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Transcriptional Regulation of Human Oct4 by Steroidogenic Factor-1

Heung-Mo Yang,[1,4] Hyun-Jin Do,[1] Dong-Ku Kim,[2] Jin-Ki Park,[3] Won-Kyong Chang,[3] Hyung-Min Chung,[2] Sang-Yun Choi,[4] and Jae-Hwan Kim[2]***

[1]Chabiotech Co. Ltd, Seoul, South Korea
[2]CHA Stem Cell Institute, Graduate School of Life Science & Biotechnology, Pochon CHA University, Seoul, South Korea
[3]Animal Biotechnology Division, National Livestock Research Institute, RDA, Suwon, South Korea
[4]School of Life Sciences and Biotechnology, Korea University, Seoul, South Korea

Abstract Oct4 encodes a transcription factor that is involved in the maintenance of self-renewal in stem cells. Recently, the molecular mechanisms that regulate Oct4 expression have come under investigation. In this study, we demonstrate that the orphan nuclear receptor steroidogenic factor-1 (SF-1) behaves as a transcriptional activator of human Oct4 (hOct4) through direct interaction with a SF-1 binding element in the hOct4 proximal promoter. We found that Oct4 and SF-1 were co-expressed in undifferentiated human embryonal carcinoma NCCIT cells and downregulated during retinoic acid-mediated differentiation. We examined the functional role played by SF-1 in regulation of hOct4 transcription using a luciferase reporter assay and Western blot analysis. Overexpression of SF-1 increased up to about threefold hOct4 promoter activity and endogenous hOct4 protein expression. Sequence analysis of the hOct4 promoter revealed that the transcriptional activity was closely linked to Conserved Regions 1 (CR1) and 2 (CR2), which contain three putative SF-1-binding sites (1st, 2nd, and 3rd SF-1). Binding assays and mutagenesis of binding sites indicated that the 1st and 2nd SF-1 elements (in CR1 and CR2, respectively) might be important cis-regulatory elements in hOct4 promoter activity. However, differences in response to SF-1 overexpression between wild-type and mutant hOct4 promoters revealed that the 1st SF-1 element is the key binding site for SF-1-mediated transcriptional activation. Thus, our data indicate that SF-1 plays a crucial role in the regulation of hOct4 transcription through direct binding to the 1st SF-1 in CR1 of the hOct4 proximal promoter. J. Cell. Biochem. 101: 1198–1209, 2007.

Key words: embryonic carcinoma cells; Oct4 promoter; SF-1

In pluripotent cells, self-renewal and pluripotency is regulated by multiple signaling molecules and transcription factors. These include the signaling molecules such as leukemia inhibitory factor, bone morphogenic protein, fibroblast growth factor 4 (FGF4) and FGF4 receptor [Williams et al., 1988; Feldman et al., 1995; Niwa et al., 1998; Ying et al., 2003], and transcription factors such as Oct4, Nanog and Sox2 [Ben-Shushan et al., 1998; Nishimoto et al., 1999; Tomioka et al., 2002; Mitsui et al., 2003; Tokuzawa et al., 2003]. Investigation of the mechanisms underlying regulation of transcription factors is essential for understanding embryonic development and cellular differentiation.

Oct4 is a member of the POU homeodomain family transcription factors that both positively and negatively regulate expression of various genes critical for pluripotency and differentiation [Niwa et al., 2000; Kuroda et al., 2005]. Oct4 expression is restricted in pre-implantation embryos or pluripotent stem cells such as

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*Correspondence to: Jae-Hwan Kim, PhD, CHA Stem Cell Institute, Graduate School of Life Science & Biotechnology, Pochon CHA University, 606-16 Vision BLDG 4th Floor, Yeoksam 1 dong, Kangnam-Gu, Seoul 135-907, South Korea. E-mail: jaehwan_k@yahoo.com, jaehwan_k@cha.ac.kr

**Correspondence to: Sang-Yun Choi, PhD, School of Life Sciences and Biotechnology, Korea University, 5-1 Anam-dong, Sungbuk-Gu, Seoul 136-701, South Korea.
E-mail: sychoi@korea.ac.kr

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embryonic stem (ES), carcinoma (EC), and germ (EG) cells [Rosner et al., 1990]. In ES and EC cells, Oct4 expression is downregulated during embryonic development and under in vitro retinoic acid (RA) treatment. RA has multiple effects on development and differentiation [Mendelsohn et al., 1992] and RA-mediated signals are transferred to members of the nuclear hormone receptor superfamily, such as retinoic acid receptors (RARs) and retinoid X receptors (RXRs). In mice, Oct4 (mOct4) expression is regulated directly by nuclear receptors (NRs) through cis-acting elements. These NRs include germ cell nuclear factor (GCNF), RARs/RXRs, chicken ovalbumin upstream promoter-transcription factors (COUP-TF) I/II, steroidogenic factor 1 (SF-1) and liver receptor homolog 1 (LRH-1) [Ben-Shushan et al., 1995; Barnea and Bergman, 2000; Fuhrmann et al., 2001; Gu et al., 2005].

There are about 48 members of the NR transcription factor superfamily in the human genome; this group can be divided further into seven subfamilies (NR0 to NR6). About half of these lack identified ligands and are considered orphan nuclear receptors. The orphan SF-1 belongs to the NR5A subfamily (NR5A1) [Nuclear Receptors Nomenclature Committee, 1999]. It is expressed primarily in hypothalamic pituitary-adrenal/gonadal axis tissues and plays a critical role in control of the endocrine development and differentiation of steroidogenic tissues, as well as steroidogenesis and sex determination [Luo et al., 1994; Val et al., 2003]. For example, in the ventromedial hypothalamic nucleus and pituitary gonadotrope, SF-1 controls expression of the α-subunit of glycoprotein hormones [Barnhart and Mellon, 1994], the β-subunit of luteinizing hormone [Keri and Nilson, 1996] and the GnRH receptor [Duval et al., 1997]. In the adrenal cortex and gonads, SF-1 controls expression of the Mullerian-inhibiting substance [Shen et al., 1994], ACTH receptor [Cammas et al., 1997], and mitochondrial cholesterol transportation protein StAR [Sugawara et al., 1997]. Recently, it has been reported that SF-1 and RAR synergistically activate the mOct4 promoter [Barnea and Bergman, 2000]. In addition, the orphan nuclear receptor LRH-1 recognizes the SF-1 binding sequence and plays an essential role in regulating mOct4 expression in mouse ES cells at the epiblast stage of embryonic development [Gu et al., 2005].

