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The histone acetyltransferase Myst2 regulates *Nanog* expression, and is involved in maintaining pluripotency and self-renewal of embryonic stem cells

Min Seong Kim [a,b], Hye In Cho [a,b], Su Hyung Park [a,b], Joo Hyuk Kim [a], Young Gyu Chai [c], Yeun Kyu Jang [a,b,*]

[a] Department of Systems Biology, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea
[b] Initiative for Biological Function & Systems, Yonsei University, Seoul 120-749, Republic of Korea
[c] Division of Molecular and Life Science, Hanyang University, Ansan, Republic of Korea

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ABSTRACT

The histone acetyltransferase Myst2 plays an important role in embryogenesis, but its function in undifferentiated ES cells remains poorly understood. Here, we show that Myst2 plays a role in pluripotency and self-renewal of ES cells. *Myst2* deficiency results in loss of characteristic morphology, decreased alkaline phosphatase staining and reduced histone acetylation, as well as aberrant expression of pluripotency and differentiation markers. Our ChIP data reveal a direct association of Myst2 with the *Nanog* promoter and Myst2-dependent Oct4 binding on the *Nanog* promoter. Together our data suggest that Myst2-mediated histone acetylation may be required for recruitment of Oct4 to the *Nanog* promoter, thereby regulating *Nanog* transcription in ES cells.

1. Introduction

Murine embryonic stem cell (mESC) lines derived from the inner cell mass of blastocysts have the ability to self-renew and maintain a pluripotent state. The pluripotent state of ESCs is maintained by a complicated regulatory network, including transcription factors such as Oct4 (Pou5f1), Nanog, and Sox2 [1–4]. In addition, the C-Myc and Kruppel-like factor (Klf) families are important regulators in promoting the self-renewal of ESCs [5–8]. These factors activate expression of many genes associated with pluripotency, but repress differentiation-related genes [9,10].

Recent studies suggest that histone modifications, such as arginine/lysine methylation and acetylation, are involved in the regulation of the pluripotent status [11–17]. Polycomb group proteins regulate expression of many developmental genes in ESCs via their histone methyltransferase activity targeting H3-Lys27 (H3K27) and H3-Lys4 (H3K4). H3K27 methylation is generally associated with repression, while H3K4 methylation serves as an activation mark. The less compact chromatin structure induced by histone acetylation is associated with gene activation and is recognized as one common signature of undifferentiated ESCs [14,18,19]. During differentiation, histone acetylation decreases and chromatin structure becomes more compact [14,19]. Recent experimental evidence suggests that histone acetyltransferase (HAT) activity is involved in the regulation of pluripotency in ESCs [1,20–23].

Myst2/Hbo1, a MYST domain-containing HAT family protein, is involved in diverse cellular processes, including DNA replication, transcriptional regulation, and embryonic development and erythropoiesis [24]. Myst2 may influence proper binding of the pre-replicative complex via acetylation of the origin recognition complex (ORC) and MCM2 [25,26]. In *Drosophila*, chameau, a homolog of Myst2, can mediate *Hox* gene silencing via genetic interaction with PcG proteins; chameau acts as co-activator in downstream JNK signaling pathways [27,28]. In addition, Myst2 is involved in transcriptional repression. Its serine-rich domain interacts with androgen receptors and/or NF-kB in mammalian cells [29,30].

Abbreviations: AP, alkaline phosphatase; DE, distal enhancer of *Nanog* promoter; EB, embryonic body; ESC, embryonic stem cell; H3K14Ac, histone H3-Lysine 14 acetylation; H4K8Ac, histone H4-Lysine 8 acetylation; H4K12Ac, histone H4-Lysine 12 acetylation; HAT, histone acetyltransferase; hMyst2, human Myst2; KD, knockdown; LIF, leukemia inhibitory factor; NEG seq., the region downstream from the proximal enhancer of *Nanog* promoter; PE, proximal enhancer of *Nanog* promoter

* Corresponding author at: Department of Systems Biology, Yonsei University, 50 Yonsei-ro, Seodaemun-gu, Seoul 120-749, Republic of Korea. Fax: +82 2 312 5657.
E-mail address: ykjang@yonsei.ac.kr (Y.K. Jang).

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Recent studies have also reported that Myst2 is essential for embryonic development and erythropoiesis via H3K14 acetylation [31,32]. Although several lines of evidence support the function of Myst2 in mammalian development and cellular regulation, its potential role in the maintenance of pluripotency and self-renewal in ESCs remains largely unexplored.

In the present study, we investigated whether Myst2 is an important regulator of ESC self-renewal and pluripotency. We show that *Myst2* deficiency causes defects in the pluripotent state of ESCs, accompanied by reduced levels of acetylated H3K14 and H4K12. Our ChIP data reveal that Myst2 associates specifically with the proximal enhancer region of the *Nanog* promoter. Furthermore, Myst2-mediated histone acetylation is required for recruitment of the transcription factor Oct4 to its binding site in the *Nanog* promoter. Thus, our data suggest that Myst2 is required for maintaining ESC pluripotency and self-renewal via at least its control of *Nanog* expression.

