Sp5 induces the expression of Nanog to maintain mouse embryonic stem cell self-renewal

Ling Tang, Manman Wang, Dahai Liu, Mengting Gong, Qi-Long Ying, Shoudong Ye

1 Center for Stem Cell and Translational Medicine, School of Life Science, Anhui University, Hefei, PR China, 2 Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research at USC, Department of Stem Cell Biology and Regenerative Medicine, Keck School of Medicine, University of Southern California, Los Angeles, California, United States of America

These authors contributed equally to this work.

*shdye@126.com

Abstract

Activation of signal transducer and activator of transcription 3 (STAT3) by leukemia inhibitory factor (LIF) maintains mouse embryonic stem cell (mESC) self-renewal. Our previous study showed that trans-acting transcription factor 5 (Sp5), an LIF/STAT3 downstream target, supports mESC self-renewal. However, the mechanism by which Sp5 exerts these effects remains elusive. Here, we found that Nanog is a direct target of Sp5 and mediates the self-renewal-promoting effect of Sp5 in mESCs. Overexpression of Sp5 induced Nanog expression, while knockdown or knockout of Sp5 decreased the Nanog level. Moreover, chromatin immunoprecipitation (ChIP) assays showed that Sp5 directly bound to the Nanog promoter. Functional studies revealed that knockdown of Nanog eliminated the mESC self-renewal-promoting ability of Sp5. Finally, we demonstrated that the self-renewal-promoting function of Sp5 was largely dependent on its zinc finger domains. Taken together, our study provides unrecognized functions of Sp5 in mESCs and will expand our current understanding of the regulation of mESC pluripotency.

Introduction

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of the pre-implantation blastocyst [1]. ESCs were first established from mice [2, 3] and then from rats [4, 5]. ESCs can be maintained indefinitely as self-renewing populations while retaining the capacity to generate any cell type in the body; they not only have become a vital model system and powerful tool for understanding biological development and human diseases but also hold great promise for tissue repair and regeneration. Therefore, it is critical to understand more about how the ESC state is established and maintained. Extrinsic signals and intrinsic transcriptional circuitries govern ESC fate decisions. Notably, exogenous provision of leukemia inhibitory factor (LIF) maintains mESC self-renewal by activating signal transducer and activator of
transcription 3 (STAT3) [6–8]. Extensive studies have identified many Stat3 downstream targets, such as Klf4, Gbx2, Pim1, Pim3, Pramel7, c-Myc, Tfcp2ll and Sp5 [9–17]. Each can reproduce the self-renewal-promoting effect of LIF in mESCs when overexpressed. However, the specific mechanism by which they function in mESCs remains unclear.

Our previous report showed that Sp5 is a downstream target of STAT3 and overexpression of Sp5 is sufficient to maintain the undifferentiated state of mESCs in the absence of LIF [15]. Sp5, a member of the Sp1 family, is characterized by the presence of three typical zinc finger domains belonging to the specificity protein/Krppel-like factor (Sp/Klf) superfamily [18]. It binds to GC/GT-rich regions in the promoter of many genes to mediate the activation and/or repression of transcription [19, 20]. Sp5 plays key roles in many critical biological processes, including stem cell maintenance, cell proliferation, apoptosis, differentiation, and development, and represents a potential target for cancer therapy [21–25]. However, it is still unknown how Sp5 supports mESC self-renewal. Here, we showed that the effect of Sp5 on mESC self-renewal could be interrupted by Nanog knockdown. Furthermore, we demonstrate that Sp5 binds to the Nanog promoter to regulate its expression, indicating that Sp5 is an upstream activator of Nanog. In addition, we found that C-terminal zinc finger domains were indispensable for the full activity of Sp5 in mESCs. Collectively, our results provide a mechanism in which Sp5 acts as a mediator linking the LIF/STAT3 signaling pathway with Nanog to control mESC self-renewal and pluripotency.