In this study, we investigated the functional role of SF-1 in human Oct4 (hOct4) expression. We demonstrated that SF-1 transactivates hOct4 promoter activity specifically through direct interaction with a putative SF-1 binding element and elevates endogenous hOct4 protein expression level. In addition, we observed that the DNA-binding activity of a transcription factor does not necessarily relate proportionately to its transactivation activity. Our results indicate that SF-1 is a transcriptional activator of hOct4.

MATERIALS AND METHODS

Cell Culture

Human embryonal carcinoma (hEC; NCCIT; American Type Cell Collection, Manassas, VA) cells were maintained in Dulbecco's Modified Eagle's medium (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 IU penicillin and 100 μg/ml streptomycin. Cells were grown to confluence at 37°C in a humidified atmosphere with 5% CO_2 and subcultured every 3 days.

Plasmid Construction

The luciferase reporter constructs that contain regions upstream of the hOct4 promoter, that is -2601-Luc, -1588-Luc (D3 mutant) and CR1-Luc (D6 mutant), were described previously [Yang et al., 2005]. The CR2 reporter construct was prepared by PCR amplification of the CR2 region using the primers 5′-ATTGGTACCGGGGATTGGGACTGGGGGGTT-3′ (forward) and 5′-TTACTCGAGAAAAAATATCTGACTTCAGGT-3′ (reverse), then inserted into pGL3ti, upstream of the minimal promoter [Jonk et al., 1998]. We performed site-directed mutagenesis to disrupt putative SF-1 binding elements using the "QuickChange" method (Stratagene, La Jolla, CA). An *Xba*I site (bold) was introduced into the primers used for mutation of the SF-1 binding elements (mutant bases are in lower case). The primers were as follows: 1st SF-1 mutation, 5′-CGGGGCCAGAGG**TCtAGa**CTAGTGGGTGGG-3′; 2nd SF-1 mutation, 5′-GATTGTCCAGCC**tctaga**ATTGTCCTGCCC-3′; and 3rd SF-1 mutation, 5′-CCAGGCCCATTC-**tctaGa**TGAGCACTTGTT-3′. Since SF-1 is a highly conserved protein in different species and human SF-1 shares 100% identities to mouse SF-1 (mSF-1) DNA binding domain, NLS, and transactivation domain amino acid

sequences, full-length mSF-1 cDNA was obtained from reverse transcription-PCR (RT-PCR) of total RNA from mouse ovary, using the primers 5′-GACGGATCCATGGACTATTCGTACGACGAG-3′ (forward) and 5′-GGAAAGCTTTCAAGTCTGCTTGGCTTGCAGC-3′ (reverse), followed by insertion into Flag-tagged pcDNA3.1+, as described previously [Yang et al., 2005]. A dominant negative form of SF-1 (SF-1 DN; Fig. 2B) [Wei et al., 2002] was generated by deletion of amino acids 443 to 462 by PCR amplification of the mSF-1 expression vector using the reverse primer 5′-GACCTCGAGTCAGTGCTTGTGGTACAGGTA-3′, followed by insertion into Flag-tagged pcDNA3.1+. All cloned PCR products and reporter plasmids were verified by sequencing.

Transient Transfection and Reporter Assays

Transient transfection of 2×10^5 NCCIT cells with plasmid DNA (1.0–1.4 μg per well) was performed in 6-well culture plates using the Exgen500 in vitro Transfection Reagent (Fermentas, Hanover, MD) according to the manufacturer's protocols. Reporter plasmid luciferase activities were normalized against the β-galactosidase activity of a co-transfected internal control plasmid (pcDNA3.1/hygro/LacZ; Invitrogen, La Jolla, CA). Cells were harvested 48 h after transfection and luciferase activities measured using the Bright-Glo Luciferase assay system (Promega, Madison, WI) and a Genios luminometer (TECAN, Salzburg, Austria). Transfection and reporter assays were performed in duplicate and repeated independently at least three times.

Electrophoretic Mobility Shift Assay (EMSA)

In vitro translation of SF-1 and SF-1 DN was performed using the TNT Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's protocols and expression was confirmed by Western blot analysis. In each binding reaction, 3 μl in vitro translation product was mixed with radiolabeled oligonucleotides corresponding to the SF-1 binding element in 15 μl reaction mix (10 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA, 10% glycerol, 1 μg poly dG:dC, 1 mM dithiothreitol, 1 nM phenyl-methylsulphonyl fluoride, and 0.5 μg BSA). For competition or supershift experiments, reactions were incubated with unlabeled competitor oligonucleotide or anti-SF-1 antibody (Upstate, Charlottesville, VA) prior to addition of the probe. DNA–protein complexes were separated from free probe by native 5% polyacrylamide gel electrophoresis (PAGE), dried and visualized by autoradiography. The sequences of probes used for EMSA are presented in Figure 4A.

Western Blot Analysis

In vitro-translated SF-1 and SF-1 DN proteins were separated by 10% SDS–PAGE, transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ), blocked in 5% non-fat milk. In vitro-translated SF-1 and SF-1 DN proteins were incubated with anti-SF-1 polyclonal antibody (1:5,000; Upstate). To determine Oct4 expression as well as SF-1, whole lysates were prepared from control or SF-1 overexpressed NCCIT cells and the blots were incubated with anti-SF-1 antibody, striped and then incubated again with anti-Oct4 monoclonal antibody (1:5,000, Santa Cruz) or anti-β-actin monoclonal antibody (1:5,000, Sigma, St. Louis, MO) separately. All membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min and detected by using an ECL detection kit (Santa Cruz).

RNA Preparation and RT-PCR

In order to analyze the expression patterns of SF-1 and Oct4 during NCCIT cell differentiation, cells were treated with 10 μM RA (Sigma), then harvested at different time points. Total RNA was isolated with Trizol reagent (Invitrogen) and cDNAs for SF-1, Oct4, and β-actin were synthesized from 3 μg total RNA using oligo dT primer (Promega) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). RT-PCR products were normalized using β-actin amplification. The following primer sequences were used: Human SF-1 (372 bp), 5′-TGCCATCAAGTCTGAGTAC-3′ (forward) and 5′-TAGATGTGGTCGAACACC-3′ (reverse); Human Oct4 (250 bp), 5′-CGTGAAGCTGGAGAAGGAGA-3′ (forwawrd) and 5′-CAAGGGCCGCAGCTTACACA-3′ (reverse); and Human β-actin (433 bp), 5′-CAACATGGATGATGATATCG-3′ (forward) and 5′-TGGATAGCAACGTACATGG-3′ (reverse). PCR amplifications were performed as follows: hSF-1, 40 cycles of 30 s at 94°C, 30 s at 45°C, and 30 s at 72°C; hOct4, 30 cycles of 30 s at 94°C, 30 s at 53°C, and 30 s at 72°C; and hβ-actin, 35 cycles of 30 s at 94°C, 30 s at 50°C, and

30 s at 72°C. Reaction products were visualized on 1.5% agarose gels.

