overexpressed, suggesting that the expression levels of OCT4 and ESE-1 may be coordinated in an opposite manner. Promoter-reporter assays revealed that induced OCT4 promoter activity in NCCIT cells was significantly down-regulated by ESE-1 overexpression in a dose-dependent manner. The inhibitory effect of ESE-1 on OCT4 promoter activity was relieved by co-expression of an ESE-1 mutant lacking the transactivation domain, but not by mutants lacking other domains. Serial deletion and site-directed mutagenesis of the OCT4 promoter revealed that a potential ETS binding site (EBS) is present in the conserved region 2 (CR2). ESE-1 interacted with the EBS element in CR2 and enrichment of CR2 significantly increased upon RA-mediated differentiation of NCCIT cells, suggesting that this binding is likely to be involved in ESE-1-mediated repression of OCT4 promoter activity upon differentiation. Taken together, the results of this study reveal the molecular details of the mechanism by which the oncogenic factor ESE-1 regulates expression of the stem cell transcription factor OCT4 in pluripotent NCCIT cells.

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1. Introduction

Pluripotent stem cells possess the unique capability to self-renew and differentiate, both during embryonic development and in the adult organism. Coordinated transcription factor networks are the master regulators of stem cell pluripotency and differentiation [1,2]. In embryonic stem (ES) cells, both down-regulation and overexpression of OCT4 lead to loss of pluripotency and induction of differentiation, suggesting that a precise level of OCT4 must be sustained to maintain pluripotency and implying that OCT4 levels are tightly regulated [3]. Recently, OCT4 has been implicated in multiple types of cancer. For instance, OCT4 is expressed by glioma cells, in which it promotes colony formation, and in bladder cancer cells, overexpression of OCT4 enhances migration and invasion [4,5]. Likewise, OCT4 increases transmigration capacity in melanoma cells, leading to higher invasiveness and aggressiveness [6]. These observations suggest that deregulation and dysfunction of OCT4 may contribute to malignant transformation and the establishment of a "cancer stem cell" phenotype. Cancer stem cells are a subset of cancer cells with the ability to self-renew as well as generate the heterogeneous cancer cell lineages that constitute tumors [1,2]. For instance, germ cell tumors (GCTs), a heterogeneous group derived from pre-malignant and noninvasive intracellular germ cell neoplasias, exhibit a gene expression profile similar

Abbreviations: ESE-1, epithelium-specific ETS transcription factor-1; CR2, conserved region2; EBS, ETS binding site; ES, embryonic stem; EC, embryonic carcinoma; ETS, E26 transformation-specific; GCT, germ cell tumors; RA, retinoic acid.

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to that of pluripotent stem cells [7], suggesting that complex stem cell transcription factor networks play crucial roles in the regulation of tumorigenesis, as well as in stem cell pluripotency.

The human E26 transformation-specific (ETS) family of transcription factors consists of 27 members, which regulate multiple biological processes such as cell proliferation, cell differentiation, and oncogenesis [8,9]. The ETS family is characterized by a highly conserved 84 amino acid DNA-binding domain known as the ETS domain, which is usually located within the carboxyl-terminal region of proteins as a winged helix-turn-helix structural motif [10]. The ETS domain mediates binding to purine-rich sites, commonly containing the core consensus sequence GGAA/T, within the promoter and enhancer regions of target genes [8,11,12]. The epithelium-specific ETS transcription factor-1 (ESE-1, also called ELF3, ESX, jen, and ERT) is expressed in various cancer-derived cell lines, as well as epithelial cell-rich tissues, and exerts multiple roles in pathophysiological processes [11,13,14]. ESE-1 contains several functional domains in addition to the ETS domain. The pointed domain (PTD), adjacent to the N-terminal transactivation domain (TAD), is involved in protein–protein interactions [8]. The A/T hook domain mediates binding to the minor groove of AT-rich tracts of double-stranded DNA, and the PEST domain plays a role in determining protein stability by modulating targeting by proteases [13,14]. In addition, ESE-1 contains a serine- and aspartic acid-rich (SAR) domain that contains a p21-activated kinase-1 (PAK1) phosphorylation site [11,15,16]. Null mutation of Elf3, the mouse homolog of human ESE-1, increases the risk of embryonic death in utero, suggesting that Elf3 plays an important role in embryonic development [17]. However, the cause of $Elf3^{-/-}$ embryonic lethality during gestation remains unknown. Thus, elucidation of the exact role of ESE-1 in early embryonic development could provide new insights into the contribution of this epithelial-specific transcription factor to the regulation of various differentiation pathways, as well as contribute to our understanding of the mechanisms of stem cell-derived tumorigenesis and stem cell pluripotency.