Materials and methods

Cell culture

46C mESCs [26], which were provided by Qi-Long Ying (University of Southern California, USA), were cultured on 0.1% gelatin-coated dishes at 37˚C in 5% CO2. The basal media for routine maintenance was Dulbecco’s Modified Eagle Medium (DMEM, TransGen Biotech, China) supplemented with 10% Fetal Bovine Serum (FBS, ExCell Bio, Australia), 1× MEM non-essential amino acids (Invitrogen, USA), 2 mM GlutaMax (Invitrogen, USA), 1× sodium pyruvate (Invitrogen, USA), 0.1 mM β-mercaptoethanol (Invitrogen, USA), 1× penicillin/streptomycin (Invitrogen, USA), and 100 units/ml LIF (Millipore, USA). 293T cells were cultured in the same 10% FBS-DMEM except in the absence of LIF.

Overexpression and knockdown plasmid construction

The coding region of Sp5 was cloned from mESC cDNA with KOD Kit (Novagen, Japan) and inserted into the PiggyBac vector. Overlapping PCR was used to generate Sp5 mutants. For RNA interference in mESCs, shRNA constructs were designed to target 21 base-pair gene-specific regions of Sp5 and were then cloned into plko.1-TRC (AgeI and EcoRI sites). The targeted sequences are as follows:

\[
\begin{align*}
Sp5 \text{sh} #1 & : \text{GGATTCAAAGGATTTGCTTTC;} \\
Sp5 \text{sh} #2 & : \text{GGACTTTGCACAGTACCAGAG;} \\
Sp5 \text{sh} #3 & : \text{GACTTTGCACAGTACCAGAGC;} \\
Nanog \text{sh} #1 & : \text{GGAGTATCCCAGCATCCATTG;} \quad \text{and} \\
Nanog \text{sh} #2 & : \text{GACTAGCAATGGTCTGATTCA}.
\end{align*}
\]

Cell transfection and virus production

For gene overexpression, mESCs were transfected with 2 μg PiggyBac vectors inserted with genes plus 2 μg transposase vector using LTX (Invitrogen, USA) according to the manufacturer’s instructions. For the knockdown experiment, Plko.1-TRC-based lentiviral vectors and
packaging plasmids (pCMV-VSVG and psPAX2) were transfected into 293T cells using LTX (Invitrogen, USA). Supernatant was collected after 48 h and passed through 0.45 μm filters (Millipore, USA). mESCs were cultured in the viral supernatant in the presence of 8 μg/ml polybrene (Sigma, USA) for 24 h. Selection began the next day by adding 2 μg/ml puromycin or 8 μg/ml blasticidin for 1 week.

**Generation of Sp5-knockout mESCs**

The gRNA sequences designed according to the exon region of Sp5 were ligated into the pX330 vector (Addgene) and transduced into cells using LTX (Invitrogen, USA). Selection was continued for 1 week by adding 2 μg/ml puromycin. The targeted sequences are as follows:

- Sp5 gRNA1F: CACCGCTGCACTGAGTCGTT CCGG
- Sp5 gRNA1R: AAACCCGGACGACCTCAGTCGC
- Sp5 gRNA2F: CACCGCTCCGGAACGACTCACTGC
- Sp5 gRNA2R: AAGGCCAGTGACTGCTTCCGGAC
- Sp5 gRNA3F: CACCGGGCGGCTGTGGCCGCTC
- Sp5 gRNA3R: AA ACGAGGACGGCCACAGCGGCCC

**Embryoid body (EB) formation**

For the EB formation assay, 1×10^7 mESCs were grown using low-attachment dishes in standard mESC basal media in the absence of LIF or inhibitors. The aggregates were allowed to grow for 6 days and collected for qRT-PCR analysis.