Statistical Analysis

The data for promoter activity were analyzed by ANOVA using General Linear Models procedures of the Statistical Analysis System (SAS Institute, Gary, NC). Means were compared by Duncan's multiple range procedure for multiple comparisons. In all experiments, $P < 0.05$ was considered statistically significant.

RESULTS

SF-1 and Oct4 Are Co-Expressed in Undifferentiated NCCIT Cells and Downregulated Upon RA Treatment

To investigate the functional relationship between Oct4 and SF-1, we analyzed their expression in undifferentiated and differentiated NCCIT cells. To induce differentiation, NCCIT cells were treated with 10 μM RA and collected at different timepoints (1, 2, 4, 6, 8, 10 days). To examine the expression level of SF-1 and Oct4 in NCCIT cells, we performed Northern and Western blot analyses. Unfortunately, endogenous SF-1 mRNA and protein were undetectable throughout the experiments while Oct4 mRNA and protein were gradually decreased upon RA treatment (data not shown). To identify the presence of SF-1 as well as Oct4, we employed RT-PCR analysis and detected SF-1 and Oct4 in undifferentiated and differentiated NCCIT cells. The mRNA expression level of SF-1 and Oct4 was reduced gradually upon RA treatment. Following 8 days RA treatment, Oct4 expression had decreased dramatically and SF-1 expression was almost undetectable (Fig. 1). Co-expression of these genes and their concomitant downregulation during RA-induced differentiation support the idea that Oct4 and SF-1 may have a functional relationship and a coordinated regulation mechanism.

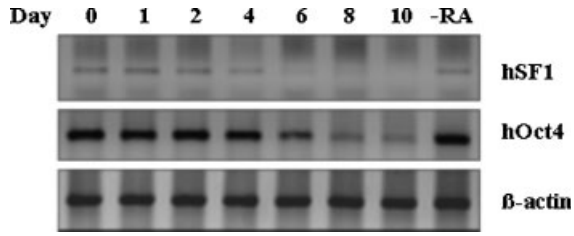

Fig. 1. Expression of SF-1 and Oct4 during RA-mediated differentiation of NCCIT cells. NCCIT cells were treated with 10 μM RA for the times indicated. '−RA' is only solvent (Ethanol) treated group during 10 d. Reverse transcription was performed on 3 μg total RNA, followed by PCR amplification with hSF-1- and hOct4-specific primers. PCR products were analyzed on 1.5% agarose gels. Human β-actin mRNA expression was used as the loading control.

hOct4 Promoter Can Be Activated by Exogenous SF-1 Overexpression in NCCIT Cells

In order to examine the role of SF-1 in transcriptional regulation of hOct4, we used a construct containing the upstream promoter region of hOct4 (−2.6 kb) that was reported previously (Fig. 2A) [Yang et al., 2005], as well as a SF-1 expression vector (Fig. 2B). SF-1 expression was confirmed by Western blot analysis (Fig. 2C). To monitor the effect of SF-1 on hOct4 promoter activity, we performed a transient co-transfection of NCCIT cells with the hOct4 promoter-containing luciferase reporter (-2601-Luc) and increasing amounts of the SF-1 expression plasmid. With increasing expression of SF-1, the hOct4 promoter reporter exhibited a dose-dependent increase in transcriptional activity, which reached about three-fold higher levels than in the absence of SF-1 expression (Fig. 3A). To confirm that the hOct4 promoter stimulation was SF-1 specific, we generated the expression vector SF-1 DN, containing a deletion of the transactivation domain (AF-2; amino acids 443 to 462), which is required for transcriptional activation [Lala et al., 1997]. SF-1 DN expression was confirmed by Western blot analysis (Fig. 2C). We performed a transient co-transfection of NCCIT cells with the Oct4 promoter-containing luciferase reporter (-2601-Luc), the SF-1 expression vector and increasing amounts of the SF-1 DN expression vector. The SF-1-mediated stimulation of luciferase activity was inhibited gradually by increasing levels of SF-1 DN expression (Fig. 3B). In the absence of SF-1 expression, overexpression of SF-1 DN decreased Oct4 promoter activity in a dose-dependent manner (Fig. 3C). Furthermore, to investigate whether endogenous hOct4 protein level is correlated with the overexpression of SF-1 in NCCIT cells, Western blot analysis was performed using equal amount of whole cell lysate after transfection (Fig. 3D). Endogenous SF-1 protein was rarely detectable in NCCIT cells transfected