ETS factors interact with TATA-less promoters, and the transcription factors Etsrp71, Elf3, and Spic affect the transcription of the Oct3/4 gene, whose embryonic expression is regulated by such promoters [12,18,19]. Therefore, we investigated whether ESE-1 could act as an upstream effector to regulate OCT4 transcription in NCCIT pluripotent embryonic carcinoma (EC) cells. These findings contribute to our understanding of the role of stem cell-related oncogenic factors in tumorigenesis and pluripotency.

2. Material and methods

2.1. Plasmid construction

We used a luciferase reporter construct containing OCT4 promoter (-2601-Luc) and a pGL3-ti minimal promoter-reporter construct containing the human OCT4 promoter conserved region 2 (CR2-ti-Luc), as described previously [20–22]. Site-directed mutagenesis was performed, using the QuickChange kit (Stratagene, La Jolla, CA, USA), to replace the putative ESE-1 binding element (also called the EBS) ($^{-1482}$AGGAAG^{-1477}) within CR2 (−1512 to −1316) with the XbaI restriction site (TCTAGA; CR2*). The resultant sequence was inserted upstream of the gene-encoding luciferase, under the control of the minimal ti promoter (adenovirus major late promoter TATA box and mouse terminal deoxynucleotidyl transferase gene initiator sequence [20]), to generate the CR2*-ti-Luc construct. The ESE-1 cDNA clone was purchased from Open Biosystems. The ORF of ESE-1 was amplified using the following primers: ESE-1 wild type (WT), 5′-AATGAATTCATGGC TGCAACCTGTGAGATT-3′ (forward) and 5′-ATT GCGGCCGCTCAGTT CCGACTCTGGAGA-3′ (reverse). ESE-1 deletion mutants were generated by QuickChange mutagenesis using the following primers: ESE-1 (ΔPTD), 5′-GAGAAGGCCAGCTGGACTTCCAGCTCT TCT-3′ (forward) and 5′-AGAAGAGCTGGAAGTCCAGCTGGCCTT CTC-3′ (reverse); ESE-1 (ΔTAD), 5′-CAGCTGCGAGACCTCGACCAGG GCAGCCCC-3′ (forward) and 5′-GGGGCTGCCCTGGTCGAGGTCTCG CAGCTG-3′ (reverse); ESE-1 (ΔSAR), 5′-TGTGGCGCAGGAGCCGAT GGTTTTCGTGAC-3′ (forward) and 5′-GTCACGAAAACCATCGGCTCCT GCGCCACA-3′ (reverse). The PCR products were cloned into FLAG-tagged pcDNA3.1+ vector, as described previously [22]. All cloned PCR products and reporter plasmids were verified by sequencing.

For preparation of short hairpin RNA (shRNA) expression vectors, two target sequences for ESE-1 RNA interference were generated using the Gene Link shRNA Design Guidelines website (http://www.genelink.com/sirna/shrnai.asp) for maximum silencing efficiency, following previously described instructions [23]. Two double-stranded oligonucleotides were generated by annealing the following pairs of oligos: ESE-1 shRNA-1, 5′-GATCCGGATGGCATG GCCTTCCAG*GAAGCTTG*CTGGAAGGCCATGCCATCCTTTTTTGGAAGC-3′ (sense) and 5′-GGCCGCTTCCAAAAAAGGATGGCATGGCCTTCCAG*CAA GCTTC*CTGGAAGGCCATGCCATCCG-3′ (antisense); ESE-1 shRNA-2, 5′-GATCCGACGCAGGTTCTGGACTGG*GAAGCTTG*CCAGTCCAGAACCT GCGTCTTTTTTGGAAGC-3′ (sense) and 5′-GGCCGCTTCCAAAAAA GACGCAGGTTCTGGACTGG*CAAGCTTC*CCAGTCCAGAACCTGCGTCG-3′ (antisense). (ESE-1 sense and antisense sequences are underlined; hairpin loop structure containing the HindIII restriction site is italicized.) The resultant double-stranded oligos were inserted into the pGSH1-GFP shRNA vector (Genlantis, San Diego, CA, USA). The pGSH1-GFP-Luc shRNA vector (Genlantis) was used as a control.

