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Regulation of OCT4 gene expression by liver receptor homolog-1 in human embryonic carcinoma cells

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ABSTRACT

We demonstrate the regulation of OCT4 gene expression mediated by liver receptor homolog-1 (LRH-1) in human embryonic carcinoma cells. LRH-1 and OCT4 are co-expressed in undifferentiated NCCIT cells and decreased during retinoic acid-induced differentiation. Dose-dependent overexpression of LRH-1 transactivated the OCT4 promoter activity and its dominant negative form with a deletion of activation function-2 motif reduced the activity even in the presence of LRH-1. The OCT4 promoter contains potent three LRH-1 binding sites; one within conserved region (CR) 1 and two within CR2. Mutagenesis of each binding site affected the decrease in OCT4 promoter activity and the 2nd binding site mutant most significantly reduced the transcriptional activity, compared to that of 1st and 3rd binding site mutants, respectively. Simultaneous disruption of 2nd and 3rd binding sites led to significant down-regulation of the activity even in the presence of 1st binding site-containing CR1. Moreover, mutation of each binding element within native or exogenous minimal promoter-driven CR1 or CR2 also decreased the promoter activity to some different extent, suggesting that three binding elements may be implicated in the induction of the full-activity of OCT4 promoter. In vivo binding assay revealed the 2nd and 3rd binding motifs within CR2 were more enriched than the 1st one within CR1. Taken together, our study indicates that LRH-1 acts as a transcriptional activator in the regulation of OCT4 gene expression through the cooperative interaction with three binding sites directly or/and indirectly.

1. Introduction

OCT4, a member of the POU homeodomain family transcription factors, plays a critical role as a key regulator of pluripotency in early stages of mammalian development [1,2]. The level of OCT4 expression is crucial to sustain self-renewal of stem cells and any up-regulation or down-regulation induces divergent cell fates [3]. OCT4 is highly expressed in embryonic carcinoma (EC) cells as well as pluripotent embryonic stem (ES) cells and is rapidly down-regulated during retinoic acid (RA)-induced differentiation [4,5]. Moreover, deregulated OCT4 expression was found in various human solid tumors as well as germ cell tumors [6–8]. It has been proposed that aberrant expression of OCT4 may affect the tumorigenic process and have a role in cancer stem cell development [9,10]. Recently, several studies demonstrated that OCT4 overexpression increases tumorigenic progression such as colony formation, motility and migration of cancer cells [11–13].

Human OCT4 promoter 5′-upstream sequence has been cloned and clustered as four conserved regions (CR1–4) that are homologous in the mouse and bovine [14,15]. And, the cis-regulatory elements have been characterized to understand the molecular mechanism of OCT4 transcriptional activity. CR2, 3, and 4 act as positive-regulatory elements, while a putative negative cis-acting element is located between CR1 and CR2 [16]. In mouse, OCT4 expression is regulated by several nuclear receptors (NRs) including steroidogenic factor (SF-1), germ cell nuclear factor, retinoic

Abbreviations: LRH-1, liver receptor homolog-1; AF-2, activation function-2; CR, conserved region; EC, embryonic carcinoma; ES, embryonic stem; RA, retinoic acid; NRs, nuclear receptors; SF-1, steroidogenic factor; LRH-1 DN, LRH-1 dominant negative; PP, proximal promoter; PE, proximal enhancer.