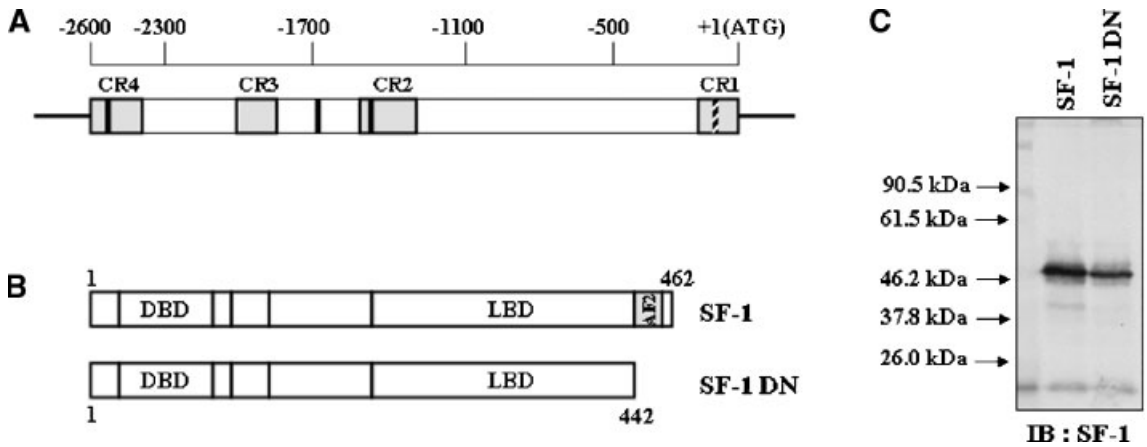

Fig. 2. Schematic representation of hOct4 promoter and SF-1 domain structures. **A**: Schematic representation of the hOct4 5′ upstream region. The nucleotide numbers represent distance from the translational start site +1 (ATG). Conserved regions (CR1-4) are identified as bold and boxed. The dark and hatched lines indicate putative enhancer regions. **B**: The domain structure of SF-1 and SF-1 dominant negative (SF-1 DN). SF-1 contains DNA- and ligand-binding domain (DBD and LBD). SF-1 DN is activation domain (AF2; amino acids 443 to 462) deleted form of SF-1. **C**: Western blot analysis was performed to confirm in vitro synthesis of SF-1 and SF-1 DN. Anti-SF-1 antibody was used for hybridization.

with a control vector (Fig. 3D, lane 1), possibly due to low expression level. Following transient transfection with SF-1 expression vector, we observed more than twofold induction of endogenous hOct4 (wild type and an isoform) expression upon SF-1 overexpression (Fig. 3D, lane 2). These data indicate that SF-1 can act as a transcriptional activator of hOct4 expression.

Direct SF-1 Binding to the hOct4 Promoter

When LRH-1 is expressed in undifferentiated mES cells, it can recognize three SF-1 binding elements located in proximal promoter and enhancer regions (PP and PE, respectively) of the mOct4 promoter [Gu et al., 2005]. The sequence of these sites is identical in the mOct4 and hOct4 promoters (data not shown). Using oligonucleotides containing the putative SF-1 binding elements (termed 1st, 2nd, and 3rd SF-1 and numbered from the translation start site +1(ATG); Fig. 4A), we performed SF-1 binding assay. EMSA indicated that oligonucleotides corresponding to the 1st and 2nd SF-1 elements formed a major complex with SF-1 (Fig. 4B, lanes 1 and 6) and SF-1 DN (Fig. 4B, lanes 5 and 10), whereas only weak binding activity was observed for the 3rd SF-1 site (Fig. 4B, lanes 11 and 15). The specificity of these protein–DNA complexes was verified by addition of a 100-fold molar excess of unlabeled oligonucleotide (Fig. 4B, lanes 2 and 7) and by a supershift assay with anti-SF-1 antibody (Fig. 4B, lanes 3 and 8). These results provide strong evidence that SF-1 is capable of transactivating the hOct4 promoter directly, and also suggest that the 1st and 2nd SF-1 elements may be crucial for this interaction.

Identification of SF-1 Binding Sites That Are Critical for hOct4 Promoter Activity

To establish the functional significance of each putative SF-1 binding element, we performed sequential site-directed mutagenesis, generating the 1st, 2nd, or 3rd SF-1 mutants in hOct4-1588-Luc (Fig. 5). In comparison to hOct4-1588-Luc, we observed a 31, 51, and 26% decrease in promoter activity in the 1st, 2nd, or 3rd SF-1 mutants, respectively. These data suggest that the 2nd SF-1 binding element may play an important role in hOct4 transcriptional activation. We further examined transcriptional activity of the mutants in the presence of exogenous overexpression of SF-1 and observed a significant and dose-dependent increase in transcriptional activity of the 2nd and 3rd SF-1 mutants (Fig. 6C,D, respectively), as well as in the activity of hOct4-1588-Luc (Fig. 6A). In contrast, exogenous SF-1 overexpression did not stimulate transcriptional activity of the 1st SF-1 mutant (Fig. 6B). These data suggest that although both the 1st and 2nd SF-1 elements demonstrated binding activity, the 1st SF-1-binding site appears to be critical for regulation of SF-1-mediated hOct4 transcription activity. In addition, these results imply that the binding activity of a transcription

factor does not necessarily relate directly to the level of transcriptional activity.

Effects of SF-1 on Conserved Regions 1 and 2 (CR1 and CR2)

To examine the direct effect of SF-1 on each SF-1 binding element, we used luciferase reporter constructs with either CR1 or CR2. The CR1 plasmids contained the 1st SF-1 element (CR1-Luc) [Yang et al., 2005] or the 1st SF-1 mutant (CR1*-Luc; Fig. 7A). The CR2 plasmids contained the 2nd and 3rd SF-1 elements (CR2-ti-Luc) or the 2nd SF-1 mutant and 3rd SF-1 elements (CR2*-ti-Luc; Fig. 7B). CR2-ti-Luc was constructed by inserting CR2 upstream of the gene encoding luciferase, which was driven by the minimal ti promoter (adenovirus major late promoter TATA box and mouse terminal deoxynucleotidyl transferase gene initiator sequence) [Jonk et al., 1998]. Each of the reporter constructs was co-transfected into NCCIT cells with increasing amounts of SF-1 expression vector. Although SF-1 effected a dose-dependent stimulation of CR1-Luc