2.2. Cell culture and differentiation

Human EC NCCIT cells (American Type Cell Collection) were grown and differentiated for 10 days of 10 μM retinoic acid treatment (RA, Sigma–Aldrich, St. Louis, MO, USA), as described previously [22,23].

2.3. Transient transfection, RNA preparation, and quantitative reverse transcription–polymerase chain reaction (qRT–PCR)

NCCIT cells (3×10^5) were transfected with pGSH1-GFP-ESE-1 shRNA 1/2 or FLAG-tagged ESE-1 using the 25-kDa L-polyethylenimine (L-PEI) transfection reagent (Polysciences, Warrington, PA, USA), as described previously [24]. pGSH1-GFP-Luc shRNA and FLAG-tagged pcDNA3.1+ vectors were used as controls. Total RNA was isolated from NCCIT cells (naive or differentiated; transfected with ESE-1 or ESE-1 shRNA 1/2) and subjected to qRT–PCR, as previously described [23]. The primers for quantitative RT-PCR were as follows: ESE-1 (amplicon, 261 bp), 5′-CACTGATGGCA AGCTCTTC-3′ (forward) and 5′- GGAGCGCAGGAACTTGAAG-3′ (reverse) [25]; OCT4 (97 bp), 5′-CCCCTGGTGCCGTGAA-3′ (forward) and 5′-GCAAATTGCTCGAGTTCTTTCTG-3′ (reverse); GAPDH (226 bp), 5′-GAAGGTGAAGGTCGGAGTC -3′ (forward) and 5′-GAA GATGGTGATGGGATTTC-3′ (reverse). GAPDH cDNA was amplified from all samples as a normalization control. PCR conditions were as follows: 94 °C for 10 min; 40 cycles of 94 °C for 15 s and 60 °C for 30 s; and 72 °C for 30 s. Relative quantification of the expression levels was determined using the $2^{-\Delta\Delta Ct}$ method [26].

2.4. Transient transfection and reporter assays

Transfection and reporter assays were performed using NCCIT cells, as described previously [27]. In all figures, data are expressed relative to the activity of pGL3-basic, which was used as a negative control.

2.5. Western blot analysis

HEK293T cells were transfected with FLAG-tagged ESE-1 WT, ΔPTD, ΔSAR, and ΔTAD expression vectors using a 25-kDa L-PEI (Polysciences) and harvested 48 h after transfection, as previously reported [24]. Western blot was performed using an anti-Flag monoclonal (1:2500, Sigma), as previously described [23].

2.6. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed using naive, ESE-1 overexpressed and differentiated NCCIT cells, as described previously with minor modifications [23]. Primer sequences were as follows: [−2613 to −2396, depicted as 'a': (forward) 5′-GGGGAACCTGGAGGATGGC AAGCTGAGAAA-3′ and (reverse) 5′-GGCCTGGTGGGGGTGGGAGG AACAT-3′], [CR2 (−1513 to −1316), depicted as 'b': (forward) 5′-T GAGGGGATTGGGACTGGGG-3′ and (reverse) 5′-TATCTGACTTCAG GTTCAAA-3′], [−237 to −136, depicted as 'c': (forward) 5′-GAGGG GCGCCAGTTGTGTCTCCCGGTTTT-3′ and (reverse) 5′-GGGAGGTG GGGGGAGAAACTGAGGCGAAGG-3′].

2.7. Statistical analysis

All experiments were performed independently at least three times. Data shown in figures are means ± standard deviations (SD) over all trials. The data were analyzed by *t*-test or ANOVA with Duncan's multiple range procedure for multiple comparisons, using the SigmaPlot 10 program (Systat Software, San Jose, CA, USA). In all experiments, $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Expression analysis of ESE-1 and OCT4 in NCCIT cells upon differentiation, overexpression, and knockdown of ESE-1

