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Nspc1 regulates the key pluripotent Oct4–Nanog–Sox2 axis in P19 embryonal carcinoma cells via directly activating Oct4

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ABSTRACT

Nspc1 is an identified transcription repressor. However, transiently up-regulated or down-regulated Nspc1 in P19 embryonal carcinoma cells affects expression levels of Oct4, Sox2 and Nanog in a positive correlation. Luciferase activity assays verified that Nspc1 regulates the Oct4 promoter in a dose dependent manner. ChIP assay shows that Nspc1 activates Oct4 by directly binding to the (−1021 to −784) region of Oct4 promoter. Dominant negative analysis indicated the activation is dependent on the retinoid acid response element (RARE). We demonstrated Nspc1 has a positive role in maintaining the pluripotency of P19 cells by directly regulating Oct4.

1. Introduction

Pluripotent stem cells are undifferentiated cells that can give rise to several lineages of differentiated cell types, which are characterized by the ability to self-renew and maintain pluripotency [1]. Among the well-established pluripotent cells, embryonal carcinoma cells (ECCs) are derived from teratocarcinomas and have been well characterized as pluripotent cell lines that can be maintained as undifferentiated cells and induced under controlled conditions to differentiate in vitro to any cell type of all three germ layers [2], providing an attractive cell model system for studying pluripotent stem cells. The mouse P19 EC cell line was derived from a teratocarcinoma in C3H/He mice, produced by grafting an embryo at 7 days of gestation into testes of an adult male mouse [3]. The cells contain a normal karyotype and express varieties of endogenous genes including Oct4, Sox2 and Nanog. P19 cells stimulated by retinoic acid (RA) can differentiate into neural ectoderm and endoderm-derived cells, such as nerve cells, glial cells and fibroblasts [4].

The proliferation and differentiation of the stem cell is a highly complex process regulated involving regulation and coordination of gene expression at multiple steps. Among them, Polycomb group (PcG) gets the rapid progress [5]. PcGs are epigenetical chromatin modifiers that can transcriptionally repress their targets during development [6]. Recent studies have shown that PcG family proteins play an important role in stem cell self-renewal and cell fate decision-making process. For example, Bmi-1, the famous PcG family member, the normal expression of this protein is a prerequisite for stem cell self-renewal. Bmi-1 can promote stem cell to be self-renewal by inhibiting CDKIs INK4A/ARF (encoding p16, p14/p19) and p21Waf1/Cip1 transcription, and has become recognized as one of stem cell maintenance gene [7–9].

Nervous system polycomb 1 (Nspc1, also named as polycomb group factor 1, Pcgf1), shares high homology with PcG proteins Bmi-1 and Mel-18 [10]. Our previous studies demonstrated that human NSPc1 gene encodes a protein that acts as a transcriptional repressor [11]. NSPc1 has a positive role in promoting tumor cell cycle transition and cell proliferation through down regulating the CDK inhibitor p21Waf1/Cip1 via the RARE element [12]. Interestingly, a previous gain-of-function screens in mouse ES cells characterized Nspc1 was capable of promoting the continued expression of the ES cell pluripotency markers and partially rescued mouse ES cells growth in the absence of LIF [13]. Recently, Nspc1/Pcgf1 was identified as an epigenetic regulator involved in hematopoietic cell differentiation [14]. However, till now, the mechanisms involving the direct target genes of Nspc1 in ECCs and its transcriptional regulation mechanism are poorly understood.

In the present study, we show a novel role of Nspc1 in maintenance of pluripotency of P19 EC cells. We found that undifferentiated pluripotency P19 cells highly express Nspc1 and its expression is obviously down-regulated upon RA-induction. The expression of pluripotency stem cell markers including Nanog, Oct4 and Sox2 are positively regulated by Nspc1 in P19 cells. Nspc1

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selectively activates and directly binds to Oct4 proximal promoter in RA dependent way. Thus, our work indicated an essential role of polycomb protein Nspc1 in stem cell pluripotency and differentiation regulation.

2. Materials and methods

2.1. Cell culture and RA-induced neural differentiation

The P19 EC cell line was purchased from ATCC. P19 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 8% fetal bovine serum (Gibco), 5 mM L-glutamine, and 100 U/ml penicillin and 100 mg/ml streptomycin under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. For RA-induced differentiation, P19 cell aggregates were formed by placing 2×10^6 cells in a 60 mm bacteriological dish (Petri dish) (Falcon) with addition of 1×10^{-6} M *all-trans*-RA (Sigma) for 4 days. Subsequently, the aggregates were replated on tissue culture dishes (Corning) for further differentiation.