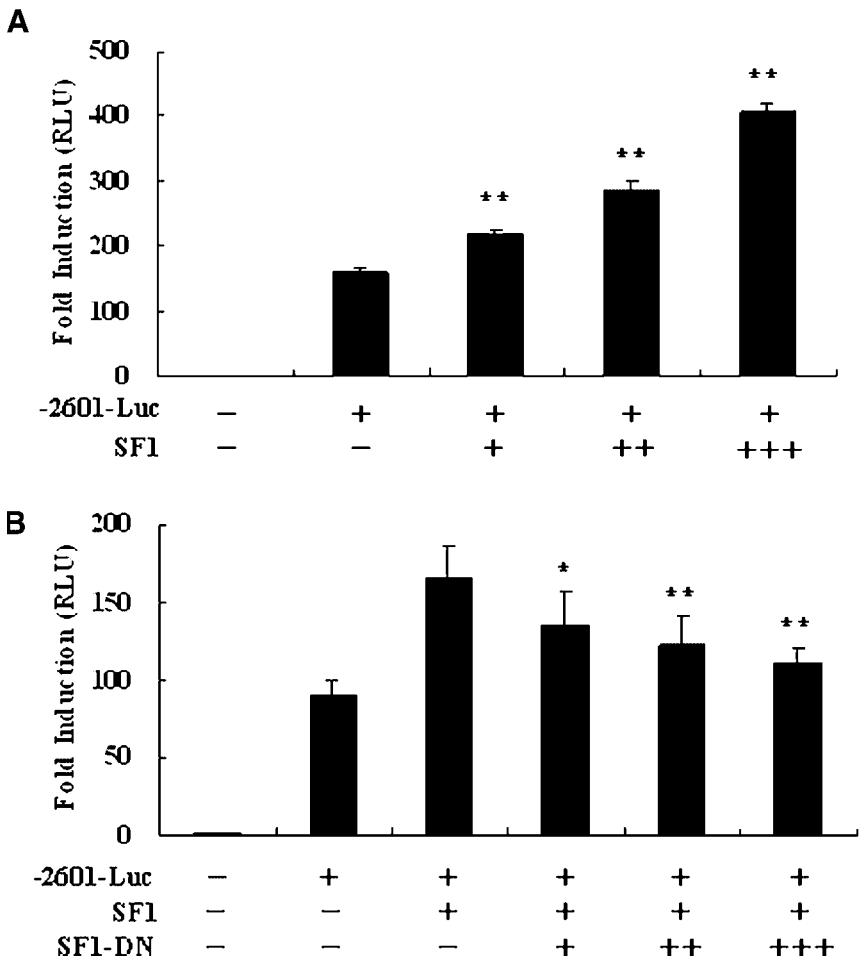

Fig. 3. SF-1 acts as a transactivator of hOct4 promoter activity. **A**: NCCIT cells were transiently co-transfected with the hOct4-2601-Luc (0.3 μg) and SF-1 expression vector (0.2, 0.5, 0.8 μg). **B**: hOct4-2601-Luc (0.3 μg) and 0.2 μg SF-1 expression vector were co-transfected into NCCIT cells with SF-1 DN expression vector (0.2, 0.5, 0.8 μg). **C**: NCCIT cells were co-transfected with hOct4-2601-Luc (0.3 μg) and SF-1 DN expression vector (0.3, 0.5, 0.8 μg). Transfection efficiency was normalized relative to β-galactosidase activity obtained from co-transfection with pcDNA3.1/hyg/LacZ (0.1 μg). The luciferase activity of the hOct4 5′ upstream regions is shown as fold induction relative to that of the empty pGL3-Basic. The data shown are the mean ± SD from three independent experiments that were performed in duplicate. Statistical analyses compared the activity from co-transfection with SF-1 and/or SF-1 DN expression vector to that of empty vector. $^{**}P < 0.001$, $^{*}P < 0.05$. **D**: Western blot analysis was performed using equal amount of NCCIT whole cell lysates (20 μg per lane) transfected with a control or SF-1 expression vector (each 3 μg). Blot was hybridized with anti-SF-1 antibody and then with either anti-Oct4 or anti-β-actin antibody. Band intensity was measured by ImageJ program (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/). Relative fold induction was indicated as numbers in the box, which is normalized to β-actin expression level.

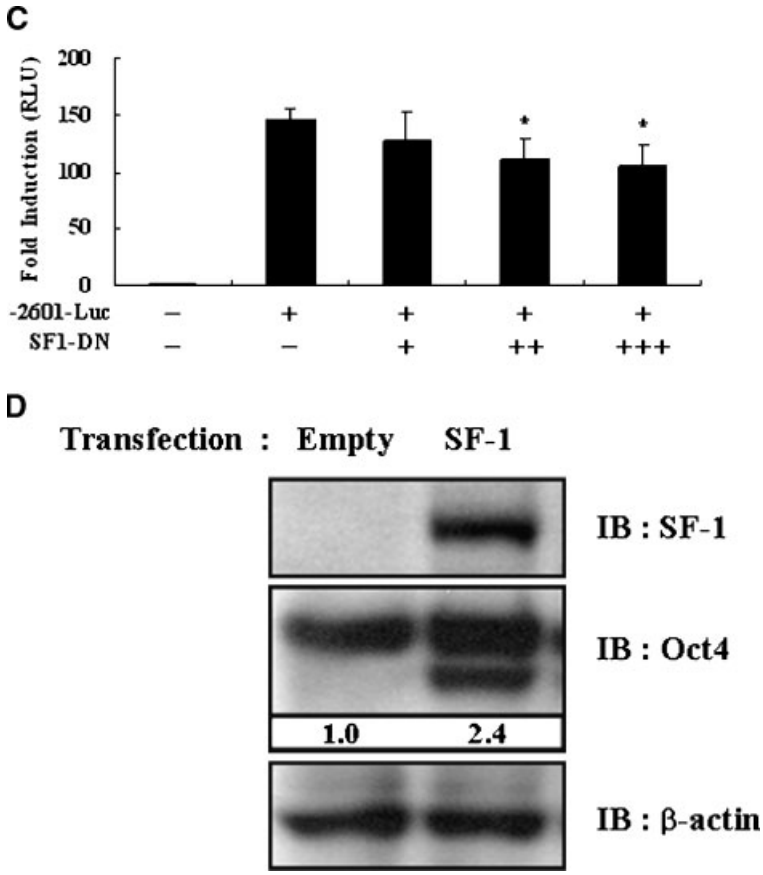

Fig. 3. (*Continued*)

luciferase activity, which increased >threefold at the highest SF-1 level investigated, it failed to recover CR1*-Luc activity (Fig. 7A). In contrast, the promoter activity of CR2-ti-Luc only increased slightly with increasing SF-1 expression, while CR2*-ti-Luc activity rather decreased slightly even with increasing SF-1 expression (Fig. 7B). These data suggest that in NCCIT cells, the 1st SF-1 binding element in CR1 plays a key role in SF-1-mediated stimulation of hOct4 promoter activity.

DISCUSSION

This study represents the first demonstration of a functional role for the orphan nuclear receptor SF-1 in activation of hOct4 expression. We showed direct interaction between SF-1 and the three SF-1 binding elements located in the hOct4 promoter. In addition, through site-directed mutagenesis, we determined that to a varying extent, each of these binding sites acts as a positive cis-regulatory element. Overexpression of SF-1 in the presence of mutant hOct4 promoters or minimal promoters containing only CR1 or CR2, revealed that the 1st SF-1 binding element plays a key role in SF-1-mediated stimulation of hOct4 promoter activity.