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acid receptors and retinoid X receptors, chicken ovalbumin upstream promoter-transcription factors and liver receptor homolog1 (LRH-1) via the interaction with cis-regulatory elements within CRs [4,17–19]. In human, OCT4 promoter can be also activated by SF-1 through directly binding to their binding sites and also endogenous OCT4 protein expression was increased by SF-1-mediated transactivation [20]. Update, about 48 members of NR transcription factor superfamily are identified in the human genome and divided further into seven subfamilies (NR0–NR6) [21]. Some of them lack identified ligands, which are termed orphan NRs. SF-1 belongs to the fourth member of the NR5A subfamily (NR5A4) and is most homologous to LRH-1, especially in the DNA-binding domain and responding to the similar DNA binding element [22]. LRH-1 belongs to the NR5A subfamily (NR5A2) and regulates gene transcription by binding as a monomer to nuclear receptor half site sequences [23,24]. LRH-1 is required for maintaining OCT4 expression at the epiblast stage of embryonic development and its deletion leads to embryonic lethality [19]. In the adult tissues, LRH-1 has well established functions in bile acid synthesis, cholesterol metabolism and steroidogenesis [25–27]. LRH-1 can act as a key regulator of the steroidogenic lineage in mesenchymal stem cells by regulating steroidogenic enzymes and play a role in steroid hormone production in Leydig cells [28]. In addition, several studies demonstrated the critical involvement of LRH-1 in gastric, pancreatic and breast cancers [29–31].

In this study, we analyzed the expression pattern of human OCT4 and LRH-1 in human EC NCCIT cells, which have an intermediate phenotype between seminoma and embryonal carcinoma cells and are a useful tool for studying the relationship between seminoma and non-seminoma tumorigenesis [32]. We also investigated the transcriptional regulation of OCT4 mediated by LRH-1 through interaction with SF-1/LRH-1 binding element(s) to contribute to the understanding the molecular regulation mechanism of OCT4 expression mediated by LRH-1 in tumorigenic as well as pluripotent processes of germ cell-derived cancer cells.

2. Material and methods

2.1. Cell culture and differentiation

Human EC NCCIT cells (American Type Cell Collection, Manassas, VA) were cultured as described previously [33]. To induce differentiation, NCCIT cells were treated with 10 μM RA (Sigma, Saint Louis, MO) and harvested at different time points (0, 2, 4, 6, 8, and 10 days) for real-time PCR analysis.

2.2. Plasmid construction

We used a variety of the luciferase reporter constructs of OCT4 promoter wild types and mutants [−1588-Luc, CR1-Luc, CR2-ti-Luc, −1588*-Luc (1st SF-1/LRH-1 binding site mutant), −1588**-Luc (2nd binding site mutant), −1588***-Luc (3rd binding site mutant), CR1*-Luc (1st binding site mutant), CR2*-ti-Luc (2nd binding site mutant), which have been described previously [16,20]. Additional site-directed mutagenesis was performed to disrupt the 3rd binding site to generate a reporter vector [CR2**-ti-Luc (3rd binding site mutant)]. And also, both 2nd and 3rd SF-1/LRH-1 binding sites were also mutated and ligated into −1588**-Luc and CR2*-ti-Luc reporter vectors to generate −1588****-Luc and CR2***-ti-Luc (2nd/3rd binding site mutants) by using the QuickChange Site-Directed Mutagenesis method (Stratagene, La Jolla, CA). To generate those mutants, the following primers (mutant bases are in lower case) containing a Pvu I site are used: 5′-CCAGGCCCAgTCgatcGTTGAGCACTTG-3′ (forward) and 5′-CAAGTGCTCAACgatcGAcTGGGCCTGG-3′ (reverse). Full-length human LRH-1 cDNA was obtained from the PCR amplification of human cDNA clone (Open Biosystems, Lafayette, CO) and inserted into one or three copies of Flag-tagged pcDNA3.1+. A dominant negative form of LRH-1 (LRH-1 DN) [34] was generated by deletion of amino acids 484 to 495 by PCR amplification and inserted into Flag-tagged pcDNA3.1+. All cloned PCR products and reporter plasmids were verified by sequencing.

