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Transcription pausing regulates mouse embryonic stem cell differentiation

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ABSTRACT

The pluripotency of embryonic stem cells (ESCs) relies on appropriate responsiveness to developmental cues. Promoter-proximal pausing of RNA polymerase II (Pol II) has been suggested to play a role in keeping genes poised for future activation. To identify the role of Pol II pausing in regulating ESC pluripotency, we have generated mouse ESCs carrying a mutation in the pause-inducing factor SPT5. Genomic studies reveal genome-wide reduction of paused Pol II caused by mutant SPT5 and further identify a tight correlation between pausing-mediated transcription effect and local chromatin environment. Functionally, this pausing-deficient SPT5 disrupts ESC differentiation upon removal of self-renewal signals. Thus, our study uncovers an important role of Pol II pausing in regulating ESC differentiation and suggests a model that Pol II pausing coordinates with epigenetic modification to influence transcription during mESC differentiation.

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1. Introduction

Understanding the mechanisms governing self-renewal and differentiation of pluripotent embryonic stem cells (ESCs) is critical for designing efficient and safe protocols for their use in regenerative medicine. The hallmarks of pluripotency; i.e. maintaining unlimited self-renewal potential while being equipped to activate differentiation programs upon signals are regulated through a set of ESC transcription factors and epigenetic marks (Bernstein et al., 2006; Marks et al., 2012; Young, 2011). These together allow for continuous activation of stem cell factors and repression of developmental regulators. Many mechanisms have been proposed to regulate this unique ability of ESCs, including the deposition of "bivalent" histone marks and RNA polymerase II (Pol II) pausing at initial stages of transcription (Marks et al., 2012; Tee et al., 2014).

Pol II pausing occurs soon after transcription initiation and is mainly regulated by two pausing factors DSIF (DRB-Sensitivity Inducing Factor) and NELF (Negative Elongation Factor) (Rougvie and Lis, 1988; Yamaguchi et al., 1999). Release of Pol II to productive elongation is facilitated by recruitment of P-TEFb (positive transcription elongation factor b) that phosphorylates the C-terminal domain of Pol II as well as the pausing factors, leading to NELF dissociation and DSIF being converted to an elongation-stimulating factor (Cheng and Price, 2007; Liu et al., 2015; Peterlin and Price, 2006).

RNA Pol II pausing is suggested to be an important checkpoint in early transcription where signals can be integrated for rapid and synchronous gene activation (Adelman and Lis, 2012; Levine, 2011). Accordingly, genomic studies have found enrichment of paused Pol II at genes involved in signal transduction, developmental control and cell proliferation (Min et al., 2011). In mice, loss of the B subunit of NELF (NELF—B) leads to embryonic lethality at the inner cell mass stage (Amleh et al., 2009). In addition, deletion of *Nelf-b* in mESCs causes proliferation defects together with a blunted response to differentiation signals (Williams et al., 2015). Although these studies suggest crucial roles of NELF-mediated pausing in mouse embryonic development, the interpretation of these results could be complicated by other functions of NELF-B, such as the physical and functional interaction of NELF-B with the DNA repair protein BRCA1 (Aiyar et al., 2007; Nair et al., 2016; Ye et al., 2001). Thus, the direct role of Pol II pausing in mammalian embryonic development remains to be elucidated.

In our previous studies, we have reported an essential function of the pausing factor DSIF in the development of zebrafish hematopoietic stem cells by characterizing a missense mutation in the DSIF subunit gene *spt5* (Yang et al., 2016). In vitro assays have shown that the mutation-caused single amino acid change in zebrafish SPT5 protein specifically disrupts the pausing function of DSIF without affecting its elongation stimulating activity (Guo et al., 2000). Here, based on the high conservation between zebrafish and mammalian SPT5, we incorporated the

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same mutation into mESCs by CRISPR/Cas9 genome editing. Using global run-on sequencing (GRO-seq), we verified the genome-wide reduction of Pol II pausing in *Spt5* mutant mESCs. Although mutant mESCs can be maintained at the self-renewal culturing conditions, these cells show genome-wide transcriptional changes and have severe defects during differentiation. Importantly, we identified a tight correlation between pausing status and local chromatin environment. Our results suggest that genes with unfavorable chromatin environment may rely more on paused Pol II to make them permissive for future activation during differentiation.

2. Material and methods

2.1. Cell culture and generation of ESC clones

ESCs were routinely cultured without feeders in mESC medium (DMEM + 15% fetal bovine serum, 1 mM sodium pyruvate, 50 μM b-mercaptoethanol, MEM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin and 1000 U/ml LIF) or in serum free N2B27 medium supplemented with 1 μM MEK inhibitor (Cayman) and 3 μM CHIR99021 (Cayman) (Ying et al., 2008) on 0.2% gelatin-coated plates. ES-E14TG2a (E14) ESCs were used for generation of $Spt5^{V1008D}$ clones. Cas9, sgRNA and donor DNA plasmids were introduced to E14 ESCs using the Neon transfection kit (Fisher), and integration of clones were confirmed using PCR followed by Sanger Sequencing. See supplemental information for further details.

