

RESEARCH ARTICLE

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Differential effects of AdOx on gene expression in P19 embryonal carcinoma cells

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Abstract

Background: Pluripotent cells maintain a unique gene expression pattern and specific chromatin signature. In this study, we explored the effect of the methyltransferase inhibitor adenosine dialdehyde (AdOx) on pluripotency maintenance and gene expression in P19 embryonal carcinoma cells.

Results: After AdOx treatment, the pluripotency-related gene network became disordered, and the early developmental genes were released from the repression. Remarkably, AdOx caused contrasting effects on the expression of two key pluripotency genes, *nanog* and *oct3/4*, with the reduction of the repressive histone marks H3K27me3, H3K9me3 and H3K9me2 only in the *nanog* gene.

Conclusions: Key pluripotency genes were controlled by different mechanisms, including the differential enrichment of repressive histone methylation marks. These data provided novel clues regarding the critical role of histone methylation in the maintenance of pluripotency and the determination of cell fate in P19 pluripotent cells.

Background

Pluripotent cells, including embryonic stem (ES) cells, embryonic germ cells, and embryonal carcinoma cells, are characterized by their ability to differentiate into all somatic cell types under appropriate conditions [1]. Accordingly, pluripotency-related genes are kept active in pluripotent cells, and early developmental genes are maintained in a 'poised' state (i.e., repressed but ready for activation) [2]. Consistent with the wide array of developmental fates, the chromatin in pluripotent cells is also highly adaptable [3]. The unique gene expression pattern and chromatin signature in pluripotent cells are controlled by a transcription factor network that involves Oct3/4, Sox2, and Nanog [1]. These three core factors are tightly regulated, and even limited fluctuation in their expression may cause significant changes in cell fate [4,5]. In ES cells, the pluripotency-related genes have relatively high levels of the active histone mark H3K4me3 in their chromatin. In contrast, the chromatin that contains poised developmental genes is associated with a combination of H3K4me3 and repressive

H3K27me3 marks [2]. Many histone methyltransferases have been proven to be important for normal embryogenesis [6]. Nevertheless, how histone methylation participates in the maintenance of pluripotency and coordinates the precise expression of the core transcription factors remains largely unknown.

P19 pluripotent cell line is derived from mouse embryonal carcinoma that has the ability to contribute to many normal embryonic tissues after blastocyst infection [7-10]. When exposed to nontoxic concentrations of dimethyl sulfoxide (DMSO) in culture conditions, P19 cells can differentiate into muscle cells. When induced with *all trans*-retinoic acid (RA), they can be directed into neuronal lineage cells [11]. AdOx indirectly inhibits S-adenosylmethionine (SAM)-dependent methyl-transfer by inhibiting the hydrolysis of the by-product S-adenosylhomocysteine [12]. AdOx has been broadly used for the functional analysis of protein methylation [13]. It has been shown that AdOx treatment during RA induction interferes with the neuronal differentiation of P19 cells [14].

Here, we show that pre-treatment of AdOx blocks RA-induced P19 cell neuronal differentiation. The impact of AdOx on the expression of pluripotency genes was investigated. We found that in contrast to decreased Oct3/4, the expression of other key