2.3. RNA preparation and real-time PCR

Total RNAs of naive and differentiated NCCIT cells were isolated with Trizol reagent (Invitrogen, La Jolla, CA) and cDNAs were synthesized from 5 μg total RNA as described previously [35]. The following primer sequences were used: Human β-actin, 5′-GCG GGAAATCGTGCGTGACATT-3′ (forward) and 5′-GATGGAGTTGAA GGTAGTTTCGTG-3′ (reverse); Human OCT4, 5′-CCCCTGG TGCCGTGAA-3′ (forward) and 5′-GCAAATTGCTCGAGTTCTTTCTG-3′ (reverse); and Human LRH-1 5′-TACCGACAAGTGGTACATGGAA-3′ (forward) and 5′-CGGCTTGTGATGCTATTATGGA-3′ (reverse). β-Actin cDNA was amplified from all samples as a normalizing control and relative quantification of the expression levels was determined using the 2-ΔΔCT method [36].

2.4. Transient transfection and reporter assays

Transfection and reporter assays were performed using NCCIT cells, as described previously [37]. Luciferase activities of reporter plasmids were normalized against the β-galactosidase activity of a co-transfected internal control plasmid (pcDNA3.1/hygro/LacZ; Invitrogen).

2.5. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using NCCIT cells overexpressing LRH-1, as described previously to analyze the in vivo interaction [35]. Chromatin obtained from NCCIT cells was precleared with salmon sperm DNA and protein G-plus agarose beads (Santa Cruz Biotech., Santa Cruz, CA) for 5 h at 4 °C. Precleared chromatin (1 ml) was rotated overnight at 4 °C with 10 μg of the mouse monoclonal anti-LRH-1 antibody (Abcam, Cambridge, UK) or normal mouse IgG (Santa Cruz). Chromatin/antibody complexes were incubated with 60 μl of Protein G-plus agarose beads for 6 h and immunoprecipitated DNA was then used as the template for quantitative PCR using primers specific to the OCT4 promoter using the Greenstar qPCR premix (Bioneer). The following primer sequences were used: 1st SF-1/LRH-1 binding site (106 bp), 5′-CTCCACCCATC CAGGGGGCGG-3′ (forward) and 5′-AGGCCCTGGGTGGGGAAAACC -3′ (reverse); 2nd SF-1/LRH-1 binding site (147 bp), 5′-AGAGCT GCCCCCTCTGGGGA-3′ (forward) and 5′-GGCCCTGGGTGGGGA AAACC-3′ (reverse); 3rd SF-1/LRH-1 binding site (124 bp), 5′-GGGATTCTGTGTGAGGGGATTGGG-3′ (forward) and 5′-CCCCA-GAGGGGGCAGCTCTAAC-3′ (reverse).

3. Result and discussion

3.1. Expression analysis of LRH-1 and OCT4 in NCCIT cells during RA-mediated differentiation

To find the functional relationship between LRH-1 and OCT4 at a transcription level, we first analyzed the expression pattern in human EC NCCIT cells during RA-mediated differentiation processes. To induce differentiation, NCCIT cells were treated with 10 μM RA for different time points (0, 2, 4, 6, 8, and 10 days). To examine the expression level of LRH-1 and OCT4 in NCCIT cells, we performed real-time PCR analysis (Fig. 1). LRH-1 transcript