2.2. Plasmids, small interfering RNAs, transfections

To construct the mouse Nspc1 expression vector, the ORF of Nspc1 from P19 was amplified. The PCR primers are as follow: mNspc1-FWD: 5′-CGG GAT CCC TTG TAC CAC CGG TAA CTA AAT G-3′; mNspc1-RVS: 5′-CCG CTC GAG TTA ATC TGA TTT GTG GCA GTA AAG G-3′. The PCR products were cloned into the pcDEF expression vector (Invitrogen, USA) and the constructs were verified by DNA sequencing. For luciferase reporter assay, the 5′UTR of Oct4-1192 bp (−1192 ∼ +18), Oct4-402 bp (−402 ∼ +18), Nanog-2.4 kb (−2400 ∼ +50), Nanog-332 bp (−332 ∼ +50), Nanog-153 bp (−153 ∼ +50), TubulinβIII-738 bp (−738 ∼ +14) and TubulinβIII-402 bp (−402 ∼ +14) were amplified from genomic DNA of P19. The PCR products were then cloned into the firefly luciferase reporter vector (pGL3-Luc) as previously described [15]. pGL3-RARE is a gift [16]. Small interfering RNAs (siRNAs) against mouse Nspc1 (5′-CAA TAC AGC TGG CTA ACA AT-3′) [17], and siRNA negative control (scrambled siRNA) were purchased from GenePharma (shanghai, China). Cells were transfected at 24 h after plating using Lipofectamine 2000 (Invitrogen, USA) and were harvested at 36 h.

2.3. Isolation of RNA and quantitative real-time PCR

Total RNA isolated from cell cultures were routinely used for reverse transcription polymerase chain reaction (RT-PCR) or quantitative real-time PCR (qPCR). Total RNA was prepared by using EasyPure RNA Kit (TransGen Biotech, China), following the manufacturer's instructions. For RT-PCR, the cDNAs were synthesized using TransScript II First-Strand cDNA Synthesis SuperMix (TransGen Biotech, China) with total RNA as templates. PCR was performed with the following primers, annealing temperature (Ta) and number of PCR cycles (N):

mNspc1-up, 5′-GTCCGGGTGAAGATCAAAGA-3′ and mNspc1-down, 5′-CTGTCTAAGCCTCGGGACTG-3′ (Ta: 56 °C, N: 32); mSox2-up, 5′-GCGGAGTGGAAACTTTTGTCC-3′ and mSox2-down, 5′-CGGGAAGCGTGTACTTATCCTT-3′ (Ta: 60 °C, N: 35); mNanog-up, 5′-TCTTCCTGGTCCCCACAGTTT-3′ and mNanog-down, 5′-GCAAGAA TAGTTCTCGGGATGAA-3′ (Ta: 60 °C, N: 35); mOct4-up, 5′-TAGGTG AGCCGTCTTTCCAC-3′ and mOct4-down, 5′-GCTTAGCCAGGTTC GAGGAT-3′ (Ta: 56 °C, N: 35); mTubulinβIII -up, 5′-GATGATGAC GAGGAATCGGAAG-3′ and mTubulinβIII-down, 5′-AGAGGTGGCT AAAATGGGGAGG-3′ (Ta: 60 °C, N: 35); mrppo-up, 5′-TTCATTGTG GGAGCAGAC-3′ and mrppo-down, 5′-CAGCAGTTTCTCCAGAGC-3′ (Ta: 56 °C, N: 32); For qPCR, three samples were collected at each time point. Each sample was analyzed in triplicate with rppo as the inner control by real-time PCR with SYBR Green Supermix (Promega, USA) on Applied Biosystems step one plus instruments. Amplification data were collected by Mastercycler ep realplex and analyzed by the realplex2.0 software (Eppendorf).