**qRT-PCR**

Total RNA was extracted using the TRIzol Up Plus RNA Kit (TransGen Biotech, China). cDNA was synthesized from 1 μg total RNA using the TransScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal, TransGen Biotech, China) according to the manufacturer’s instructions. qRT-PCR was carried out with Top Green qPCR SuperMix (TransGen Biotech, China) in a PikoReal Real-Time PCR machine (Thermo Scientific, USA). Target gene expression was normalized to β-actin expression. The primers used are listed in S1 Table.

**Western blot analysis**

Cells were lysed in ice-cold RIPA cell buffer (Sigma, USA) supplemented with protease inhibitors (TransGen Biotech, China). Proteins were separated on a 10% PAGE gel (made in-house) and electrotransferred onto a PVDF membrane. Probing was performed with specific primary antibodies and HRP-conjugated secondary antibodies. The primary antibodies used were HA (3724S, Cell Signaling Technology, 1:1000), Flag (M2, Sigma, 1:1000) and α-Tubulin (SC-8035, Santa Cruz Biotechnology, 1:1000).

**Alkaline phosphatase activity assay**

The alkaline phosphatase activity of mESCs cultured on gelatin-coated plates was detected using the Alkaline Phosphatase Kit (Sigma, USA).

**Immunofluorescence staining**

Cells were fixed in 4% paraformaldehyde for 30 min and incubated at 37°C in blocking buffer (PBS containing 5% BSA and 0.2% Triton X-100). Cells were incubated in the presence of primary antibodies at 4°C overnight and then washed three times with PBS. Cells were then
incubated with the Alexa Fluor 488 (Invitrogen, 1:1000) secondary antibody for 1 h at 37˚C. Nuclei were stained with Hoechst (Invitrogen, 1:5000). The primary antibodies and dilutions used were Oct4 (sc-5279, Santa Cruz Biotechnology, 1:200), SSEA1 (sc-21702, Santa Cruz Biotechnology, 1:100) and Nanog (ab808692, Abcam, 1:100).

Luciferase reporter assay
Different fragments of the Nanog promoter were inserted into the pGL3-basic plasmid (Promega, USA) and co-transfected into PB or PB-Sp5 293T cells with a Renilla luciferase plasmid (Promega, USA). Cells were harvested after 48 h and the luciferase activity of the lysate was measured using the Dual-Luciferase Reporter Assay System (Promega, USA).

Chromatin immunoprecipitation (ChIP) assay
ChIP assays were performed as previously described [27, 28]. Briefly, PB or PB-Sp5 mESCs were grown to near confluency in 15-cm dishes. Cells were fixed in 1% formaldehyde. Sheared chromatin was prepared, precleared with protein G-agarose, and immunoprecipitated with anti-HA antibody overnight at 4˚C. Immune complexes were captured using protein G-agarose and formaldehyde cross-links in the eluted complexes were reversed. DNA was analyzed by real-time PCR. All related sequences are included in S2 Table (as described in detail previously [29]).