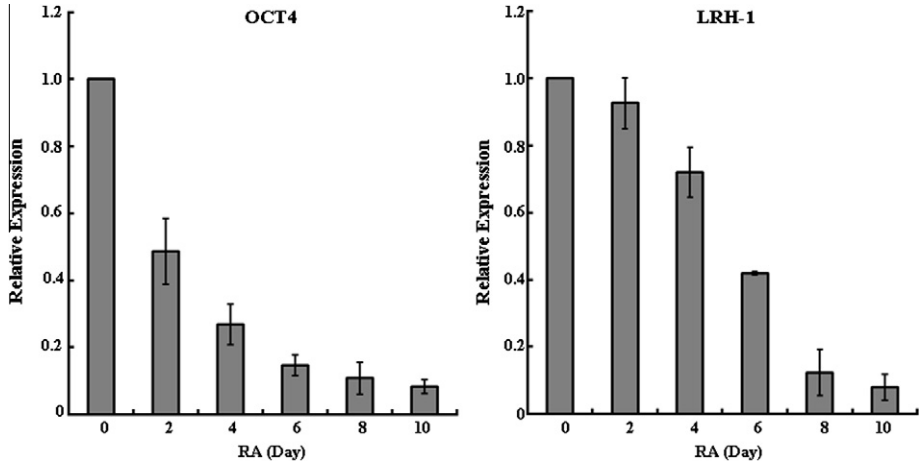

Fig. 1. Expression analysis of LRH-1 and OCT4 in NCCIT cells during RA-mediated differentiation. Real-time PCR analysis of OCT4 and LRH-1 expression. β-Actin was used as an internal control for normalization of cDNA content. The data represent the means and SD from three independent experiments that were performed in duplicate.