2.4. Cell extracts and Western blotting

Cell lysates were subjected to Western blot analysis with the following antibodies and dilutions: mouse monoclonal anti-Nspc1 (1:5000) [18], rabbit anti-Oct4 (1:3000; Bioworld BS1262), rabbit anti-Nanog (1:2000; SAB 21423), rabbit anti-Sox2 (1:3000; Bioworld AP0014), rabbit anti-TubulinβIII (1:2000; SAB 21617), mouse anti-β-Actin (1:4000;CWBIO CW0264a) and rabbit anti-GAPDH (1:4000; CWBIO CW0101).The signals from the primary antibody were amplified by horse radish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (Bio-Rad, Hercules, CA, USA), and detected with Enhanced Chemi-luminescence Plus (ECLplus, Amersham Pharmacia Biotech, USA).

2.5. Luciferase reporter assays

The mouse Oct4 promoter (−1192 bp and −402 bp), mouse Nanog promoter (−2.4 kb, −332 bp and −153 bp) and mouse TubulinβIII promoter (−738 bp and −402 bp) constructs construct ligated to pGL3-basic vector. pRL-TK was purchased from Promega. Regulation activity assays were performed as described before [11]. In short, exponentially growing P19 cells were seeded onto 24-well plates at a density of 2×10^5 cells/well, 1 day prior to transfection. Cells were transfected with pcDEF-Nspc1 (500 ng/well), together with reporter plasmid pGL3 promoter series (200 ng/well) and pRL-TK (50 ng/well) as an internal control. Total amounts of transfected DNA were equalized by the addition of empty vector (pcDEF-control). Each transfection reaction was performed in triplicate. After 24 h post transfection, cells were harvested and assayed for luciferase activity by using the Dual-luciferase reporter assay system (Promega, USA). Results were obtained from three different transfection experiments after normalization for the internal control of TK activity. Experimental variations are indicated as means ± SDs.

2.6. Chromatin immunoprecipitation (ChIP) assay

Approximately 5×10^7 subconfluent P19 cells were collected, and native protein–DNA complexes were cross-linked by treatment with 1% formaldehyde (SIGMA) for 15 min. The ChIP assay was generally carried out as reported earlier [12]. Briefly, equal aliquots of isolated chromatin were subjected to immunoprecipitation with rabbit polyclonal anti-Nspc1 antibody [11], mouse monoclonal anti-Nspc1 [18], or control preimmune IgG. The DNA fragments associated with specific immunoprecipitates or with negative control serum were isolated and used as templates for subsequent PCR. The primers for the Oct4–ChIP1 region (−211 to +18) were: up primer, 5′-GTGAGAGGACCTTGAAGGTTGA-3′; down primer, 5′-GCTCACCTAGGGACTGTTTC-3′; The primers for the Oct4–ChIP2 region (−402 to −190) were: up primer, 5′-GTAAG-CAAGAACTGAGGAGTGG-3′; down primer, 5′-TCAACCTTCAAG GTCCTCTAAC-3′; The primers for the Oct4–ChIP3 region (−1021 to −784) were: up primer, 5′-CTGGGGAAGTCTTGTGTGAGG-3′; down primer, 5′-TGGGAGGTGGGTAGAGAGAAG-3′.

2.7. Statistical analysis

We used Microsoft Excel Program to calculate SD and statistically significant differences between samples with Student's *t*-test. The asterisks in each graph indicate statistically significant

changes with P values calculated by Student's t-test: $^{*}P < 0.05$, $^{**}P \leqslant 0.01$ and $^{***}P \leqslant 0.001$. P-values <0.05 were considered statistically significant.

3. Results

3.1. Nspc1 is repressed at early time points and disappeared at middle time point during RA induction of P19 cells

P19 cells were known to differentiate to neural cells by RA treatment in vitro. We confirmed that RA-induced P19 cell neural differentiation displayed at least three phases (Fig. 1A): the first phase (1–2 days following RA treatment) in which P19 cells lost their pluripotency, marked by disappearance of the expression of the pluripotency marker Nanog, Oct4 during RA induction; the terminal differentiation phase (6–7 days following RA treatment) in which differentiated neuronal cell populations appeared, evidenced by elevated expression of neuron-specific marker Tubulin βIII (Fig. 1C), and an Intermediate phase (3–5 days following RA treatment). Both the RNA and protein levels of Nspc1 are highly expressed in undifferentiated P19 cells but they decreased dramatically at day 4–6 (middle time point) following RA treatment (Fig. 1B and C). Furthermore, retinoic acid (RA)-induced neural differentiation of pluripotent P19 cells correlates with decreased expression of Nspc1, Nanog and Oct4, however increased expression of Tubulin βIII (Fig. 1C), suggesting the reduction of Nspc1 was one of the early events during differentiation of the P19 pluripotent cells and Nspc1 might participate in the maintenance of P19 cells pluripotency and neural differentiation.