First, we analyzed the expression patterns of OCT4 and ESE-1 in pluripotent human EC NCCIT cells that had been treated with 10 μM RA for 10 days to induce differentiation. qRT-PCR analysis revealed high levels of OCT4 expression in undifferentiated NCCIT cells, which significantly decreased after RA treatment. By contrast, ESE-1 expression significantly increased in the differentiated cells (Fig. 1A), suggesting that expression levels of OCT4 and ESE-1 are regulated in an opposing manner that depends on differentiation status. To further analyze the role of ESE-1 in OCT4 gene expression, we examined the effect of overexpression and knockdown of ESE-1 on OCT4 gene expression, as determined by qRT-PCR (Fig. 1B). Expression of endogenous OCT4 was significantly increased by shRNA-mediated knockdown of ESE-1 and decreased by overexpression of ESE-1, suggesting that ESE-1 negatively regulates OCT4 gene expression in NCCIT cells. RA plays an important role in the regulation of biological events such as embryogenesis, differentiation, and homeostasis [28,29]. OCT4 expression is repressed during RA-mediated differentiation in both EC and ES cells [30]. ESE-1 expression is up-regulated by RA treatment during differentiation of leukemic cells [31]. In addition, treatment of EC cells with RA represses the expression of several oncogenic factors and further suppresses the tumorigenicity, suggesting a link between enforced pluripotency and transformation [32]. In this report, we first describe the relationship between expression of OCT4 and ESE-1 in pluripotent cells upon RA-mediated differentiation, and demonstrate the effect of ESE-1 knockdown on OCT4 expression.

3.2. OCT4 promoter activity was repressed by ESE-1 in a manner dependent upon the N-terminal TAD of ESE-1

To determine whether ESE-1 regulates OCT4 expression at a transcription level in NCCIT cells, an OCT4 promoter–reporter vector (-2601-Luc) was co-transfected along with ESE-1 WT and its deletion derivatives (Fig. 2A). Ahead of these experiments, the expressions of the constructs were confirmed by Western blot analysis (Fig. 2B). Promoter–reporter assays revealed that transcription of OCT4 significantly decreased in a dose-dependent manner when ESE-1 was overexpressed, suggesting that ESE-1 acts as a potent repressor of the OCT4 promoter (Fig. 2C). ESE-1 deletion mutants such as ΔPTD or ΔSAR retained the repressive effect on OCT4 promoter activity and decreased OCT4 expression to the same extent as ESE-1 WT. However, OCT4 promoter activity was restored by overexpression of an ESE-1 mutant lacking the TAD (ΔTAD), even in the presence of exogenous ESE-1 WT (Fig. 2D). These findings support the notion that the reduction in transcription of OCT4 described above was due to expression of ESE-1 and furthermore, that the ESE-1 TAD mediates the repressive effect on the OCT4 promoter. These data suggest that the high level of OCT4 promoter activity in naive NCCIT cells was down-regulated by ESE-1 via a mechanism that involves the N-terminal TAD.

3.3. Effects of ESE-1 on serial deletion mutants of the OCT4 promoter

The OCT4 promoter contains four regions that are conserved between human and mouse [CR1–4; 22,33]. We previously reported that deletion of the upstream region of the OCT4 promoter spanning bases −1588 to −1427, which partially overlaps with CR2, resulted in significant down-regulation of promoter activity, suggesting the presence of important regulatory element(s) [22]. Therefore, we analyzed these sequences using the TFSEARCH database (http://mbs.cbrc.jp/research/db/TFSEARCH.html) and found a putative ETS binding element in this region. First of all, we co-transfected a series of deletion mutants of the OCT4 promoter into NCCIT cells along with ESE-1 expression vector (Fig. S1A). The activity of the -1588-Luc construct was significantly diminished by co-expression of exogenous ESE-1. By contrast, constructs containing deletions further downstream retained activity regardless of exogenous ESE-1 expression (Fig. S1B). This observation suggests that the region between −1588 and −1427 contains an EBS, with the consensus sequence (GGAA/T) recognized by a variety of ETS family members, and that ESE-1 may interact with this putative regulatory region.

3.4. OCT4 promoter CR2 is important for transcriptional regulation by ESE-1 via the direct interaction in vivo

To further investigate the importance of the putative EBS (containing the GGAA consensus sequence) within CR2, we used the CR2–ti-Luc construct, described previously [21]. In a derived construct, the putative EBS ($^{-1482}$AGGAAG^{-1477}) within CR2 (−1512 to −1316) was replaced with the XbaI restriction site (TCTAGA; CR2*), and the resulting sequence was inserted upstream of the gene-encoding luciferase, under the control of the minimal ti promoter [20], to yield the CR2*-ti-Luc construct. We co-transfected each of these reporter vectors into NCCIT cells along with ESE-1 expression vector, and observed the effect on transcriptional activity (Fig. 3A). In the presence of ESE-1, the activity of the CR2-ti-Luc was reduced, whereas the CR2*-ti-Luc construct retained its basal activity, suggesting that the ESE-1 negatively controls OCT4 promoter activity via the putative EBS within CR2 (Fig. 3B).