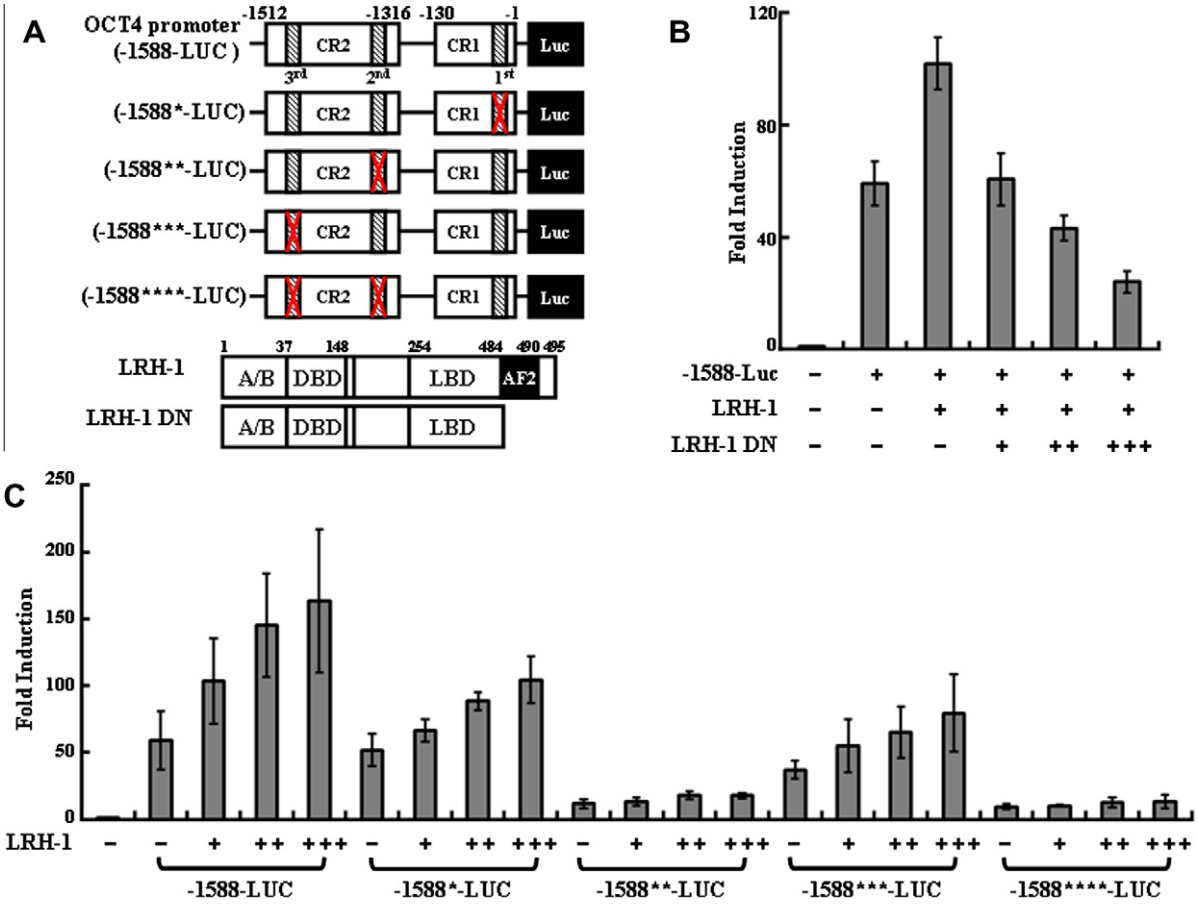

Fig. 2. The effect of dose-dependent overexpression of LRH-1 on OCT4 promoter activity in NCCIT cells. (A) *Upper:* Schematic representation of the OCT4 promoter 5′-upstream region (−1588-Luc) and mutants with disrupted 1st, 2nd, 3rd, or 2nd/3rd binding site(s) (−1588*-Luc, −1588**-Luc, −1588***-Luc and −1588****-Luc). The nucleotide numbers represent distance from the translational start site +1 (ATG). Conserved regions (CR1–2) are identified as bold and boxed. The boxes indicate LRH-1 binding sites and flaking sequences and X indicates disruption by site-directed mutagenesis. *Lower:* Domain structure of LRH-1 and LRH-1 DN. LRH-1 contains A/B, DNA binding, and ligand binding domains (DBD and LBD). LRH-1 DN is a form of a deletion of AF-2 motif. (B) The OCT4 promoter reporter (−1588-Luc) was co-transfected with LRH-1 and the increasing amount of LRH-1 DN expression vectors. (C) Each LRH-1 binding site mutant was cotransfected with LRH-1 in dose-dependent manner and transcriptional activity was calculated relative to the expression of pGL3-basic as a negative control. The data represent the means and SD from three independent experiments that were performed in duplicate.

has decreased dramatically after 2 days of RA treatment, while OCT4 transcript gradually decreased upon RA treatment, suggesting that LRH-1 may act as an upstream effecter on OCT4 gene expression. The concomitant expression pattern of LRH-1 and OCT4 in NCCIT cells suggested that LRH-1 might play a role in OCT4 expression as an upstream effecter.

RA and its derivatives are involved in the regulation of biological events such as embryogenesis, differentiation, and homeostasis [38]. OCT4 expression is downregulated during RA-mediated differentiation in both EC and ES cells [1]. In mice, RA treatment induces the down-regulation of LRH-1 as well as OCT4 expression [19]. We demonstrated that in human, LRH-1 was also co-expressed with OCT4 in undifferentiated NCCIT cells and decreased during RA-induced differentiation, supporting that LRH-1 and OCT4 have an interactive regulatory mechanism at a transcription level.

3.2. The effect of dose-dependent overexpression of LRH-1 on OCT4 promoter activity in NCCIT cells

In order to examine the effect of LRH-1 in OCT4 transcriptional activity, we used a reporter construct containing the upstream promoter region of OCT4 (−1588-Luc) that was reported previously [20]. In addition, mouse LRH-1 could interact with SF-1/LRH-1 binding elements within OCT4 promoter [19], those elements were compared with human, found to be identical each other, and further designated as the SF-1/LRH-1 binding elements. LRH-1 DN was generated by a deletion of the activation function-2 motif (AF-2; amino acids 484–490), which is required for transcriptional activation [21,34], and ligated into Flag-tagged pcDNA3.1 + expression vector (Fig. 2A). The OCT4 promoter reporter (−1588-Luc) was co-transfected with the increasing amounts of LRH-1 and LRH-1 DN in NCCIT cells. The over-expression of exogenous LRH-1 activated the transcriptional activity of the OCT4 promoter reporter (−1588-Luc) but the promoter activity was gradually decreased by the over-expression of LRH-1 DN even in the presence of wild-type LRH-1 (Fig. 2B). This result indicates that LRH-1 specifically activates the transcriptional activity of OCT4.