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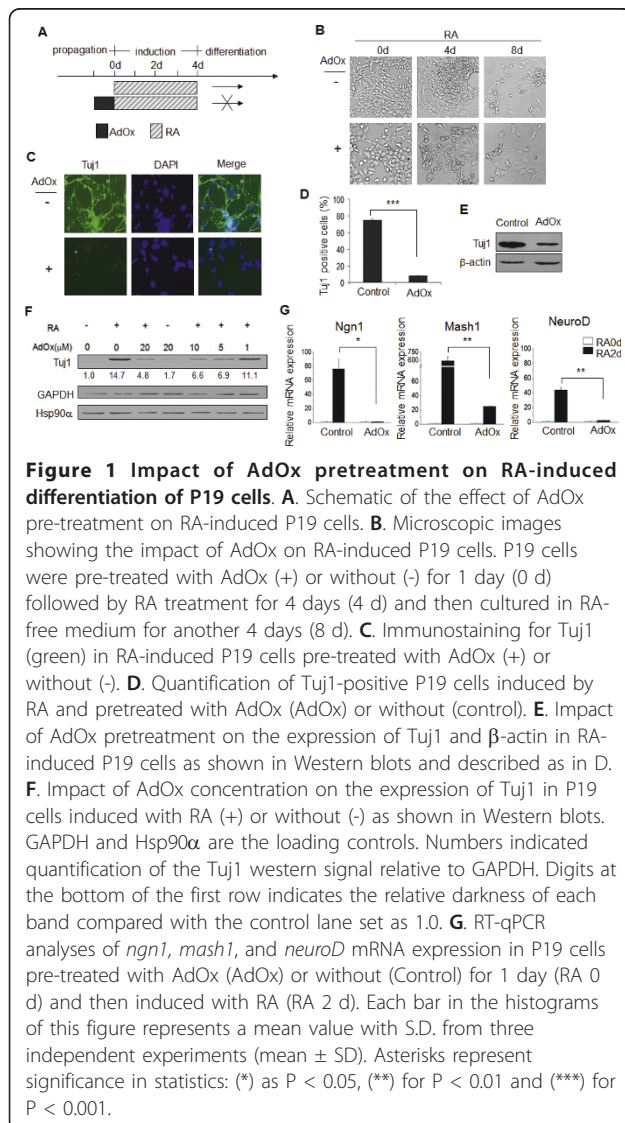
pluripotency-related genes is elevated or unchanged in P19 cells treated with AdOx. We then specifically examined the opposing effects of AdOx on the expression of *nanog* and *oct3/4*, which were supported by the differential repressive histone methylation of these genes. These results provide an example on the differential control of pluripotency-related genes by histone methylation in P19 cells.

Results

AdOx reduces the neuronal lineage potential of P19 cells

To explore whether AdOx affects the maintenance of pluripotency, we examined the neuronal differentiation potential of AdOx-treated P19 cells. P19 cells were pre-treated with AdOx for 1 day and then induced by RA as shown in Figure 1A. Our data showed that AdOx pre-treated cells neither aggregated by day 4 nor formed

neuron-like networks by day 8, in contrast to AdOx-null RA-treated control cells (Figure 1B, bottom *vs.* top panels). Furthermore, immunostaining showed that AdOx significantly reduced the expression of neuron-specific tubulin-isotype β -tubulin III (Tuj1) in RA-induced P19 cells (Figure 1C, bottom *vs.* top panels). In contrast to the almost three quarters of RA-induced cells that express Tuj1 protein and have extended neurites, only 8% of AdOx-treated cells were Tuj1-positive, and they had fewer neurites (Figure 1D). Similar results demonstrating the inhibitory effects of AdOx on RA-induced expression of Tuj1 were obtained by Western blotting (Figure 1E). The reduction of Tuj1 was dependent on the dosage of AdOx (Figure 1F). Next, we investigated whether the neuronal differentiation program can be initiated by AdOx pre-treatment. The hierarchical expression of the transcription factors Ngn1, Mash1, and NeuroD is essential for neuronal fate determination [15]. We found that the RA-induced expression of these early neuronal transcription factors was remarkably reduced or completely abolished at the mRNA level in the presence of AdOx (Figure 1G). These results indicated that the potential for neuronal fate is severely reduced in AdOx-pretreated P19 cells.