3.2. The exogenous expression levels of Nspc1 affected the expression levels of Nanog, Oct4, Sox2 and Tubulin βIII

In order to determine the role of Nspc1 in neural differentiation of P19 cells, we used the vector pcDEF constructed with mouse Nspc1 cDNA to mediate the induction of Nspc1 in the cells without RA treatment. We found that elevated exogenous expression of Nspc1 alone in P19 cells through pcDEF-Nspc1 infection was able to activate the expression increasing of pluripotency markers including Nanog, Oct4 and Sox2, while induced the decreasing of neuron-specific marker Tubulin βIII in P19 cells (Fig. 2A and B). Meanwhile we analyzed the expression levels of differentiation markers in siRNA-Nspc1 infected P19 cells, including Nanog, Oct4, Sox2 and Tubulin βIII. Knockdown of Nspc1 in P19 cells resulted in a rapid decrease of pluripotency markers, on the other hand resulted in a rapid increase of Tubulin βIII (Fig. 2C and D). It indicated that Nspc1 plays a positive regulation role for the expression of three stem cell master genes in P19 cells.

3.3. Nspc1 activates Oct4 and Nanog promoter in a dose dependent manner

To elucidate the molecular mechanisms of the regulation of Nanog, Oct4 and Tubulin βIII by Nspc1, we examined the effect of over-expressed and knocked-down of Nspc1 on the activity of the Oct4, Nanog and Tubulin βIII promoter by using dual luciferase report assay. We mainly focused on the −1192 bp region of the Oct4, the −2.4 kb region of the Nanog, and the −738 bp region of the Tubulin βIII in the following activity assays. As shown in Fig. 3, the over-expression of Nspc1 in P19 cells led to from 4% to 18% increase of relative luciferase activity of Nanog compared with control (Fig. 3C), while over-expression of Nspc1 led to a stronger increase (16–86%) on the Oct4 promoter (Fig. 3A). Furthermore, we found that Nspc1 activates Oct4 and Nanog promoter-mediated luciferase expression both in a dose-dependent manner in P19 cells (Fig. 3A and C). The same dose-dependent assay was also performed in P19 cells with siRNA knocked-down of Nspc1 (Fig. 3B and D). Knocked-down of Nspc1 in P19 cells has a repression effect on the Oct4 promoter (7–15% decrease) (Fig. 3B), and has also a repression effect on the Nanog promoter (4–22% decrease) (Fig. 3D). Though It seemly that Nspc1 could activate Tubulin βIII promoter activity, but it was not in a dose-dependent manner (Fig. 3E). These results eliminated that Nspc1 could both activate the transcription activity of Oct4 and Nanog in a dose dependent manner, but had a stronger effect on the Oct4 promoter.

3.4. Nspc1 directly binds and stimulates Oct4 promoter

According to previous work [12,19], we speculated several Nspc1 putative binding sites at the regions of −1192 to +18 bp in the Oct4 promoter (Fig. 4A). We used ChIP assays to determine the putative Oct4 promoter regions that mediate Nspc1 binding to endogenous Oct4 promoter at physiological conditions. The amount of promoter DNA associated with the IP chromatin was