To examine whether LRH-1 can respond to the SF-1/LRH-1 binding elements within the OCT4 promoter, we performed sequential site-directed mutagenesis of 1st, 2nd, or 3rd SF1/LRH-1 binding site within OCT4 promoter (−1588-Luc) and delivered them into NCCIT cells (Fig. 2A). While the transcriptional activity of the OCT4 promoter reporter (−1588-Luc) increased in the presence of exogenous dose-dependent overexpression of LRH-1, The activity decreased in each binding site mutant to different extent of range (Fig. 2C). In detail, 2nd SF-1/LRH-1 binding site mutant (−1588**-Luc) most significantly down-regulated the transcriptional activity in OCT4 promoter (−1588-Luc), while 1st and 3rd binding site mutants (−1588*-Luc and −1588***-Luc) decreased the activity to less extent, suggesting that the 2nd binding site act as a major element in the activation of OCT4 promoter mediated by LRH-1. Moreover, simultaneous disruption of 2nd and 3rd binding sites led to dramatic down-regulation of the activity even in the presence of 1st binding site-containing CR1, supporting the significance of 2nd binding element in the OCT4 promoter activity. In human, bovine and murine systems, the OCT4 promoter upstream sequences contain four conserved regions (CR1–4) that possess important regulatory elements [15]. It has been reported that LRH-1 promotes OCT4 expression directly by binding evolutionary conserved elements in the proximal promoter (PP) and proximal enhancer (PE) in mouse [19,38].

3.3. The effects of LRH-1 on CR1 or CR2-driven transcriptional activity

To examine whether LRH-1-mediated OCT4 promoter stimulation directly occurs through the SF-1/LRH-1 binding site(s) within CR1 and CR2, we generated a variety of native or exogenous minimal promoter-driven reporter constructs containing wild-type or cognate SF-1/LRH-1 binding element mutants (Fig. 3).

First, we examined the transactivational effect of LRH-1 on the 1st SF-1/LRH-1 binding element by using luciferase reporter