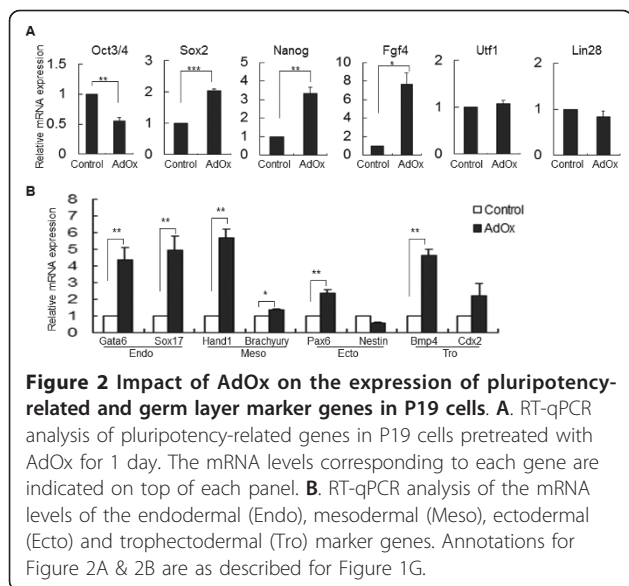


The impact of AdOx on the expression of pluripotency-related genes in P19 cells

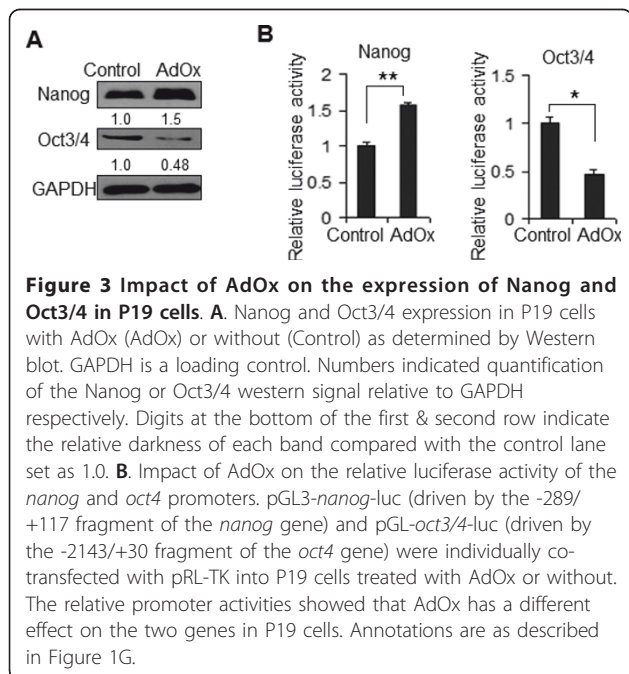
It is important to determine whether the effect of AdOx is correlated with the maintenance of pluripotency. We thus examined the expression of pluripotency-related factors, including Oct3/4, Sox2, Nanog, Fgf4, Utf1, and Lin28. The analysis showed that Oct3/4 mRNA was reduced, which is consistent with the reduced differentiation potential. Unexpectedly, Sox2, Nanog, and Fgf4 were markedly up-regulated after AdOx treatment (Figure 2A). As a control for the pluripotency-related genes, we examined the impact of AdOx on the mRNA expression of three germ layer-marker genes as well (Figure 2B), because these genes are maintained in a 'poised' state in pluripotent cells that are expressed only when the three layers are established during early embryonic development [2]. The mRNA levels of almost all of these genes in P19 cells increased with AdOx treatment, whereas the mRNA level of *nestin*, an ectoderm marker, did not obviously change. Collectively, these data show that the expression pattern of pluripotency-related genes is disordered in AdOx-treated P19 cells and that early development-related genes are released from their poised states

AdOx has opposite effects on the expression of Nanog and Oct3/4

Next, we focused on the differential regulation of pluripotency-related genes by AdOx. We examined the