Results and discussion
Identification of Sp5 downstream targets in mESCs
To facilitate the identification of Sp5 targets that contribute to mESC self-renewal, we transduced HA-tagged Sp5 (PB-Sp5) using the PiggyBac (PB) transposon-based vector into 46C mESCs and Western blot analysis was used to confirm the enhanced expression of Sp5 (Fig 1A). After culture in serum-containing medium in the absence of LIF for eight days, PB-Sp5 mESCs were continuously propagated while retaining typical mESC morphology and alkaline phosphatase (AP)-positive staining (Fig 1B). Immunofluorescence showed positive expression of the pluripotency marker OCT4 (Fig 1C). At the transcriptional level, as assessed by qRT-PCR, overexpression of Sp5 maintained most of its pluripotency genes (Oct4, Sox2 and Nanog), but suppressed the differentiated genes (Gata4, Gata6 and T) compared to the PB empty vector (Fig 1D) (as described in detail previously [15]). Many reports have demonstrated that Klf2/4/5, Nanog, Esrrb, Gbx2, c-Myc, Tfcp2l1, Tbx3 and Pim1/3 [9, 11, 13, 14, 30–37] can replace LIF to support mESC self-renewal when overexpressed. To investigate whether Sp5 maintains the undifferentiated state of mESCs through regulation of these genes, we used qRT-PCR to detect their expression levels in PB and PB-Sp5 mESCs cultured under LIF/serum-containing conditions. Overexpression of Sp5 upregulated Nanog and Klf2 expression, but not that other factors (Fig 1E). To further confirm the two downstream targets of Sp5, we constructed a lentiviral vector expressing Sp5-specific shRNA sequences and observed that knockdown of Sp5 downregulated the Nanog transcript, but not Klf2 (Fig 1F). Additionally, we designed three guide RNAs of Sp5 to knockout the Sp5 gene in 46C mESCs using the CRISPR/Cas9 system. After selection, we picked and expanded 18 colonies cultured in LIF/serum-containing media. The disruption of both Sp5 alleles was confirmed in two clones by genomic DNA sequencing (S1 Fig). As expected, the expression of Nanog was lower in Sp5-null mESCs compared with wild type cells (S1 Fig), but all were maintained in an undifferentiated state and stained positive for AP activity when cultured under LIF/serum-containing conditions (S1 Fig). This is not surprising, as the pluripotency of Nanog-null and Sp5-knockdown mESCs
can be maintained in LIF/serum media [15, 38]. Taken together, these results suggest that Nanog is a downstream target of Sp5.

Nanog is a direct downstream target of Sp5

To further determine whether Nanog is a direct target of Sp5, we performed ChIP-qPCR to examine whether Sp5 directly binds to the Nanog promoter and found that Sp5 was enriched
and located in several segments of the Nanog promoter (including sites 1, 3, 5, 8, 9, and 15) (Fig 2A). In support of this, we inserted four different regions of the Nanog promoter, including -6000~0, -2342~0, -1500~0 and -322~0, into the PGL3 vector. These luciferase reporter constructs were co-transfected into 293T cells with PB or PB-Sp5, respectively. The luciferase reporter assay showed that the magnitude of Nanog luciferase activity corresponded to the ChIP-qPCR analysis (Fig 2B). Overall, these results indicate that Nanog is a direct target of Sp5 in mESCs.

Nanog mediates the self-renewal-promoting effect of Sp5

To investigate whether Nanog mediates the function of Sp5 in promoting mESC self-renewal, we downregulated Nanog expression in PB-Sp5 46C mESCs with lentiviruses encoding two short-hairpin RNAs (shRNAs) specific to Nanog mRNA (Nanog<sup>sh#1</sup> and Nanog<sup>sh#2</sup>). qRT-PCR analysis confirmed that the Nanog transcript level was decreased (50–70%) in these cells (Fig 3A). After culture in serum-containing medium in the absence of LIF for eight days, scramble control-infected PB-Sp5 mESCs were continually passaged and retained classical mESC morphology, positive AP activity, and expressed the pluripotency markers OCT4 and SSEA1 (Fig 3B and 3C), whereas knockdown of Nanog induced differentiation in PB-Sp5 mESCs, as they expressed lower levels of the pluripotency markers (Oct4, Sox2, Klf4, Esrrb and Tfcp2L1), but higher levels of the differentiation-associated genes (Gata4, Gata6, Mixl1, Sox1 and Cdx2)
Sp5 relies on Nanog to promote mESC self-renewal.

(A) qRT–PCR analysis of Nanog expression in PB-Sp5 mESCs infected with Nanog knockdown lentiviruses and cultured in LIF/serum-containing media. The transcript level was normalized to the scramble shRNA control. Data represent the mean ± s.d of three biological replicates. **p < 0.01 vs scramble shRNA control.

