GATA-1 directly regulates Nanog in mouse embryonic stem cells

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ABSTRACT

Nanog safeguards pluripotency in mouse embryonic stem cells (mESCs). Insight into the regulation of Nanog is important for a better understanding of the molecular mechanisms that control pluripotency of mESCs. In a silico analysis, we identify four GATA-1 putative binding sites in Nanog proximal promoter. The Nanog promoter activity can be significantly repressed by ectopic expression of GATA-1 evidenced by a promoter reporter assay. Mutation studies reveal that one of the four putative binding sites counts for GATA-1 repressing Nanog promoter activity. Direct binding of GATA-1 on Nanog proximal promoter is confirmed by electrophoretic mobility shift assay and chromatin immunoprecipitation. Our data provide new insights into the expanded regulatory circuitry that coordinates Nanog expression.

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1. Introduction

The pluripotent state of mouse embryonic stem cells (mESCs) is reportedly orchestrated by a core set of transcriptional factors exclusively expressed in pluripotent cells [1]. One such factor, Nanog, is of particular interest in terms of its expression pattern and functional role. The homeoprotein Nanog is highly expressed in mESCs and is rapidly downregulated during in vitro differentiation. Increased expression of Nanog can maintain mESCs in an undifferentiated state regardless of the presence of leukemia inhibitory factor (LIF)/STAT3 signaling that is conventionally regarded as essential [2]. These findings endow Nanog to be a hallmark of pluripotency. Further analysis reveals a heterogeneous expression pattern for Nanog in mESCs [3], and the mosaic distribution of Nanog confer a variable resistance to differentiation upon individual mESCs [4]. Nanog is not involved in the housekeeping machinery of pluripotency but rather stabilize mESCs via resisting global differentiation activity. Another report indicates that Nanog regulates pluripotency in a dose-dependent manner [5].

Collectively, these findings establish that the expression level of Nanog signals the fate of mESCs.

It has been proved that the mosaic distribution of Nanog in mESCs was determined at transcriptional level [4]. Given this, to characterize the transcriptional regulation of Nanog expression in mESCs is crucial for a better understanding of how mESCs make a commitment decision. Hitherto several transcription factors have been identified to directly bind to Nanog promoter and consequently regulate Nanog expression in mESCs. Nanog expression is positively regulated by Brachury [6], Klf2, Klf4, Klf5 [7] and Nanog-Sall4 heterodimer [8] FoxD3 [9] and Zic3 [10] and Zfp143 [11] and Oct4-Sox2 heterodimer [12]. Nanog expression has also been shown to be negatively regulated by Tcf3 [13], GCNF [14], p53 [15] and GATA-4 [16]. The disequilibrium distribution of positive and negative control elements in Nanog promoter might be responsible for the highly expressed level of Nanog, nevertheless, the fluctuating level of Nanog implies that more underlying negative regulators await to be discovered.

Previous reports indicate that the tendency of mutual inhibition exists between Nanog and two endoderm markers, GATA-4 and GATA-6 [3,17,18]. Very recently, all GATA family members have been demonstrated to substitute for Oct4 to induce pluripotency suggesting an overlapping role shared by GATA members [19]. GATA-4 and GATA-6 negatively regulate Nanog, which prompts us to examine whether the other members of GATA family play a part in regulating Nanog expression in mESCs.

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2. Material and methods

2.1. Plasmid construction

For Nanog promoter luciferase reporter plasmid construction, the promoter fragment spanning −235 to +50, −332 to +50, −812 to +50 and −2500 to +50 (relative to the transcription start site) of mouse Nanog was generated by PCR amplification from genomic DNA and cloned into a pGL4.10 vector at NheI and BglII restriction sites. Site-directional mutation fragments of the mouse Nanog promoter were overlap-PCR amplified using the completed −235 to +50 reporter vector as a template. PCR products were digested with NheI and BglII and inserted into the similarly digested pGL4.10 vector. When constructing substitution mutants, the following strategy for nucleotide choice was used: a C/G base pair was changed to A/T, G/C to T/A, A/T to C/G, and T/A to G/C. The coding regions of GATA-1 was cloned from J1 mESCs cDNA and inserted into the pCMV-HA vector.

2.2. Cell culture and cell transfection

J1 mESCs were cultured on 0.1% gelatin-coated dishes, at 37 $^\circ$C in 5% CO_2. Medium for routine maintenance was GMEM (Sigma, G5414) supplemented with 10% FCS (HyClone), 1% MEM non-essential amino acids (Invitrogen), 2 mM GlutaMax (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen), and 1000 units/ml LIF (Merck Millipore). 293T cells were cultured in DMEM with 10% FBS and maintained at 37 $^\circ$C with 5% CO_2.