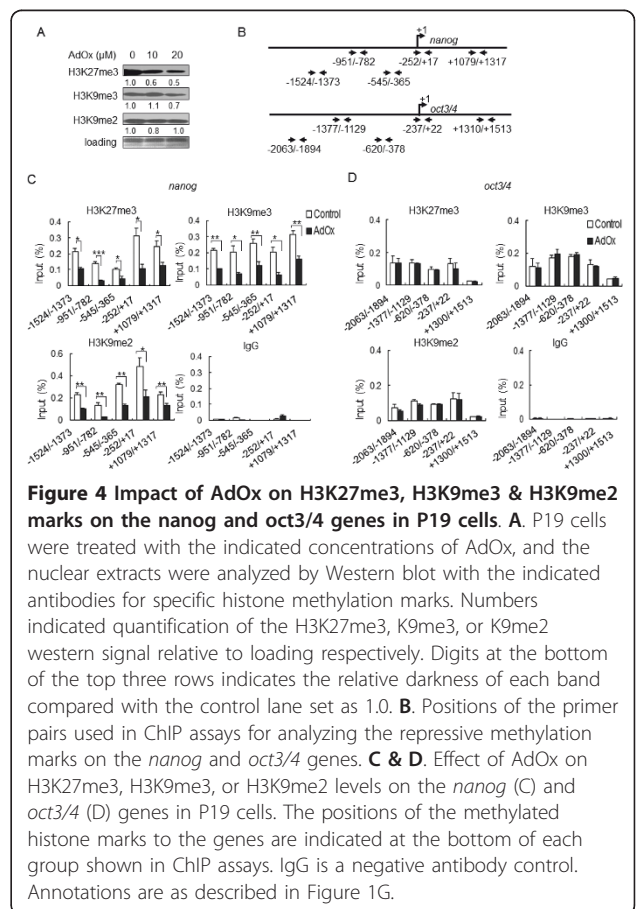
protein expression of the core transcription factors Oct3/4 and Nanog. AdOx-induced changes in the protein levels of Nanog and Oct3/4 were similar to those observed at the mRNA level (Figure 3A vs. 2A). Reporter plasmids driven by the *nanog* (-289/+117) and *oct3/4* (-2143/+30) promoters were then used to confirm the effects of AdOx. It has been reported that these two reporter plasmids can recapitulate the endogenous expression of *nanog* and *oct3/4* [16,17]. We found that the reporter activity driven by the *nanog* promoter was increased, whereas that of *oct3/4* was reduced in AdOx-



treated P19 cells (Figure 3B). Because they are consistent with the measured mRNA levels, the promoter activity data suggest that AdOx has opposite effects on the transcription of the *nanog* and *oct3/4* genes.

Impact of AdOx on the occupation of histone H3K27me3 and H3K9me3 on the *nanog* and *oct3/4* genes

Methylation of histone H3, including H3K27me3, H3K9me3, and H3K9me2, is usually associated with the gene repression. We next examined the global effects of AdOx on these repressive methylation marks and found that AdOx reduced the level of H3K27me3 and H3K9me3, but not H3K9me2, in P19 cells (Figure 4A). To examine whether the *nanog* and *oct3/4* promoters are associated with repressive methylation marks, ChIP assays were carried out for H3K27me3, H3K9me3, and H3K9me2 on five genomic sites that were indicated on the *nanog* and *oct3/4* genes (Figure 4B). The ChIP data revealed that all three repressive marks were significantly reduced around the *nanog* gene after AdOx treatment (Figure 4C), but none were affected in the *oct3/4* gene (Figure 4D). This result suggested that the AdOx-mediated reduction in repressive methylation marks on the *nanog* gene (but not the *oct3/4* gene) is correlated



with the differential regulation of these two major pluripotency-related genes by AdOx in P19 cells.

Discussion

In this study, we demonstrate that AdOx affects the maintenance of pluripotency in P19 cells. Among the pluripotency-related genes, *nanog* and *oct3/4* are differentially regulated by AdOx, and their regulation is correlated with a differential reduction in repressive histone methylation marks in these genes.

The SAM-dependent methyltransferase substrates include lipids, nucleic acids, and a variety of proteins [13]. The effects of AdOx may be universal. After treatment of P19 cells with AdOx, we observed that in addition to the aberrant pluripotent gene expression pattern, AdOx blocked the cell cycle, changed the cell shape and induced expression of the adhesive molecule E-cadherin (data not shown). All of these effects may have contributed to the loss of neuronal potential. In addition, the nonspecific release of early developmental genes may also have disturbed the potential for neuronal fate in P19 cells.