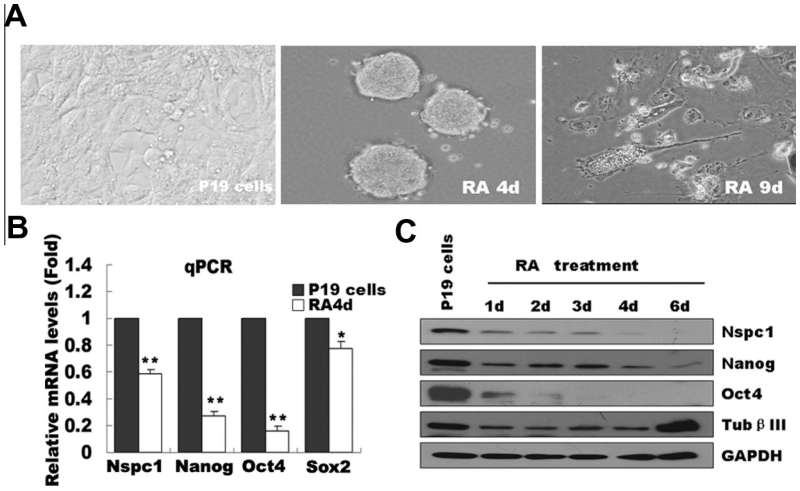

Fig. 1. Transcription factor Nspc1 highly expressed in P19 EC cells and its expression decreased dramatically at day 6 following RA treatment. (A) Pictures of the typical cell morphology of P19 EC cells, RA-induced P19 cells that were cultured into cloning formation till 4 days and RA-induced P19 cells at day 9 according to neural differentiation protocol (see Section 2) were taken at 200× magnification. (B and C) Gene expression analysis levels were performed by qPCR or Western blotting. Cell lysates were isolated at different time points as indicated to measure the expression levels of Nspc1, Oct4, Nanog, Tubulin βIII and GAPDH (loading control). *Abbreviation:* TubβIII, tubulin βIII. The asterisks indicate statistically significant changes: $^{*}P < 0.05$, $^{**}P \leqslant 0.01$.

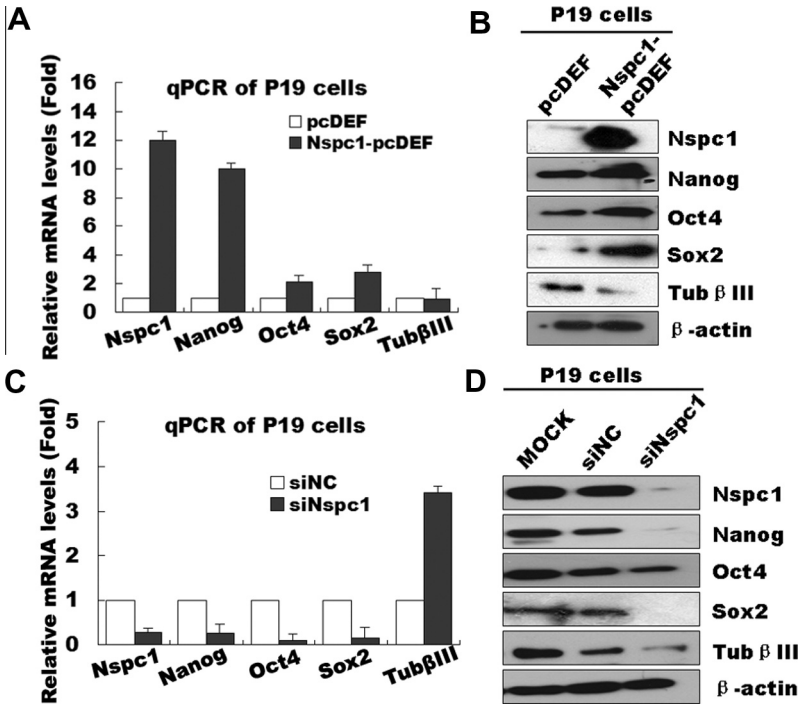

Fig. 2. Changed expression of Nspc1 affected the expression levels of pluripotency markers in P19 cells. Gene expression analysis of Nspc1, Oct4, Nanog, Sox2 and TubβIII was performed by qPCR or Western blotting after P19 cells were over-expressed Nspc1 (A and B). or Knockdown of Nspc1 (C and D). β-Actin was used as the loading control. *Abbreviation:* SiNC, si RNA negative control (scrambled siRNA).