For luciferase reporter assay, the J1 cells were seeded at 5×10^4 cells per well in 24-well plates 24 h prior to transfection. Then, 100 ng each of the promoter reporter constructs, 10 ng of pRL-TK (Promega) along with 100 ng pCMV-HA or pCMV-HA-GATA-1 were co-transfected in triplicate by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The subsequent assay was performed using the Dual Luciferase Reporter assay system (Promega) according to the supplier's recommendations. Data were collected with a VICTOR X5 Multilabel Plate Reader (Perkin Elmer). All transfections were repeated three times and shown with representative samples. The transfection efficiency was 40%.

2.3. Real-time PCR

Total RNA was isolated from J1 mESCs using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed using a SYBR PrimeScript™ RT-PCR Kit (Takara). Real-time quantification of mRNA was performed with an ABI StepOnePlus PCR system (Applied Biosystems) using SYBR Premix ExTaq II kits (Takara). Target gene expression was normalized to GAPDH expression. The primer sequences used will be available upon requested.

2.4. Western blot analysis

Cells were lysed in ice-cold RIPA cell buffer (prepared in-house) supplemented with protease inhibitors (Thermo Scientific). The proteins were separated with 12% acrylamide gels and transferred to PVDF membranes (Millipore). Probing was performed with specific primary antibodies and HRP-conjugated secondary antibodies (Beyotime technology). The primary antibodies used were Hemagglutinin (HA) (ab16B12; Abcam, 1:500), α-tubulin (ab125267; Abcam, 1:2000).

2.5. Electrophoretic mobility shift assay (EMSA)

EMSAs were performed using an Electrophoretic Mobility Shift Assay Kit (Molecular 176 Probes, Invitrogen, USA) according to the manufacturer's instructions.

Double-stranded DNA oligonucleotides labeled with FAM at the 5′ termini of the sense and antisense strands were annealed with reverse strands in annealing buffer (10 mM Tris—HCl, pH 8.0, 50 mM NaCl, and 1 mM EDTA) and purified with an agarose gel DNA extraction kit. A total of 50 ng probes were incubated with 10 µg cell lysates from 293T cells that had been transfected with pCMV-HA-GATA-1 for 30 min at room temperature. For a supershift assay, 2 µg HA antibodies was added in the mixture. Then, the mixtures were resolved on 6% non-denaturing polyacrylamide gels. The sense strand of the probe: TACCCTTTAAATCTATCGCCTT; the sense strand of mutant: TACCCTTTACCGATATCGCCTT.

2.6. Chromatin immunoprecipitation (ChIP)

ChIP was performed using a ChIP Assay Kit (Beyotime) according to the manufacturer's instructions. J1 cells were cultured to a density of 1×10^8 cells for each immunoprecipitation. Cells were cross-linked for 10 minmin at room temperature with 1% (w/v) formaldehyde and the reaction subsequently quenched with 125 mM glycine. Genomic DNA was isolated and sheared to average lengths of 300—500 bp by ultrasonic. Hemagglutinin (HA) antibody (ab16B12; Abcam) was used for GATA-1-HA ChIP. Flag antibody (ab18230; Abcam) was used in the control ChIP. ChIP enrichment was performed by qPCR. Fold-enrichment was determined by normalizing threshold cycle values of ChIP samples against control. Primer sequences used for ChIP-qPCR are GGGTAGGGTAG-GAGGCTTGA and CGGCTCAAGGCGATAGATT.

3. Results and discussion

To investigate whether other GATA members regulated Nanog transcription, we scanned the Nanog proximal promoter using TFSEARCH (cbrc.jp/research/db/TFSEARCH) for potential binding sites. Notably, we obtained four potential binding sites for GATA-1 in the query sequence (Fig. 1A). Thus we proceeded to determine whether GATA-1 was able to regulate the Nanog promoter activity. To this end, we co-transfected GATA-1 expression plasmid along with a series of Nanog promoter reporter constructs into J1 mESCs. Ectopic expression of GATA-1 caused a sharp reduction in the production of reporter gene (Fig. 1B), indicative of inhibition of Nanog promoter activity. The four Nanog promoter reporter constructs represented different promoter contexts that were designed to ensure the universality of the trans-regulating effect. Also we observed a GATA-1 dose-dependent reduction in Nanog promoter activity (Fig. 1C), thus the inhibiting effect was specific. These data argued that GATA-1 could downregulate Nanog proximal promoter activity. To test whether endogenous Nanog mRNA was influenced by artificial expression of GATA-1, we performed real-time PCR to evaluate the expression of Nanog. We noticed a slight but significant decrease of Nanog transcript in GATA-1-overexpressing J1 cells compared to control J1 cells (Fig. 1D). Collectively, these data indicated that GATA-1 negatively regulated Nanog promoter activity and consequently reduced the transcript level in J1 mESCs.

The finding that GATA-1 represses Nanog promoter activity insuffices to present a direct regulative effect. To identify the GATA-1 responsive element in the proximal promoter, we mutated the four predicted binding sites BS1, BS2, BS3 and BS4 individually and then assayed the regulative effect exerted by GATA-1 again. Site-directional mutation of BS1, BS3, or BS4 has no influence on the regulative effect of GATA1 (Fig. 2A), whereas mutation of BS2