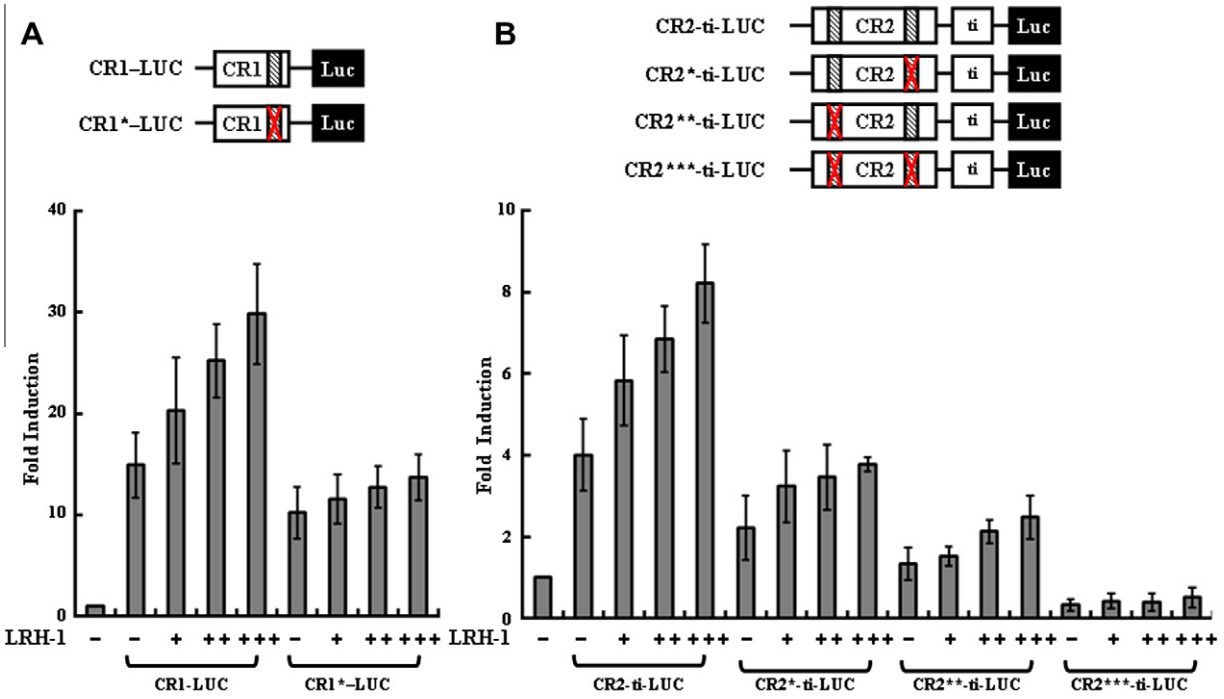

Fig. 3. The effect of dose-dependent LRH-1 overexpression on CR1 and CR2 in NCCIT cells. (A) *Upper:* Schematic representation of the OCT4 minimal promoter-containing CR1 and a mutant with disrupted 1st binding site (CR1-Luc and CR1*-Luc). The box indicates the 1st LRH-1 binding site and site-directed mutagenesis of the 1st LRH-1 binding site is shown by an X. *Lower:* Each reporter vector was cotransfected with the increasing amounts of LRH-1. (B) *Upper:* Schematic representation of the exogenous minimal ti reporter vector (CR2-ti-Luc) and mutants with disrupted 2nd, 3rd, or 2nd/3rd binding site within CR2 [marked as asterisk(s) such as CR2*-ti-Luc, CR2**-ti-Luc, or CR2***-ti-Luc]. The boxes indicate 2nd, 3rd, 2nd/3rd binding sites and site-directed mutagenesis of each binding site is shown by an X, respectively. *Lower:* Each reporter vector was cotransfected with the increasing amounts of LRH-1. Transcriptional activity was calculated relative to the expression of pGL3-basic as a negative control. The data represent the means and SD from three independent experiments that were performed in duplicate.

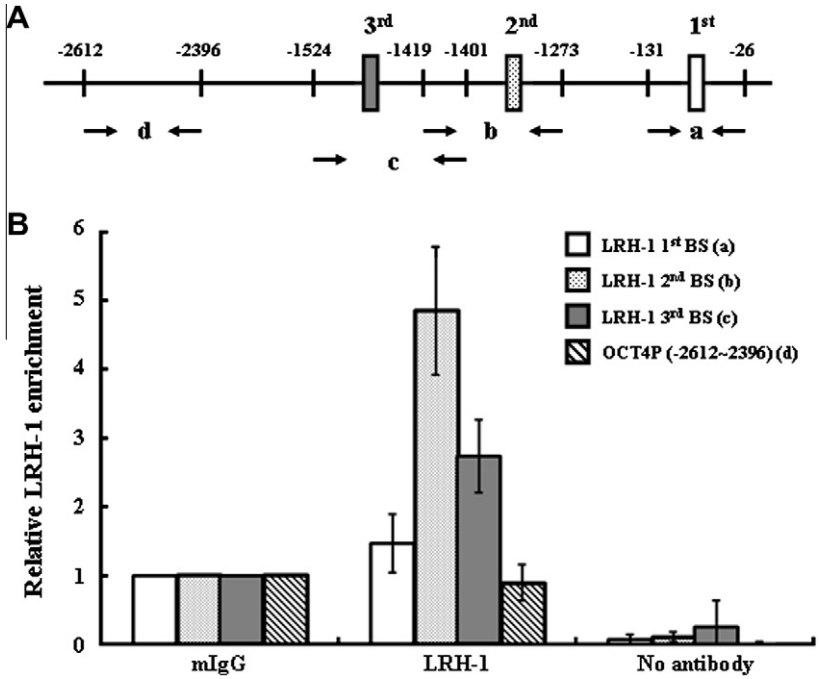

Fig. 4. Binding analysis of LRH-1 to human OCT4 promoter region in vivo. (A) Schematic diagram of the locations of the OCT4-specific primers corresponding to 1st, 2nd, or 3rd binding and flanking sequences. And an additional primer was also designed for the upstream region of OCT4 promoter as a negative control. (B) Cross-linked genomic DNA from NCCIT cells was immunoprecipitated with anti-LRH-1 antibody and amplified by real-time PCR. Input DNA, anti-mouse IgG-precipitate, and no antibody samples were used as positive and negative controls. The data represent the means and SD from three independent experiments that were performed in duplicate.