RA and its derivatives are involved in the regulation of biological events such as embryogenesis, differentiation, and homeostasis [Mendelsohn et al., 1992; Pikarsky et al., 1994]. Oct4 expression is downregulated during RA-mediated differentiation in both EC and ES cells [Okamoto et al., 1990]; in mice, RA treatment induces downregulation of SF-1 expression, inhibiting SF-1-mediated Oct4 expression [Barnea and Bergman, 2000]. We observed that in undifferentiated NCCIT cells, Oct4 and SF-1 are co-expressed and become downregulated simultaneously during RA-induced differentiation (Fig. 1). This finding suggests both a functional and regulatory relationship between Oct4 and SF-1.

Oct4 is a member of the POU transcription factor family and is critical for maintaining stem cell characteristics as a regulator of

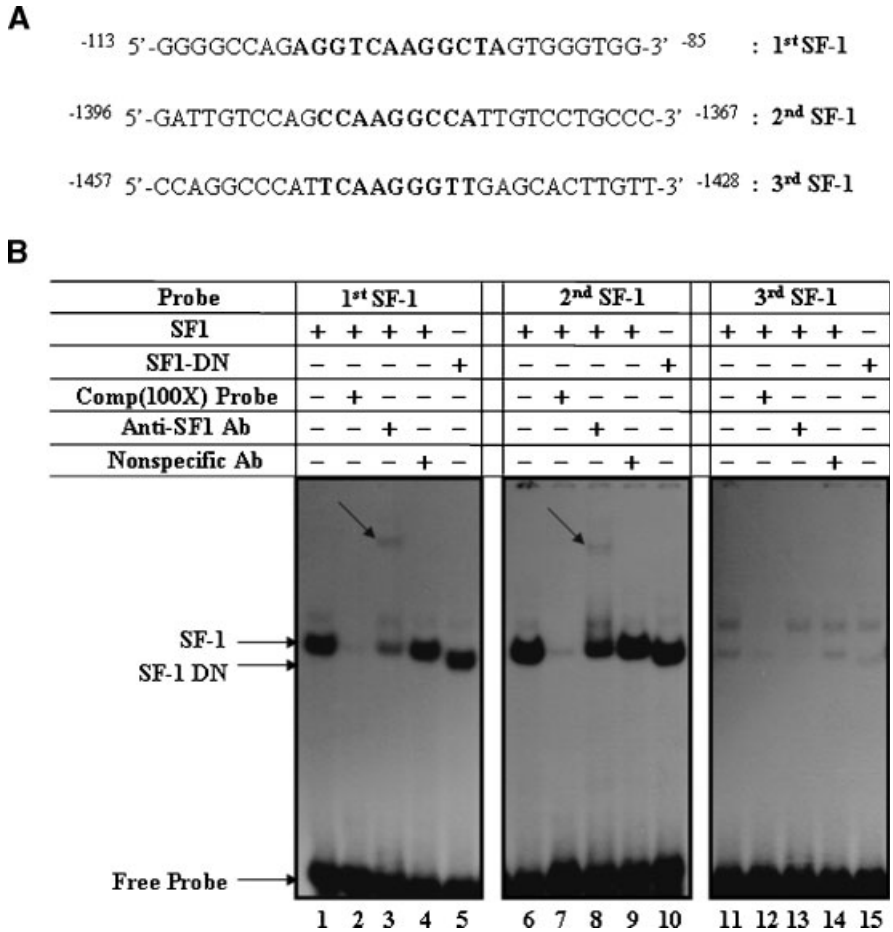

Fig. 4. SF-1 binds to the putative binding elements within the hOct4 promoter. **A**: The hOct4 promoter sequences corresponding to the SF-1 binding elements (1st-, 2nd-, and 3rd SF-1) are shown in bold. The nucleotide numbers represent distance from the translational start site +1 (ATG). **B**: Radiolabeled oligonucleotides were incubated with equal amounts of in vitro-synthesized SF-1 and SF-1 DN. Protein–DNA complexes were indicated by the arrows. A 100-fold molar excess of each unlabeled probe was used as a competitor. A supershift assay was performed using 1 μg of polyclonal rabbit anti-SF-1 antibody or nonspecific polyclonal rabbit antibody. The supershifted bands are indicated by diagonal arrows.

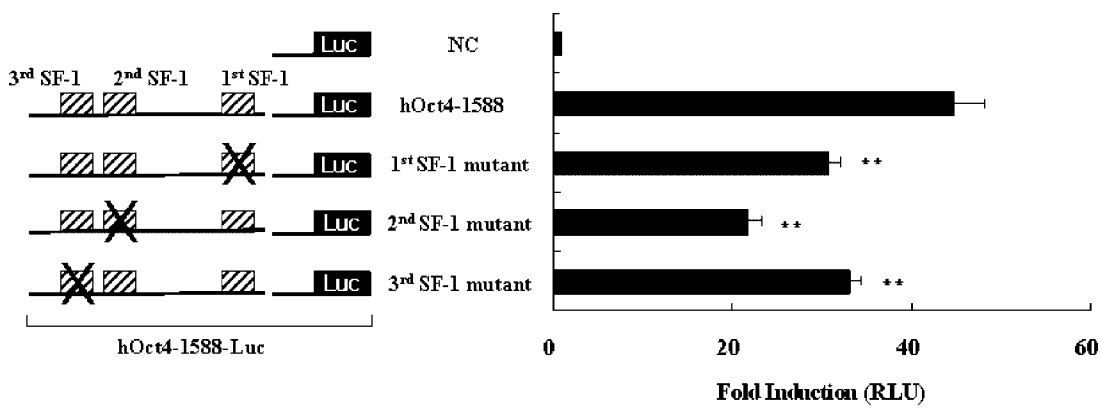

Fig. 5. Effect of SF-1 binding elements on transcriptional activity of the hOct4 promoter. The SF-1 binding elements are depicted by hatched boxes and the X indicates disruption by site-directed mutagenesis. Each construct (1 μg) was co-transfected into NCCIT cells with pcDNA3.1/hyg/LacZ (0.1 μg). The luciferase activity of the hOct4 5′ upstream regions is shown as fold induction relative to that of the empty pGL3-Basic. The data shown are the mean ± SD from three independent experiments with duplicates for each experiment. Statistical analyses compared the activity of the mutant construct to that of hOct4-1588-luc. $^{**}P < 0.001$