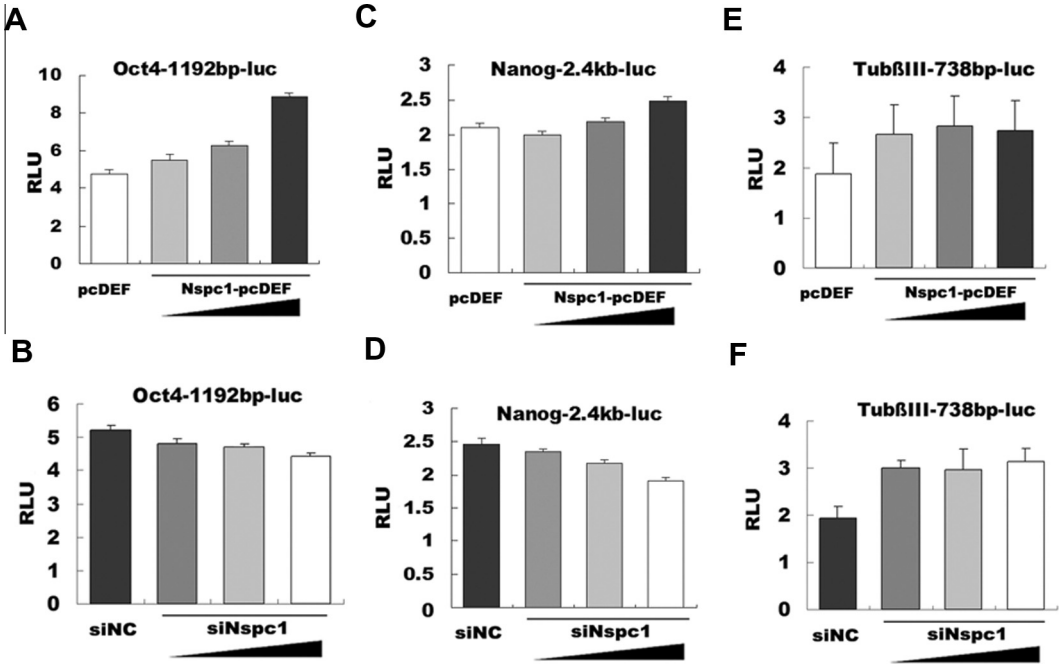

Fig. 3. Nspc1 stimulates Oct4 and Nanog promoter in a dose dependent manner. (A) P19 cells were co-transfected with increased amounts of pcDEF-Nspc1 plasmid (200, 400 and 600 ng/well) together with the Oct4-1192 bp reporter plasmid (200 ng/well) and pRL-TK plasmid (50 ng/well). (B) P19 cells were co-transfected with increased amounts of siRNA-NSPc1 (200, 400 and 600 ng/well) together with the Oct4-1192 bp reporter plasmid and pRL-TK plasmid as above. (C) P19 cells were co-transfected with increased amounts of pcDEF-Nspc1 plasmid (100, 300 and 500 ng/well) together with the Nanog-2.4 kb reporter plasmid and pRL-TK plasmid as above. (D) P19 cells were co-transfected with increased amounts of siRNA-Nspc1 (100, 300 and 500 ng/well) together with the Nanog-2.4 kb reporter plasmid and pRL-TK plasmid as above. (E) P19 cells were co-transfected with increased amounts of pcDEF-Nspc1 plasmid (100, 300 and 500 ng/well) together with the TubβIII-738 bp reporter plasmid and pRL-TK plasmid as above. (F) P19 cells were co-transfected with increased amounts of siRNA-Nspc1 (100, 300 and 500 ng/well) together with the TubβIII-738 bp reporter plasmid and pRL-TK plasmid as above. The luciferase activities were measured at 24 h following transfection.

quantitated by RT-PCR with primers specific to three Oct4 promoter region: ChIP1 (−211 to +18 bp), ChIP2 (−402 to −190 bp) and ChIP3 (−1021 to −784 bp). It showed obviously binding activities of Nspc1 to the Oct4 proximal promoter region around the RARE region (−1021 to −784 bp) (Fig. 4B) in P19 pluripotent cell samples. To test whether Nspc1 specifically activates