In this study, we examined the expression of six pluripotency-associated genes in P19 cells and their changes in response to AdOx treatment. *Utf1* and *Lin28* were not responsive to AdOx. In contrast, *Sox2*, *Nanog*, and *Egf4* showed higher expression levels following AdOx treatment, whereas the expression of *Oct3/4* was reduced. *Nanog*, *Oct3/4* and *Sox2* are all critical transcription factors for pluripotency maintenance, and *Sox2* functions to maintain the identity of neuronal progenitors, suggesting that the core pluripotency transcription factors perform distinct roles during embryonic development [18]. Recently, it was reported that *Sox2* and *Oct3/4* have opposite effects in defining mesoderm and neural ectoderm [19]. We found that *Nanog* and *Oct3/4* were differentially regulated by AdOx, which suggests that they are controlled by different mechanisms. Consistent with this finding, *Nanog* and *Oct3/4* play non-overlapping roles in the establishment of the primitive endoderm and the epiblast [20]. These clues led us to study the histone methylation of these two genes.

To maintain pluripotency, the crucial pluripotency transcription factors are maintained at relatively high but precisely controlled levels [4,5]. In ES cells, the localized chromatin regions of poised developmental genes have both the active histone mark H3K4me3 and the repressive H3K27me3 mark, whereas the pluripotency-related genes are mainly occupied by relatively high levels of H3K4me3 [2]. However, we found repressive histone methylation marks at pluripotency-related genes at low levels, implying that they potentially have a role in the modulation of pluripotency-related genes. The

expression of *Nanog* in ES cells requires the histone demethylase *Jmjd2C* to antagonize the repressive H3K9me2 [21]. Our ChIP data revealed that AdOx treatment preferentially causes decreased levels of the three repressive histone methylation marks H3K9me2, H3K9me3, and H3K27me3 on the 5' proximal region of the *nanog* gene, whereas it has no effects on the *oct3/4* gene. In other words, the repressive histone methylations are more involved in the regulation of *nanog* than in the regulation of *oct3/4* in P19 cells. As reported previously, both *nanog* and *oct3/4* are under the control of very similar composite sox-oct elements consisting of neighboring binding sites for *Sox2* and *Oct3/4* [16,22]. These observations raised interesting questions: how is the differential regulation of *nanog* and *oct3/4* achieved? Is the underlying mechanism related to functional differences between *Nanog* and *Oct3/4*? We expect that further investigations in the fields of ES cells and developing embryos would confirm our results and answer the above questions. Because AdOx may also influence the active histone methylation marks, the methylation of DNA and on non-histone proteins [13], more specific manipulations on the relevant histone methyltransferases would indeed be helpful in elucidating the differential regulation of *nanog* and *oct3/4*.

Conclusions

We have shown that the expression of *Nanog* and *Oct3/4* are differentially regulated by AdOx, with the repressive histone marks reduced only on the *nanog* gene. These data shed light on the critical role of histone methylation in the maintenance of pluripotency and the determination of cell fate in pluripotent cells.

Methods

Cell culture and treatment

P19 cells were cultured in minimum essential medium (MEM) (Invitrogen) supplemented with 10% (v/v) fetal calf serum. For neuronal differentiation, P19 cells were cultured in medium containing 0.5 μ M RA (all-trans-retinoic acid, Sigma) for 4 days, and the aggregates were plated as a monolayer and cultured for another 4 days in the absence of RA [23]. AdOx pre-treatment involved adding 20 μ M of AdOx (Sigma) to P19 cells for 1 day. Then, AdOx was removed, and the cells were cultured with RA.