constructs of OCT4 promoter CR1 and a mutant (CR1-Luc and CR1*-Luc) providing own minimal promoter activity that was previously reported by Yang et al. [20]. Each reporter construct was co-transfected with the increasing amounts of LRH-1 expression vector into NCCIT cells. Over-expression of LRH-1 increased the transcriptional activity of OCT4 promoter (CR1-Luc) but failed to fully recover OCT4 mutant promoter activity (CR1*-Luc) (Fig. 3A), suggesting that 1st SF-1/LRH-1 binding site may affect the OCT4 promoter activity to some extent.

In addition, since the 2nd and 3rd SF-1/LRH-1 binding sites are present within CR2, a reporter construct (CR2-ti-Luc) and mutants (CR2*-ti-Luc, CR2**-ti-Luc, CR2***-ti-Luc) were generated by inserting CR2 wild-type and mutants [2nd, 3rd, or 2nd/3rd binding site(s) disrupted] into the 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)[39,40] (Fig. 3B). To eliminate the potent effect of 1st binding site present in CR1 containing minimal promoter activity, exogenous minimal ti promoter was inserted downstream of CR2. Each reporter construct was co-transfected with the increasing amounts of LRH-1 expression vector into NCCIT cells. While the transcriptional activity of reporter construct (CR2-ti-LUC) was activated along with the increase in LRH-1 expression, CR2**-ti-Luc with 3rd binding site disrupted significantly decreased, compared with the 2nd one (CR2*-ti-Luc), which exhibited some different pattern from the activity in OCT4 promoter (−1588) mutants (Fig. 2), suggesting that 1st and 3rd binding sites may compensate for each other to sufficiently activate the OCT4 promoter region (−1588 to −1 from ATG) activity. However, disruption in both 2nd and 3rd binding sites (CR2***-ti-Luc) completely abolished the activities (Fig. 3B), indicating that all three binding sites are required for the maximal activation of OCT4 promoter activity. Our study revealed that the 2nd LRH-1 binding site in the PE corresponding to mOCT4 promoter is the key regulatory element for LRH-1-mediated transcriptional activation of the OCT4 promoter. Moreover, the 1st and 3rd SF-1/LRH-1 binding elements have also influence on the transcriptional activity cooperatively.

3.4. Interaction of LRH-1 with SF-1/LRH-1 binding elements within the OCT4 promoter region in vivo

To address the direct interaction of LRH-1 with OCT4 promoter in vivo, we tested the ability of LRH-1 protein to bind to these three binding elements in native chromatin of NCCIT cells by a ChIP assay (Fig. 4). Chromatin was prepared from undifferentiated NCCIT cells transfected with LRH-1 expression vector, and LRH-1-bound chromatin was enriched with an anti-LRH-1 antibody. The three SF-1/LRH-1 binding sites were analyzed by real-time PCR using the OCT4 promoter-specific primers to amplify the regions specific to 1st binding element within CR1, 2nd and 3rd within CR2. The results revealed that 2nd and 3rd binding motifs within CR2 were more enriched than 1st one within CR1. It has been also demonstrated that endogenous mouse LRH-1 is directly bound to the OCT4 PP and PE in vivo and supported a function for LRH-1 in the regulation of OCT4 expression in embryonic stem cells [19].

In conclusion, we have demonstrated LRH-1 plays a role as a transcriptional activator of OCT4 expression through interaction with SF-1/LRH-1 binding elements in human EC NCCIT cells.

Acknowledgments

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