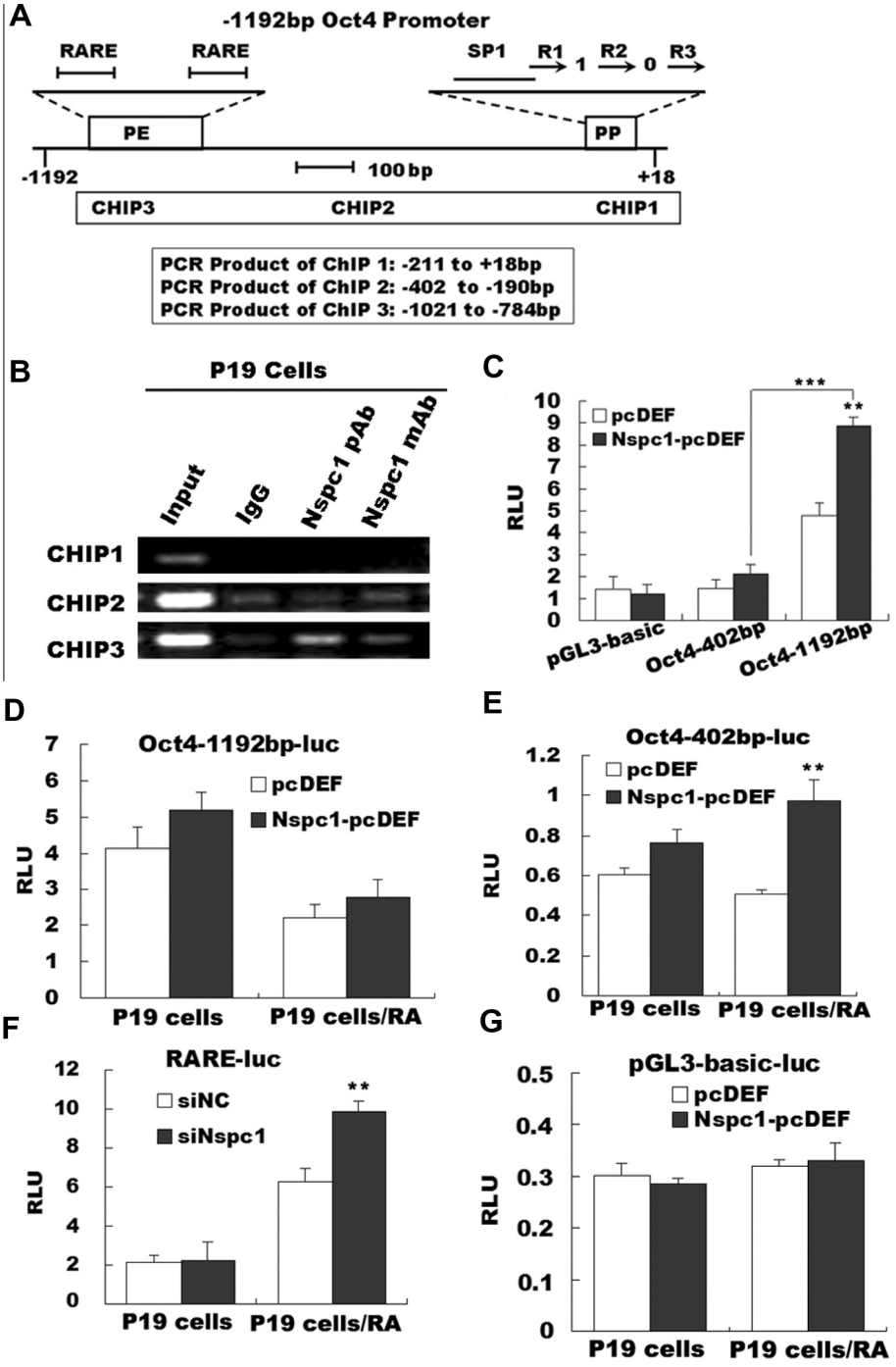

Fig. 4. Nspc1 directly binds to and stimulates Oct4 proximal promoter. (A) The predicted positions of putative Nspc1 binding sites in mouse Oct4 promoter by bioinformatics analysis and the positions of primers designed for ChIP assays. (B) ChIP assays in P19 cells. The predicted size of the PCR product was 229 bp (ChIP1), 212 bp (ChIP2) or 237 bp (ChIP3). (C) The Oct4 promoters with different length were constructed into luciferase reporter plasmid. The different reporter plasmid (200 ng) and control pRL-TK plasmid (50 ng) were transfected into P19 cells with the pcDEF-Nspc1 expression vector (500 ng) or a empty expression vector (500 ng). Protein lysates were prepared at 24 h following transfection, and used to measure dual luciferase enzyme activity. (D and E) The activities of Oct4 promoters with different length were activated at different degree during RA-induced P19 cell differentiation. The different reporter plasmid, over-expression and control plasmid were transfected into P19 cells as above, followed by RA-induced differentiation for 3 days, then the luciferase activities were measured. (F) In P19 cells, RARE-Luc activity is detected by using luciferase reporter assay between Nspc1 depletion and control with or without RA. When Nspc1 depletion, P19 cells were more easily induced by RA signaling. (G) Over-expression of Nspc1 with or without RA had no obvious repression to the pGL3-basic vector. *Abbreviations*: PE, proximal enhancer; PP, proximal promoter; RARE, RA responsive element; RLU, relative luciferase unit. The asterisks indicate statistically significant changes: $^{*}P < 0.05$, $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$.