(B) Immunofluorescence of PB-Sp5 cells infected with scramble control and Nanog shRNA lentiviruses cultured under serum-containing conditions in the absence of LIF for eight days. Scale bar, 100 μm.

(C) AP staining images of scramble control and Nanog shRNA mESCs overexpressing PB-Sp5 cultured under serum-containing conditions. Scale bar, 100 μm.

(D) qRT–PCR analysis of the expression of mESC pluripotency markers (Oct4, Sox2, Klf4, Esrrb and Tfcp2l1) and differentiation-associated genes (Gata4, Gata6, Mixl1, Sox1 and Cdx2) in scramble and Nanog knockdown mESCs transfected with PB-Sp5 cultured in the absence of LIF. Data represent the mean ± s.d of three biological replicates. *p < 0.05, **p < 0.01 vs scramble control.
compared to scramble control cells (Fig 3D). These results suggest that Sp5 relies on Nanog to promote mESC self-renewal.

Previous reports have shown that Nanog inhibits differentiation of the primitive endoderm by repressing the expression of the differentiation markers Gata4 and Gata6 in mESCs [33, 34]. To determine whether Sp5 has a similar effect on primitive endoderm specification, we performed an EB formation assay to recapitulate early mouse embryonic development using PB, PB–Sp5, wild type 46C or Sp5 KO mESCs. qRT–PCR analysis showed that PB–Sp5 efficiently suppressed the expression of primitive endoderm markers (Gata4 and Gata6) compared with PB (Fig 3E). In contrast, Sp5 KO EBs showed higher levels of Gata4 and Gata6 compared to wild type mESC-derived EBs (Fig 3F). These results suggest that Sp5 represses primitive endoderm commitment, similar to Nanog.

Previous reports have also indicated that enhanced Nanog can replace LIF to maintain mESC self-renewal [33, 34, 39]. We next constructed a Flag-tagged Nanog-overexpressing vector and transfected it into Sp5 shRNA mESCs (S2 Fig). LIF was then withdrawn from the LIF/serum conditions for eight days to test whether Nanog promotes Sp5 shRNA mESC self-renewal. These mESCs were continually expanded. Over multiple passages, they exhibited a tightly packed classical mESC morphology and positive AP activity (S2 Fig). qRT–PCR analysis also revealed high-level expression of the pluripotency genes but low-level expression of genes associated with differentiation (S2 Fig). These results further illustrate that Nanog is located downstream of Sp5.

Sp5 largely depends on its zinc finger domains to maintain mESC self-renewal

Sp5 belongs to the Sp1 transcription factor family [40, 41] and contains three highly conserved zinc finger domains located near its C-terminus. It is also closely related to the BTEB/KLF gene family [20, 42]. KLF family members, such as Klf2, Klf4 and Klf5, have the ability to phenocopy LIF stimulation to maintain the undifferentiated state of mESCs when overexpressed [11, 30–32]. A previous report demonstrated that the C-terminal zinc finger domains are required for the full activity of Klf5s and that deletion of two zinc fingers can abolish the efficiency of their self-renewal-promoting activity [43]. However, it remains unknown whether Sp5 has such features. To determine whether the zinc fingers are also required for the self-renewal-promoting effect of Sp5, we generated PB system-mediated expression constructs encoding full-length (FL) and three different mutant mouse Sp5 proteins lacking the first (Δ301–320), second (Δ342–365) or third (Δ356–378) zinc finger (Fig 4A). These Flag-tagged genes were successfully expressed in 46C mESCs, as confirmed by Western blot (Fig 4B). As expected, 46C mESCs transfected with the PB empty vector showed widespread differentiation and no longer expressed AP under serum-containing conditions. However, the full-length and mutant Sp5 transfectants were passaged under serum-containing conditions without overt differentiation for eight days (Fig 4C). Notably, the Sp5 mutant-transfected mESCs produced less AP-positive colonies than the PB–Sp5FL mESCs and some colonies exhibited a comparatively flatter morphology (Fig 4C and 4D). Finally, to confirm whether the zinc fingers are essential for the full effect of Sp5, we next tested their ability to regulate the expression of Nanog. As expected, the deletion mutant exhibited a lower level of Nanog expression in Sp5-
overexpressing mESCs compared to PB-\(Sp^{5}\) FL mESCs (Fig 4E). Taken together, these results suggest that the three zinc finger domains are important for the full activity of Sp5 and that the deletion of each impairs the self-renewal-promoting effect of Sp5 in mESCs.