Antibodies

The following antibodies were used: monoclonal antibody against Neuronal Class III β -tubulin (*Tuj1*) from Covance, Inc. (MMS435P); antibody against β -actin (ab8226) from Abcam; antibody against HSP90 α from Stressgen (SPS-771); antibodies against GAPDH (MAB347) and *Sox2* (AB5603) from Chemicon; antibody

against Nanog from BETHYL (A300-397A); antibody against Oct3/4 from Santa Cruz (sc-5279); antibodies against trimethyl-Histone H3 (Lys27, 07-449), trimethyl-Histone H3 (Lys9, 07-442), dimethyl-Histone H3 (Lys9, 07-212), and IgG (12-370) from Upstate Biotech.

Quantitative Real Time RT-PCR analysis

Quantitative Real time RT-PCR assays were carried out as previously described [24]. The relative expression of genes was normalized against GAPDH, using the comparative CT method according to the manufacturer's instructions (Roter-Gene RG-3000A Real-time PCR System, Corbett Research, Australia). Primers used in the PCR assays are shown in Table 1. The experiments were repeated at least three times, with statistical analyses for each individual experimental set. All the values in the experiments were expressed as the mean \pm S.D.

Western blotting

Whole cell extracts (WCEs) were prepared as described previously [23]. The pellets were washed twice with RIPA buffer and then resuspended in 500 μ l RIPA buffer (containing protease inhibitors). After adding SDS to 1%, samples were sonicated for 5 seconds on ice on high; the clear solutions were then stored as "nuclear extracts". The samples were separated in SDS-polyacrylamide gels and analyzed as described previously [25]. The density of bands was analyzed and normalized with internal controls (GAPDH or loading) using AlphaImager™ 2200 (Alpha Innotech Corp).

Immunofluorescence

The cells were first fixed with 2% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS for 10 min and then washed and blocked in 1% goat serum. The cells were consecutively incubated in 1:100 diluted mouse anti-Tuj1 antibody at 4°C overnight and the fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:100) at 37°C for 30 min. The cells were then washed and incubated in DAPI (1:3000) for 10 min followed by two PBS washings. Images were collected using a Nikon Eclipse TE2000-U microscope. DAPI was used to stain the nuclei to count the number of cells. The percentage of cells that were immunoreactive for Tuj1 antigens was determined by capturing images of random fields. DAPI-stained nuclei and cells positive for Tuj1 protein were counted.

Luciferase reporter assays

The pGL3-*nanog*-luc (-289/+117) was from Dr. Paul Robson [16]. The pGL-*oct3/4*-luc (-2143/+30) was from Dr. Duanqing Pei [17]. P19 cells were transfected with VigoFect Reagent (Vigorous Biotech) according to the manufacturer's instructions. pGL3-*nanog*-luc or pGL-*oct3/4*-luc reporter plasmids (0.1 μ g) and 0.002 μ g of control plasmid pRL-TK were co-transfected into the cells. Twenty-four hours after transfection, the medium was replaced with fresh α -MEM (AdOx- or AdOx+). Promoter activity was measured 24 hours later. The activities of both luciferase reporters were determined by the Dual-Luciferase Reporter System (Promega) according to the manufacturer's instructions. The