To further determine whether ESE-1 can directly interact with CR2 (containing the putative EBS) in NCCIT cells, we performed ChIP assays (Fig. 4). First of all, to facilitate the recruitment of ESE-1 bound chromatin, we prepared chromatin from NCCIT cells

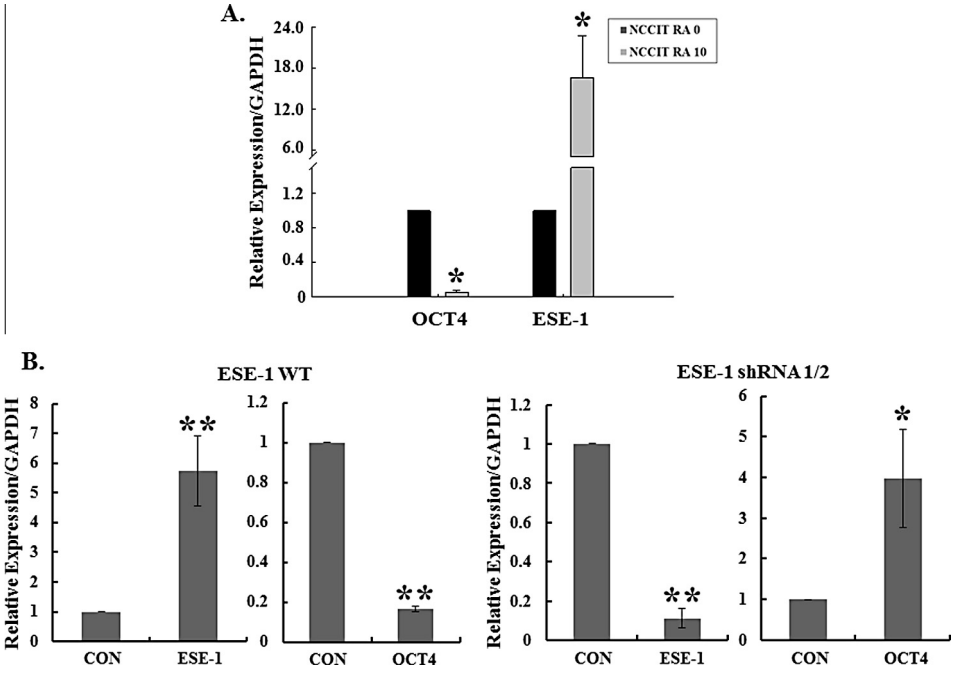

Fig. 1. Expression analysis of OCT4 and ESE-1 in NCCIT cells. (A) Levels of ESE-1 and OCT4 mRNA were measured by qRT-PCR in undifferentiated and differentiated NCCIT cells. Transcript levels were normalized to GAPDH mRNA level. (B) NCCIT cells were transfected with FLAG-tagged ESE-1 or pGSH1-GFP-ESE-1 shRNA 1/2. FLAG-tagged pcDNA3.1 and pGSH1-GFP-Luc vectors were used as controls. Levels of ESE-1 and OCT4 mRNAs were measured by qRT-PCR, normalized against the level of GAPDH in the same samples, and further normalized to the levels in control transfectants. $^{*}P < 0.05$; $^{**}P < 0.01$.