**Conclusions**

Sp5 acts as a target of the LIF/Stat3 signaling pathway and elevated expression of Sp5 recapitulates the self-renewal-promoting effect of LIF to sustain mESC pluripotency [15]. However, the detailed molecular mechanism by which Sp5 mediates its action on mESC maintenance remains unknown. In this study, we demonstrated that Nanog is the major downstream effector of Sp5 and exerts a unique function downstream of Sp5 that is necessary for the full ability of Sp5 in maintaining mESC self-renewal. In support of this, we presented evidence that
knockdown of Nanog induces differentiation in Sp5-overexpressing mESCs. We also showed that Sp5 depends on its three zinc finger domains to maintain mESC identity. Thus, the intact domains of Sp5 are essential for the Sp5-mediated induction of Nanog and maintenance of mESC pluripotency. In the future, understanding how Sp5 interacts with other genes or signaling pathways to induce Nanog and how the latter cooperates with other downstream targets of Sp5 to maintain the pluripotent state of mESCs may facilitate the development of new and better culture conditions for the derivation of authentic mESCs from various species.

**Statistical analysis**

All data are reported as the mean±s.d. Student’s t-test was used to determine the significance of differences. p<0.05 was considered statistically significant.

**Supporting information**

**S1 Fig. Knockout of Sp5 does not impair mESC self-renewal.** (A) Disruption of Sp5 by the CRISPR/Cas9 system was verified by sequencing genomic DNA. False regions are shown in red typeface. (B) qRT-PCR analysis of Nanog expression in Sp5-knockout (KO) 46C mESCs cultured under LIF/serum-containing conditions. The transcript level was normalized to the 46C control. Data represent the mean±s.d. of three biological replicates. **p < 0.01 vs 46C. (C) AP staining images of Sp5-KO and 46C control mESCs cultured under serum/LIF-containing conditions for more than five passages. Scale bar, 100 μm. (D) Quantification of AP-positive colonies in S1C Fig. (TIF)

**S2 Fig. Overexpression of Nanog maintains Sp5-knockdown mESC self-renewal in the absence of LIF.** (A) Flag-tagged Nanog was introduced into 46C mESCs infected with the Sp5 knockdown (KD) lentivirus. The protein level of Flag-tagged Nanog was determined by Western blot. α-Tubulin was used as a loading control. (B) Phase-contrast and AP staining images of Sp5-KD mESCs transfected with PB or PB-Nanog cultured under serum-containing conditions in the absence of LIF for eight days. Scale bar, 100 μm. (C) qRT-PCR analysis of the expression of self-renewal genes and differentiation markers in PB and PB-Nanog mESCs infected with the Sp5 KD lentivirus cultured under serum-containing conditions in the absence of LIF. Data represent the mean±s.d. of three biological replicates. *p < 0.05, **p < 0.01 vs PB. (TIF)

**S1 Table. List of primers used for qRT-PCR analysis.** (XLSX)

**S2 Table. Primer sequences and locations related to the Nanog promoter region.** (XLSX)

**Author Contributions**

**Formal analysis:** Mengting Gong.

**Investigation:** Ling Tang, Manman Wang.

**Resources:** Qi-Long Ying.

**Supervision:** Shoudong Ye.

**Validation:** Manman Wang, Dahai Liu.
Writing – original draft: Ling Tang.
Writing – review & editing: Shoudong Ye.

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