Table 1 Primers used in q-RT-PCR analysis

Gene	Forward primer	Reverse primer
<i>ngn1</i>	CCCTGAAGACGAGGTGAAAAGT	CTTGCCATTGTATTGTCAGCCG
<i>mash1</i>	TAACTCCAACCACTAACAGGC	TGAGGAAAGACATCAACCCAGT
<i>neuroD</i>	TTCCACGTCAAGCCGC	TCGGCGGATGGTTCGT
<i>sox2</i>	CCACCAATCCCATCCA	ACCGCCTAACGTACCCT
<i>oct3/4</i>	TCACTCACATCGCCAATCA	GTAGCCTCATACTCTTCTCGTT
<i>nanog</i>	GGCAGCCCTGATTCTTCTAC	CGCTTGCACTTCATCCTTT
<i>lin28</i>	CGGCCAAAAGGGAAGAACAT	CATTCCTTGGCATGATGGTCT
<i>fgf4</i>	GGCTTCGGCGGCTCTACT	AGGATTTCGTAGGCGTTGTAGTTG
<i>utf1</i>	TCCTCTTACGAGACCCGACAC	GCAACGCGGTATTCAACGA
<i>gata6</i>	AACAGCAGTGGCTCTGTCCC	CTCGGGGTTGGCGTTTT
<i>sox17</i>	TGGGCCAAAGACGAACG	TTGGGGTGGTCTGCATAT
<i>hand1</i>	CTACTTGATGGACGTGCTGG	CAACTCCCTTTCCGCTTG
<i>brachyury</i>	CCAGTCTCTGGTCTGTGAGCA	GGGGAGCCTCGAAAGAA
<i>pax6</i>	GTGAATGGGCGGAGTTATG	ACTTGGACGGGAACTGACA
<i>nestin</i>	TGAGAACTCTCGCTTGACAGC	GTATCCCAAGGAAATGCAGC
<i>bmp4</i>	CGAGCCAACACTGTGAGGA	GAGGTTGAAGAGGAAACGAAA
<i>cdx2</i>	TCAGGAGGAAAAGTGAGCTG	GGCTGTGGAGGCTGTTGTT
<i>gapdh</i>	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTT

Table 2 Primers used in CHIP-qPCR analysis

Gene	Forward primer	Reverse primer
<i>nanog</i> -1524/-1373	AGAGGCAGACAGTGGTGACGA	CCACAGCCACCATACTACTACTG
<i>nanog</i> -951/-782	GGCAAACCTTTGAACCTGGGATGTGGAATA	CTCAGCCGTCTAAGCAATGGAAGAAGAAAT
<i>nanog</i> -545/-365	GCTCTTCTCAGACTTGCCTT	GAGTTTCTCTCCATCTTCTTCTAC
<i>nanog</i> -252/+17	GGGGATGCTTTAGATCAGAGG	CCAATCAGCCTATCTGAAGG
<i>nanog</i> +1079/+1317	TGTCCTCTTTTCTGAGCTT	CACTTTCCACCTCCAAAAT
<i>oct3/4</i> -2063/-1894	GGAAGTGGGTGGGGAGGTTGTA	AGCAGATTAAGGAAGGCTAGGACGAGAG
<i>oct3/4</i> -1377/-1129	TGCTCTGGGCTTTTGGAGCTGTGTGATT	TGGCGAAAGACACTAAGGAGACGGGATT
<i>oct3/4</i> -620/-378	TCTACCAACCTGGACAACAAGATGGAA	GCCACTCTCAGTTCTTCTTACCCAC
<i>oct3/4</i> -237/+22	GTGAGGTGTCGGGTGACCCAAGGCAG	CGGCTCACCTAGGACGGTTTACC
<i>oct3/4</i> +1310/+1513	GGAGTCCCTAGGAAGGCATTAATAGTTT	GGATTCTCTCGGCTTACAGACAGACTT

relative luciferase activities, as measured in relative light units, were compared to a co-transfected internal control (pRL-TK). The assay results are shown as the means \pm S.D.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were carried out as described earlier [24]. The primers used in ChIP-qPCR assays are shown in Table 2. The cycle quantity required to reach a threshold in the linear range (Qt) was determined and compared with a standard curve for the primer set generated by five 10-fold dilutions of genomic DNA samples of known concentration. The percentage of immunoprecipitated DNA relative to input (Input %) was calculated and shown as the mean \pm SD from three independent experiments.

Statistical analysis

Statistical analyses were performed using a two-tailed Student's *t*-test. All of the data are shown as means with standard deviations. $P < 0.05$ was considered significant (*), $p < 0.01$ was considered highly significant (**), and $p < 0.001$ was considered extremely significant (***)

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Authors' contributions

LY, H-YZ, YZ, Y-FS conceived the study, designed the experiments; LY and H-YZ performed the experiments and data analysis; LY, H-YZ, YZ, Y-FS read and revised the final manuscript.

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