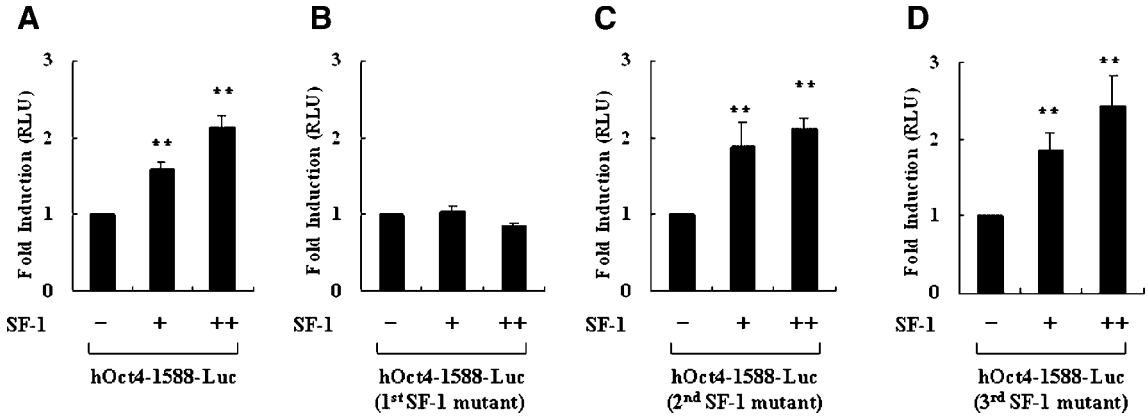

Fig. 6. The role of SF-1 binding element in SF-1-mediated transactivation of the hOct4 promoter. **A**: NCCIT cells were co-transfected with hOct4-1588-Luc (0.3 μg) and SF-1 (0.3, 0.6 μg). **B**, **C**, and **D**: NCCIT cells were co-transfected with each of the SF-1 binding site mutants within hOct4-1588-Luc construct (0.3 μg) and SF-1 (0.3, 0.6 μg). Transfection efficiency was normalized relative to the β-galactosidase activity obtained from co-transfection with pcDNA3.1/hyg/LacZ (0.1 μg). The luciferase activity of the hOct4 promoter region in the absence of SF-1 was set at 1. Activity of the hOct4 promoter in the presence of transfected SF-1 was calculated as fold induction relative to the absence of SF-1. The data shown are the mean ± SD from three independent experiments that were performed in duplicate. Statistical analyses compared the activity from co-transfection with SF-1 expression vector to that of empty vector. $^{**}P < 0.001$.

pluripotent- and differentiation-specific gene expression [Niwa et al., 2000]. The importance of its regulatory role is reflected by a complex expression pattern during mammalian development. In human, bovine and murine systems, the Oct4 promoter upstream sequences contain four conserved regions (CR1–4) that possess important regulatory elements [Nordhoff et al., 2001]. Each regulatory element shows specificity for development stage, tissue, or lineage,

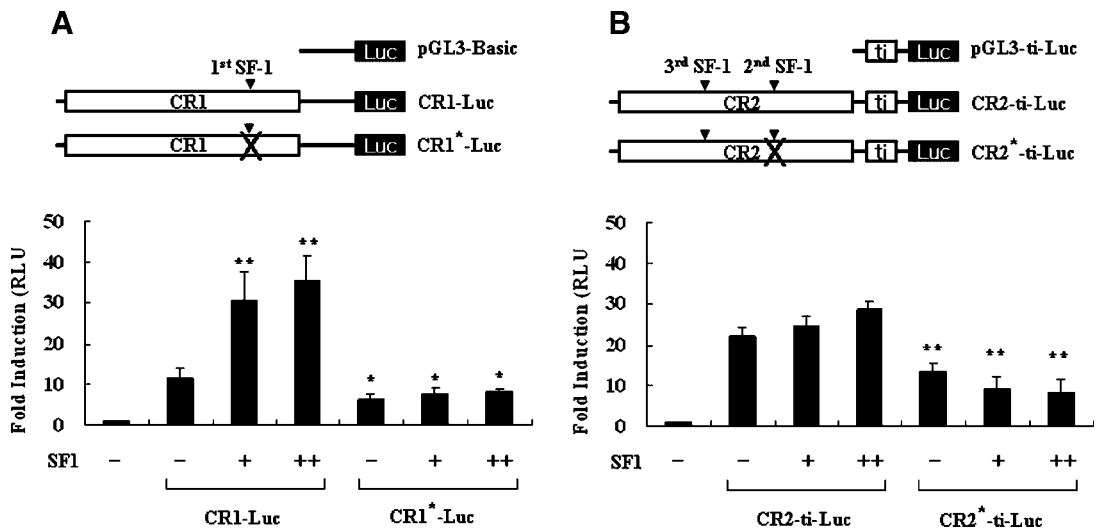

Fig. 7. The effect of SF-1 on CR1 and CR2. **A**: Schematic representation of the minimal hOct4 promoter containing CR1 and disruption of the 1st SF-1 element. Arrowheads indicate the 1st SF-1 and site-directed mutagenesis of the element is shown by an X or asterisk. NCCIT cells were co-transfected with either 0.3 μg CR1-Luc or CR1*-Luc and SF-1 (0.3, 0.6 μg). **B**: Schematic representation of hOct4-CR2 flanked by ti minimal promoter and 2nd SF-1 disrupted CR2. Arrowheads indicate the 2nd and 3rd SF-1 and site-directed mutagenesis is shown by an X or asterisk. CR2-ti-Luc or CR2*-ti-Luc (0.3 μg) were co-transfected into NCCIT cells with SF-1 (0.3, 0.6 μg). The transfection efficiency was normalized relative to the β-galactosidase activity from co-transfecting pcDNA3.1/hyg/LacZ (0.1 μg). The luciferase activity of the hOct4 regions is shown as fold induction relative to that of empty pGL3-Basic or pGL3-ti-Luc. The data shown are the mean ± SD from three independent experiments performed in duplicate. Statistical analyses compared the activity obtained from co-transfection with SF-1 expression vector to that with empty vector. $^{**}P < 0.001$, $^{*}P < 0.05$.

and the enhancers are crucial for function in vivo [Yeom et al., 1996; Kirchhof et al., 2000; Gu et al., 2005]. A recent study identified binding of Oct4 and Sox2 to a novel cis-element in the mOct4 promoter (Site 2B, 2040/−2024 in CR4) and determined that site 2B might contribute to maintaining expression of Oct4 in primitive cells [Okumura-Nakanishi et al., 2005]. In addition, several important transcription factors have been shown to bind to elements in the mOct4 PP, such as Sp1/Sp3 in the GC box [Pesce et al., 1999] and GCNF, SF-1, RAR/RXR, COUP-TF I/II and LRH-1 in the direct HRE repeat [Ben-Shushan et al., 1995; Barnea and Bergman, 2000; Fuhrmann et al., 2001; Gu et al., 2005]. However, with the exception of Sp1/Sp3 [Yang et al., 2005], little is known about which transcription factors are involved in cis-regulation of hOct4 expression.