the Oct4 promoter, luciferase reporter plasmids with different length of mouse Oct4 promoter regions were constructed and transfected into P19 cells with the pcDEF-Nspc1 expression vector or a pcDEF empty expression vector. Co-transfection of Nspc1 expression vector caused a significant increase in Oct4 promoter activity, as shown in Fig. 4C, over-expression of Nspc1 in P19 cells led to a ~86% increase of the Oct4-1192 bp relative luciferase activity compared with control, while over-expression of Nspc1 only led to ~47% increase on the Oct4-402 bp promoter. Further, the activities of Oct4 promoters with different length were inhibited at different levels during RA-induced P19 cell differentiation (Fig. 4D and E). We found when knockdown Nspc1, the stimulation

of a single RARE element reporter gene activity was significantly higher compared with control (Fig. 4F), that's to say when Nspc1 depletion, P19 cells were more sensitive to the RA signaling. Together, these results demonstrated that Nspc1 is a direct upstream activator of the key pluripotent gene Oct4 in pluripotent stem cells.

4. Discussion

It is well known that Oct4, Sox2, and Nanog form a regulatory circuitry including autoregulatory and feedforward loops for maintaining pluripotency in pluripotent stem cells [20,21]. This regulatory circle, in which all transcription factors regulate themselves as well as each other to constitute an interconnected autoregulation loop, has been accounted to be necessary to the ESCs identity. Here, we found the expression level of Nspc1 changes contemporarily with Oct4, Sox2, and Nanog during RA-induced P19 cells differentiation (Fig. 1). Meantime, the expression level of Nspc1 in pluripotency P19 cells affects positively the expression level of Oct4 and Nanog (Fig. 2) in a dose dependent manner (Fig. 3). This regulation has been further verified by the experiments of Nspc1–ChIP in P19 cells. Three representative Oct4 promoter regions were selected (Fig. 4A). The selected proximal region (−211 to +18) of the Oct4 promoter represents the core promoter region, which includes the R1, R2, R3, the SP1 binding sites, and the transcription start site [19]. We observed that the repression of Oct4 by Nspc1 does not involve disturbing the basal transcription machine, for there was no positive signal from this core promoter region in the ChIP assay (Fig. 4B). The second selected middle region (−402 to −190), which does not contain any typical binding site, represents a random or non-specific control region of the Oct4 promoter. The third selected distal region (−1021 to −784) includes two typical RA response elements (RARE), based on the reports from Djabali's lab that the polycomb group protein M33 is able to control the accessibility of the RA response elements in the vicinity of the Hox genes [22]. In our ChIP assay, a positive signal was obtained from the region (−1021 to −784) (Fig. 4B). Further luciferase analysis of the Oct4 promoter revealed that it is reasonable to believe that Nspc1 contributes to the key pluripotent Oct4–Nanog–Sox2 axis through its regulation on Oct4 expression (Fig. 4C–E), suggesting that Nspc1 must possess a role in the pluripotency regulatory loop of pluripotency. In our model, Nspc1 selectively regulate expression of the master pluripotency factor Oct4. Highly expressed Nspc1 may be sequestering or diluting the activity of the RAR/RXR receptors via RARE, thereby preventing full transcriptional repression of target genes in RA signaling pathway (Fig. 4F and G). Further, RA-initiated differentiation reduces the Nspc1 expression, which is correlated with the decreased expression of Oct4, Nanog and Sox2. Thus, Oct4 is subjected to dual regulation by RA and Nspc1, hence the effect of regulation on Oct4 is enlarged. Interestingly, the transcriptional activating activity of Nspc1 on Oct4–Sox2–Nanog axis in P19 cells is very distinctively different from its epigenetic repressor activity [17,23], which offered new visions into the polycomb family transcriptional regulation in stem cells.

Recently, over-expression of a cocktail of transcription factors (Oct4, Sox2, c-Myc and Klf4 or Oct4, Sox2, Lin-28 and Nanog) has resulted in the induction of pluripotency in somatic cells [24,25]. Our results showed Nspc1 activates the key pluripotent Oct4–Nanog–Sox2 axis in P19 EC cells, which not only define a new role for polycomb protein in maintaining the pluripotency of stem cells but also indicates a potential use of Nspc1 in the future iPSC technology development.

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