2. Materials and methods

2.1. Embryonic stem cell culture

E14Tg2a and R1 mouse ESCs (ATCC) were cultured under a feeder-free condition. They were maintained on 0.1% gelatin-coated dishes in knockout Dulbecco's Modified Eagle's Medium (KO-DMEM) supplemented with 15% ESC-qualified fetal bovine serum (GIBCO), 2 mM L-glutamine, 100 μM 2-mercaptoethanol, 100 U/ml penicillin/100 μg/ml streptomycin, and 1× non-essential amino acids, with or without 1000 U/ml leukemia inhibitory factor (LIF) (Millipore). E14Tg2a and R1 mouse ESCs stably expressing *shRNA* were cultured with medium supplemented with 2 μg/ml puromycin.

2.2. Generation of cell lines stably expressing shRNAs

Myst2 shRNAs and *Luciferase* shRNA were cloned into the lentivirus-based pLKO.1 TRC cloning vector (Addgene plasmid 10879). Myst2 shRNA#1 targets the coding sequence (CDS) of Myst2 mRNA, and Myst2 shRNA#2 targets the 3′ untranslated region of Myst2 mRNA (Supplementary Table S2). The pLKO.1 vector and packaging plasmids pMD.2G-VSVG were used for lentivirus production and infection. This protocol is available elsewhere (http://www.addgene.org/plko). Infected mouse ESCs were selected by culturing in the presence of puromycin (2 μg/ml), and then the mixture of puromycin-resistant colonies was used for further analyses.

2.3. Antibodies

The following antibodies were used for Western blotting, immunofluorescence, and quantitative ChIP (qChIP): anti-OCT4 (Santa Cruz #sc5279), anti-Nanog (Santa Cruz #sc30328), anti-Myst2 (Abcam #ab70183), anti-GAPDH (AbClon #AbC-2003), anti-FLAG (Sigma–Aldrich #F3165), anti-H3 (Abcam #ab1791), anti-H4 (Upstate #07-108), anti-acetyl-H3K9 (Upstate #07-352), anti-acetyl-H3K14 (Upstate #06-911), anti-acetyl-H4K8 (Upstate #06-760), anti-acetyl-H4K12 (Upstate #06-761), anti-acetyl-H4K16 (Upstate #07-329), and IgG (Cell signaling 2729P).

2.4. Histone preparation

Histones were prepared using standard methods, as described previously [33]. Briefly, harvested ESC or embryoid body (EB) pellets were suspended in hypotonic lysis buffer, then lysed via hypotonic swelling and mechanical shearing. Histones were purified with H_2SO_4 and precipitated with TCA. After washing with acetone, histone pellets were dissolved in distilled water.

2.5. Immunofluorescence and alkaline phosphatase (AP) staining

For immunostaining, mESCs were cultured in 12-well plates. The cell fixation, incubation with primary and secondary antibodies, and microscopic observation were performed as previously described [34].

To investigate ESC pluripotency, an Alkaline Phosphatase Detection Kit (Millipore, USA) was used to perform AP staining. ESCs were fixed with 4% paraformaldehyde for 2 min and rinsed with TBST buffer (20 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20). Then cell colonies were stained with Fast Red Violet solution/Napthol AS-BI phosphate solution for 15 min in the dark.

2.6. RNA extraction, quantitative reverse transcription PCR (qRT-PCR), and ChIP assay

Total RNA from ESCs was extracted with TRI-Reagent LS (MRC, USA), according to the manufacturer's instructions. The cDNA synthesis and qRT-PCR were performed as previously described [34]. ChIP experiments were performed using the SimpleChIP Enzymatic chromatin IP Kit (Cell Signaling), according to the manufacturer's instructions. Briefly, ESCs were fixed with 1% formaldehyde for 10 min and treated with 2 ml 10× Glycine for 5 min at room temperature. Chromatin was sonicated and immunoprecipitated using the appropriate antibodies overnight.

2.7. CCK assay and colony formation assay

Cell counting kit (CCK)-8 (Dojindo Molecular Technologies, Inc.) was used to assess cell viability. We incubated 100 μl cell suspension per well in 96-well plates (1000 cells per well) for various time intervals (1, 2, 3, or 4 days). After adding 10 μl CCK-8 solution to each well, cells were incubated for 2 h at 37 °C. Then, absorbance was measured at 450 nm using a microplate reader.

The colony formation assay was performed as described in a previous report [34].

2.8. Hanging drop culture

To test the effect of Myst2 knockdown (KD) on EB formation, hanging drop culture was performed as described previously [35]. Roughly 200 drops, each consisting of ca. 300 cells per 20 μl, were plated on the lids of 150 mm-petri dishes in regular arrays. The lids were reversed and placed on the bottom of PBS-filled petri dishes. After 2 days, EBs were harvested using 2 ml culture media. The EB suspension was then transferred into 6-well culture dishes prior to taking pictures.

2.9. Statistical analysis

Data are presented as means S.D. or S.E.M. Data for the mRNA expression level and ChIP-qPCR were analyzed by a two-tailed student *t* test or a variance (ANOVA) test followed by Bonferroni post-hoc test. Results with $P < 0.05$ were considered significantly different.

3. Results

3.1. Myst2 is downregulated during mESC differentiation

To determine whether Myst2 may be an important regulator of pluripotency in ESCs, we investigated Myst2 transcript and