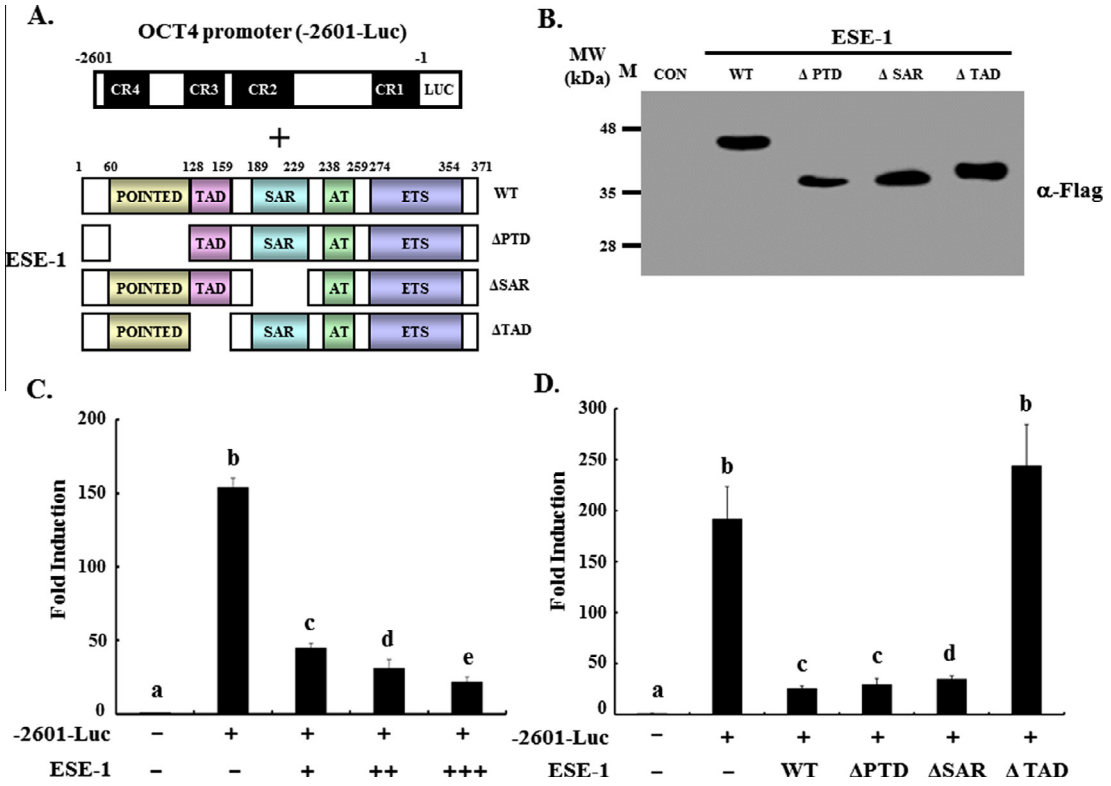

Fig. 2. OCT4 promoter activity was repressed by ESE-1 in a manner dependent upon the N-terminal TAD of ESE-1. (A) Schematic representation of the OCT4 promoter-reporter vector (-2601-Luc) and ESE-1 wild-type (WT) and deletion-mutant derivatives tagged with the FLAG epitope (WT, ΔPTD, ΔSAR, ΔTAD). Four conserved regions of the OCT4 promoter and the functional domains of ESE-1 are indicated by boxes. (B) The expression of ESE-1 WT and derivatives was confirmed by Western blotting using anti-FLAG antibody. (C) The OCT4 promoter reporter (-2601-Luc) was co-transfected into NCCIT cells along with increasing amounts of ESE-1 expression vector. (D) The OCT4 promoter reporter (-2601-Luc) was co-transfected with ESE-1 WT or deletion-mutant derivatives. Values labeled with different letters (a–e) are significantly different from one another ($P < 0.05$).