2.2. Self-renewal assays and alkaline phosphatase staining

Single cells were plated 100cells/cm^2 in 0.2% gelatin-coated 6-well plates and 24-well plates in triplicate. Every three days, cells in 6-well plates were dissociated with 0.25% Trypsin-EDTA and passaged to new plates at a concentration of 100cells/cm2. At the same time, the cells in 24-well plates were stained for alkaline phosphatase according to the manufacturer's instructions (Stemgent). The number of positive colonies is counted. The cumulative number of colonies was calculated by multiplying the colony counts by the dilution factor used for passaging. Results are plotted as mean ± SEM of three wells.

2.3. RNA isolation and q-RT-PCR analysis

Total RNA was extracted using Trizol (Fisher) followed by DNAse treatment and reverse transcribed to cDNA with Superscript 3 cDNA synthesis kit (Fisher). qPCR was performed on Roche LightCycler 480 using the iQ SYBR Green Mastermix (BioRad). Gene expression was analyzed relative to *Gapdh* using the ΔΔCt method.

2.4. GRO-seq and data analysis

Nuclei isolation, run-on and preparation of libraries were performed as previously described(Franco et al., 2015). GRO-seq data were analyzed using the groHMM package described elsewhere (Chae et al., 2015). Additional details of GRO-seq library preparation and data analysis are described in Supplemental Information. ATAC-seq was performed as previously described (Buenrostro et al., 2013).

2.5. Differentiation assays

Serum-free differentiation was performed by removal of GSK3 and MEK inhibitors from ESCs in 2i media for indicated time lengths. EBs were prepared by titrating ESCs to single cell suspension in ESC media lacking LIF in hanging drops (300–500 cells/drop) and transferred to low-attachment plates in a shaking incubator after 3 days. EBs were collected at indicated time points for further analysis. Teratomas were prepared by intraperitoneal injection of 200,000 ESCs. Teratomas were collected after 21 days and stained with H&E for histological analysis (Histoserv Inc.).

2.6. Statistical analyses

Data were analyzed by Student's *t*-test. In all figures, data are represented by mean ± SEM from ≥3 independent experiments.

2.7. Accession numbers

GRO-seq and ATAC-seq data are available on GEO under accession numbers GSE99760.

3. Results

3.1. Generation of Spt5 mutant mESCs

To identify the targets of Pol II pausing in ESCs, we aimed to generate pausing mutant mouse ESCs. Previous studies using mouse ESCs with a conditional deletion of the NELF complex subunit gene *Nelf-b* have shown a severe proliferation defect (Williams et al., 2015). Therefore, we opted for an alternative strategy to target the pausing complex DSIF subunit SPT5 (encoded by *Supt5h*). In vitro studies have shown that a single amino acid change (V1012D) in the C-terminal of zebrafish SPT5 protein specifically disrupted the pausing function of DSIF without affecting its elongation stimulation function (Guo et al., 2000). Because the residue mutated in zebrafish SPT5 is conserved in mouse (Fig. S1A), we introduced the same mutation (V1008D) in mouse SPT5 using a CRISPR/Cas9-mediated knockin approach (Fig. 1A). Multiple homozygous mESC clones carrying the V1008D mutation were successfully generated and confirmed by Sanger sequencing (Fig. 1B, S1B). All clones show comparable ESC characters to wild-type cells based on colony morphology, growth rate and alkaline phosphatase staining (Fig. 1C and data not shown), and can be maintained under both serum containing and serum-free ESC culture conditions.

To quantify the clonal self-renewal ability of $Spt5^{V1008D}$ ESCs, we monitored the expansion of cells that are able to establish alkaline-phosphatase (AP) positive colonies at clonal density over three passages. $Spt5^{V1008D}$ ESCs behaved at similar levels compared to control ESCs (Fig. 1D). In line with this, quantitative RT-PCR revealed no significant changes of expression levels of stem cell markers in mutant ESCs (Fig. 1E).

To test the self-renewal ability of $Spt5^{V1008D}$ ESCs in long term culturing, we grew wild-type and $Spt5^{V1008D}$ ESCs for over 15 passages in the serum-free culture condition (Fig. S2). Similar to wild-type ESCs, $Spt5^{V1008D}$ ESCs were able to maintain normal ESC morphology and positive AP staining at late passages (Fig. S2A). Furthermore, mutant ESCs showed comparable cell cycle profiles and expression levels of pluripotency markers (Fig. S2B–D). We conclude that the V1008D mutation in SPT5 does not cause significant defects in self-renewing ESCs.

3.2. Genome-wide reduction of pol II pausing in $Spt5^{V1008D}$ ESCs

Previous in vitro studies have shown that the same mutation in zebrafish SPT5 specifically disrupts the pausing function of DSIF (Guo et al., 2000). To understand the genome-wide impact of this mutation on Pol II pausing, we performed GRO-seq to measure the distribution of transcriptionally engaged Pol II in wild-type and $Spt5^{V1008D}$ ESCs. We first validated our GRO-seq approaches in wild-type ESCs grown under serum/LIF vs. 2i conditions. A much higher occupancy of promoter-proximal Pol II in 2i condition than serum/LIF condition was observed, indicating strong pausing in cells grown in 2i (Fig. S3A). This is consistent with previous studies showing increased pausing in 2i by Pol II ChIP-seq (Marks et al., 2012). Accordingly, calculation of pausing index (PI) by taking the ratio between Pol II around the promoter and the gene body revealed a much higher PI in 2i condition

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