Our overexpression studies revealed that SF-1 plays a role as a transactivator by increasing hOct4 promoter activity and inducing endogenous hOct4 expression (Fig. 3). However, there are some limitations to prove the importance of SF-1 in hOCT4 promoter activity by knockdown or repression of SF-1. Endogenous SF-1 expression level is extremely low to be detected in NCCIT cells, suggesting that SF-1 may not be a unique or major factor in the regulation of Oct4 expression. It is expected that hOct4 promoter activity may be still maintained just by repressing SF-1 alone, possibly due to the existence of largely unknown other transcriptional regulators involved in hOct4 promoter activity. Therefore, further investigation should be performed for the identification of nuclear factors involved in hOct4 promoter activity.

Several SF-1 binding sites have been identified in the mOct4 promoter, including SF-1(a), which is located in RAREoct element and SF(b), which is located in the upstream promoter element (UPE) between nucleotides −193 and −209 [Barnea and Bergman, 2000]. Another study identified three SF-1 response elements (DR0 in CR1 and PE1/PE2 in CR2) that are bound by the orphan nuclear receptor LRH-1 [Gu et al., 2005]. Sequence analyses revealed that the SF-1/LRH-1 binding elements DR0, PE1, and PE2 are highly conserved in human, bovine, and murine Oct4 promoters; the SF-1(a) sequence is identical to DR0, but no sequence corresponding to SF-1 (b) was identified in the hOct4 promoter. To investigate whether endogenous hOct4 promoter is directly regulated by endogenous SF-1 in vivo, ChIP assay would be highly useful. However, expression of endogenous SF-1 mRNA and protein was hardly detectable by Northern blot and IP/Western blot analyses in NCCIT cells (data not shown). In addition, hOct4 promoter contains putative three SF-1 binding sites, which are very closely localized with one another. It is possible to immunoprecipitate the DNA fragments containing all three (~1.3 kb long) or at least two SF-1 binding sites (2nd binding site exists 57 bp apart from 3rd one). Therefore, ChIP assay seems to hardly discriminate the binding affinity of each SF-1 binding site within hOct4 promoter region, since it apparently looks that all three SF-1 binding sites may have the similar binding affinity. To investigate the binding affinity for each binding site, we performed EMSA using in vitro transcribed and translated SF-1. Based on these sequence homologies, we generated probes corresponding to each of the SF-1 binding elements in the hOct4 promoter. EMSA/supershift assays confirmed that SF-1 bound to each site with differing levels of affinity. SF-1 demonstrates preferential binding to the 1st and 2nd SF-1 elements, rather than the 3rd (Fig. 4). We observed a similar in vitro binding pattern of SF-1 to the hOct4 promoter to that with the mOct4 promoter [Gu et al., 2005]. Site-directed mutagenesis of each SF-1 element resulted in a decrease in hOct4 promoter activity, although to differing extents (Fig. 5), suggesting that all of these sites, and in particular the 2nd SF-1 element, play important roles in transcriptional activity. However, overexpression studies revealed that following disruption of the 1st SF-1, even SF-1 stimulation could not restore transcriptional activity, whereas exogenous SF-1 stimulation could restore transcriptional activity following disruption of either the 2nd or 3rd SF-1 elements (Fig. 6). This suggests that the 1st SF-1 element plays the key role in SF-1-mediated hOct4 promoter activity.

Reports revealed that SF-1 does not activate transcriptional activity of human CYP1B1 or CYP11B2, even when SF-1 is bound to the SF-1 binding elements of those genes [Bassett et al., 2002; Tsuchiya et al., 2006]. This suggests that interaction with putative SF-1 binding sites might not be essential for transcriptional regulation and concurs with our data indicate that transcription factor binding is not always

proportional to transcriptional activity. In the Oct4 promoter, CR2 partially overlaps the PE region; the human, murine and bovine sequences are well conserved, sharing 92–94% identity [Nordhoff et al., 2001]. hOct4 CR2 contains other putative cis-elements that flank the 2nd and 3rd SF-1 sites, such as the E-box/Mash-2 binding site and CCCTCCC motifs, which can be bound by other transcription factors and may lead to repression or activation of hOct4 promoter activity [Nordhoff et al., 2001]. Therefore, a reduction of promoter activity may not be due entirely to disruption of the 2nd and 3rd SF-1 binding sites, as it could also be attributed to disruption of other co-localized cis-elements and their interactions with as yet unidentified transcription factors. Thus, further investigation is required to identify other cis-regulatory elements in the hOct4 CR2, as well as the factors with which they interact.

To verify that SF-1-mediated hOct4 promoter stimulation occurs through the SF-1 binding site, we generated a variety of minimal promoter reporter constructs containing CR1 (CR1-Luc), CR2 (CR2-ti-Luc), 1st SF-1 mutant CR1 (CR1*-Luc) and 2nd SF-1 mutant CR2 (CR2*-ti-Luc). Exogenous SF-1 overexpression resulted in a >threefold increase in the transcription activity of CR1-Luc containing the 1st SF-1 binding element and the effect of SF-1 disappeared following disruption of the 1st SF-1 site, whereas no significant effect was observed for both CR2-ti-Luc, which contained both the 2nd and 3rd SF-1 binding elements, and CR2*-ti-Luc (Fig. 7). In mOct4, SF-1 can also activate transcriptional activity through binding to both the PP containing CR1 and the PE containing CR2, even if PE possesses the lower SF-1-mediated transcriptional activity than PP [Gu et al., 2005]. However, our study revealed that the 1st SF-1 binding site in the PP is the key regulatory element for SF-1-mediated transcriptional activation of the hOct4 promoter, even though both the 1st and 2nd SF-1 elements demonstrated binding activity and site-directed mutation of the 2nd SF-1 resulted in a significant decrease in transcriptional activity.

In conclusion, we have demonstrated for the first time that SF-1 plays a role as a transcriptional activator of hOct4 expression through direct binding to the 1st SF-1 site in CR1 of the PP. However, further investigation is needed to elucidate the molecular regulation of hOct4 expression in maintenance of stem cell characteristics.

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