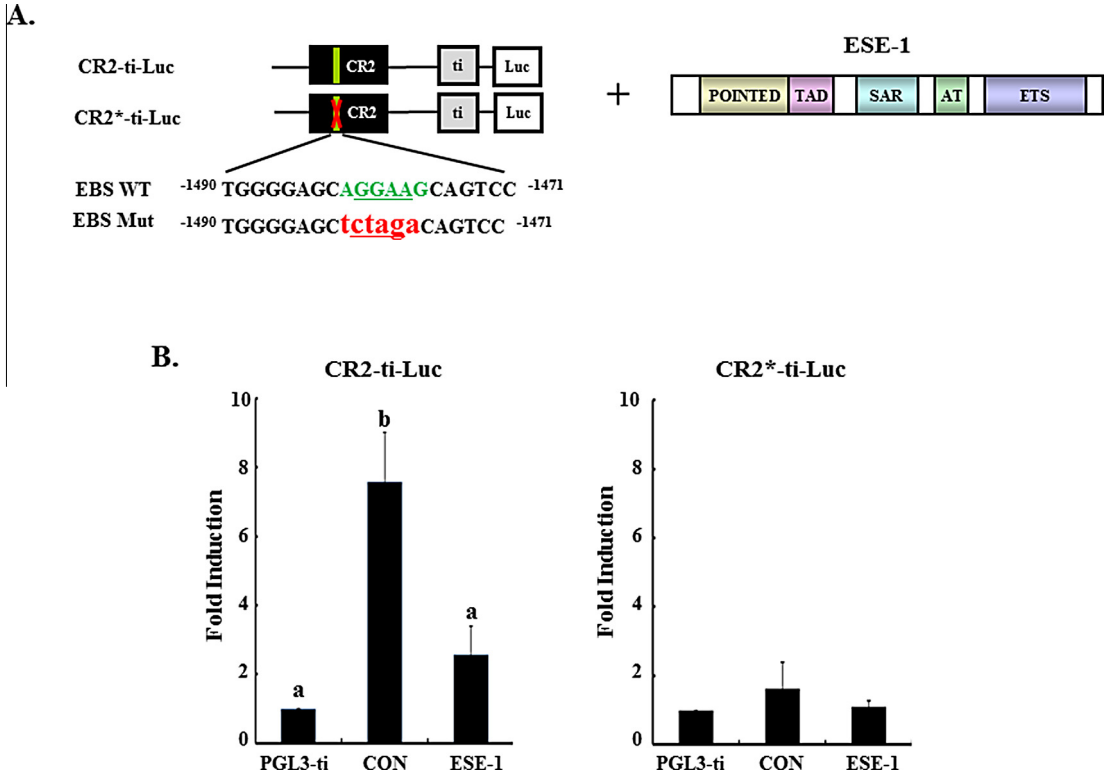

Fig. 3. OCT4 promoter CR2 is important for transcriptional regulation by ESE-1. (A) Schematic representation of the exogenous minimal ti reporter vector (CR2-ti-Luc), a mutant in which the ETS binding site (EBS) within CR2 is disrupted (CR2*-ti-Luc), and the ESE-1 expression vector. The box in black indicates the putative EBS; site-directed mutagenesis of the EBS is indicated by an X. (B) Each reporter vector was co-transfected with ESE-1 expression vector. Values labeled with different letters (a, b) are significantly different from one another ($P < 0.05$).

transfected with ESE-1 expression vector, pulled down ESE-1-bound chromatin with an anti-PEA3 antibody, and amplified the region containing the putative ESE-1-binding site by real-time PCR with CR2-specific primers. Three pairs of primers were used: one pair to amplify CR2 (−1513 to −1316, depicted as 'b'), which spans the putative EBS, and two pairs to amplify other sites (−2613 to −2396, depicted as 'a'; and −237 to −136, depicted as 'c') used as negative controls for ESE-1 recruitment (Fig. 4A). In NCCIT cell overexpressing ESE-1, region b (−1513 to −1316), which contains the putative EBS, was significantly enriched compared to regions a and c (Fig. 4B). To further functionally validate the interaction of OCT4 promoter with ESE-1 depending on differentiation status, NCCIT cells were treated with RA for 10 days and subjected to ChIP and quantitative PCR analyses. As shown in Fig. 4C, in undifferentiated NCCIT cells, there were no statically significant differences between the regions (a,b,c), respectively. However, enrichment of CR2 (region b) significantly increased, compared to other regions a and c in RA-mediated differentiated NCCIT cells (Fig. 4C). Those results demonstrate that ESE-1 can directly bind to the putative EBS within CR2; this binding is likely to be involved in ESE-1-mediated repression of OCT4 promoter activity during differentiation. Next, to examine the direct binding of ESE-1 to this element in the OCT4 promoter CR2, EMSA was performed (Fig. S2). When the nuclear extract from ESE-1 overexpressed HEK293T cells and in vitro translated ESE-1 were added to the ^{32}P-labeled EBS probe, protein-DNA complexes were observed (lane 2 and 5). The specificity of ESE-1 binding to the EBS was confirmed by an EMSA supershift (lane 3 and 6).

It has been known that ESE-1, plays a role in the activation of type II TGF-β receptor gene in differentiated mouse EC cells and lung lysozyme in epithelial cells [34,35]. ESE-1 also potently represses transcription of the type II collagen gene in human chondrocytes [36]. Moreover, ESE-1 suppresses the promoter of the basal keratin 4 gene, but activates the SPRR2A promoter [37], suggesting that ESE-1 may act as a repressor as well as activator depending on a promoter context. Furthermore, it has been demonstrated that disruption of the TAD relieves the inhibitory effect and enhances binding to DNA [38]. Recently, we have demonstrated that ESE-1 expression was significantly elevated upon RA treatment in NCCIT cells. Furthermore, we also accessed the proliferation potentials of NCCIT cells. Overexpression of ESE-1 decreased proliferation, while shRNA-mediated ESE-1 knockdown increased growth significantly, suggesting that ESE-1 delays proliferation by inhibiting progression from G1 to S and may act as a repressor, respectively, of cell-cycle progression and growth of NCCIT cells [39]. Moreover, it has been reported that suppression of ETS factors Etsrp71, Elf3, and Spic reduced the transcription of the Oct3/4 gene in the embryos, suggesting that ETS factors play important roles in preimplantation development [19]. However, little is known about the molecular mechanism of OCT4 gene expression regulated by ESE-1. Therefore, the results of this study reveal the molecular details of the mechanism by which the oncogenic factor ESE-1 regulates expression of the stem cell transcription factor OCT4 in pluripotent EC cells.

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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.2014.06.079.

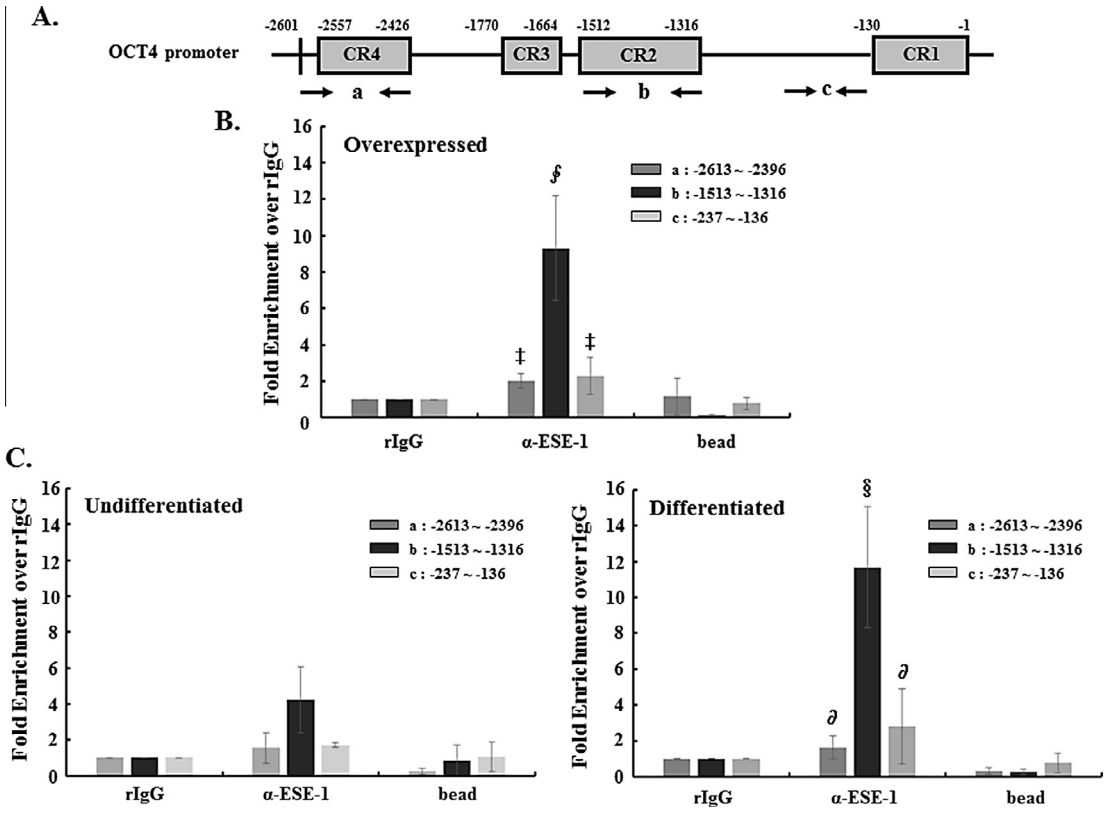

Fig. 4. ESE-1 interacts with the putative EBS in OCT4 promoter CR2 *in vivo* and enrichment of CR2 was significantly increased upon RA-mediated differentiation. (A) Schematic diagram of the locations of OCT4 promoter-specific primer pairs (B) Chromatin enrichment of the putative ESE-1 binding site in NCCIT cells expressing exogenous ESE-1. $^{\ddagger,\text{ƒ}}P < 0.05$. (C) Differentiation of NCCIT cells were induced by RA treatment for 10 days and subjected to ChIP and quantitative PCR analyses. $^{\S,\partial}P < 0.05$.

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