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The BRPF2/BRD1-MOZ complex is involved in retinoic acid-induced differentiation of embryonic stem cells

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ABSTRACT

The scaffold protein BRPF2 (also called BRD1), a key component of histone acetyltransferase complexes, plays an important role in embryonic development, but its function in the differentiation of embryonic stem cells (ESCs) remains unknown. In the present study, we investigated whether BRPF2 is involved in mouse ESC differentiation. *BRPF2* depletion resulted in abnormal formation of embryoid bodies, down-regulation of differentiation-associated genes, and persistent maintenance of alkaline phosphatase activity even after retinoic acid-induced differentiation, indicating impaired differentiation of *BRPF2*-depleted ESCs. We also found reduced global acetylation of histone H3 lysine 14 (H3K14) in *BRPF2*-depleted ESCs, irrespective of differentiation status. Further, co-immunoprecipitation analysis revealed a physical association between BRPF2 and the histone acetyltransferase MOZ in differentiated ESCs, suggesting the role of BRPF2-MOZ complexes in ESC differentiation. Together, these results suggest that BRPF2-MOZ complexes play an important role in the differentiation of ESCs via H3K14 acetylation.

1. Introduction

Embryonic stem cells (ESCs), which are derived from the inner cell mass of blastocysts, have unique properties, such as self-renewal and pluripotency. During differentiation, ESCs lose these properties and commit their lineage to three germ layers: the ectoderm, mesoderm, and endoderm. This complex process of lineage commitment is tightly regulated by epigenetic mechanisms [28], such as dramatic changes of chromatin structure associated with global histone modifications. In particular, histone acetylation supports hyperactive transcription in pluripotent stem cells and activates lineage-committed genes depending on differentiation stage, suggesting the crucial role of histone acetyltransferase (HAT) complexes in both pluripotent and differentiated states [5,13,16,22,32]. For example, previous studies show that global histone acetylation dramatically decreases during the initial stage of differentiation but rebounds as differentiation progresses [10], implying that histone acetylation plays a significant role in the function of ESCs during differentiation.

Among several subunits comprising HAT complexes, scaffolding proteins are critical for proper enzymatic activity. The scaffold protein BRPF2 (also called BRD1), a member of the BRPF family, links the MYST family of HATs with ING5 and EAF6, helping to recruit the proteins to specific target gene(s) and providing HAT histone tail specificity [4,8,29,30,31]. A previous study shows that BRPF2 regulates the differentiation of mouse ESCs (mESCs) and is required for fetal erythropoiesis via interaction with MYST2 [14]. Furthermore, *BRPF2* knock-out embryos show abnormal growth, with malformation of the neural tube and optic cups [14]. In addition, BRPF2 is implicated in adult brain function, as well as embryonic neurodevelopment, with the BRPF2 gene linked to both schizophrenia and bipolar effective disorder [17,25]. These studies indicate that BRPF2 is essential for embryonic development, especially during ectoderm differentiation. However, the function of BRPF2 in the differentiation of ESCs into multiple lineages is not fully understood. In this study, we investigated the regulatory role of BRPF2 in the differentiation of mESCs. Using *BRPF2* knock-down (KD) ESCs, we showed that depletion of BRPF2 delays differentiation of ESCs and reduces global levels of histone H3 lysine 14 (H3K14) acetylation. Therefore, our findings suggest that BRPF2 regulates differentiation of ESCs through H3K14 acetylation.

Abbreviations: Ac, acetylation; AP, alkaline phosphatase; EBs, embryoid bodies; ESCs, embryonic stem cells; HATs, histone acetyltransferases; H3K9, histone H3 lysine 9; H3K14, histone H3 lysine 14; H3K9Ac, histone H3 lysine 9 acetylation; H3K14Ac, histone H3 lysine 14 acetylation; KD, knockdown; LIF, leukemia inhibitory factor; qRT-PCR, quantitative real-time PCR; RA, retinoic acid

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2. Materials and methods

2.1. Cell culture and ESC differentiation

E14Tg2a mouse ESCs (ATCC, Manassas, VA) were cultured on 0.1% gelatin-coated dishes under feeder-free conditions in knock-out Dulbecco's Modified Eagle's Medium (GIBCO, Waltham, MA) supplemented with 15% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), 2 mM L-glutamine, 100 U/ml penicillin/100 μg/ml streptomycin, 100 μM 2-mercaptoethanol, 1 × non-essential amino acids, and 1000 U/ml leukemia inhibitory factor (MTI-Global Stem, Gaithersburg, MD). E14Tg2a mouse ESCs stably expressing shRNA were cultured in medium supplemented with 2 μ/ml puromycin. For ESC differentiation, cells were resuspended in bacterial culture dishes in leukemia inhibitory factor (LIF)-free ESC medium supplemented with 10 μM all-trans retinoic acid (RA; Sigma-Aldrich, St. Louis, MO).

2.2. Construction of stable cell lines

To generate KD constructs for BRPF2, MORF, MOZ, MYST2, and Luciferase, each shRNA was cloned into lentivirus-based pLKO.1 TRC cloning vector (Addgene plasmid 10879; kindly provided by Dr. David Root). BRPF2 shRNA#1 (shBRPF2#1) and MORF shRNA target the coding sequences (CDS) of the mRNAs, whereas BRPF2 shRNA#2 (shBRPF2#2), MOZ shRNA, and MYST2 shRNA target the 3′ untranslated region (UTR) of the mRNAs (Supplementary Table S1). BRPF2 overexpression plasmid was generated by cloning the BRPF2 coding region into flag-tagged pMSCV-puro vector or flag-tagged pMSCV-hygro vector.

Production of lentiviral particles was done as previously described [7]. mESCs were infected by viral particles from HEK293FT cells and selected by culturing in the presence of puromycin (2 μg/ml; Santa Cruz Biotechnology, Inc., Dallas, TX) or hygromycin (100 μg/ml; Invitrogen, Carlsbad, CA).

2.3. Alkaline phosphatase (AP) and immunofluorescence staining

To examine ESC pluripotency, an AP detection kit (Millipore, Billerica, MA) was used. ESCs were cultured in 6-well plates for 5 days and then stained with Fast Red Violet solution/Napthol AS-PI phosphate solution for 15 min in the dark, according to the manufacturer's protocol.

For immunostaining, mESCs were cultured in 12-well plates. Cell fixation, incubation with primary and secondary antibodies, and microscopic observation were carried out as previously described [7]. Cells were visualized by inverted fluorescent microscopy (AE31, Motic) and a digital camera system (SPOT Insight™ Camera, SPOT Imaging Solutions, Sterling Heights, MI) with the use of SPOT 5.1 Advanced Software (SPOT Imaging Solutions).

2.4. RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRI-reagent LS (MRC, Cincinnati, OH) according to the manufacturer's instructions. cDNA synthesis and qRT-PCR were performed as previously described [18]. Primer sequences are shown in Supplementary Table S2.

2.5. Histone preparation

Histones were prepared as previously described [26]. Briefly, harvested ESCs or embryoid body (EB) pellets were resuspended in hypotonic lysis buffer and then lysed via hypotonic swelling and mechanical shearing. Histones were then purified with H_2SO_4 and precipitated with TCA. After washing with acetone, histone pellets were dissolved in distilled water.

2.6. Antibodies

The following antibodies were used for western blotting, immunofluorescence staining, and co-immunoprecipitation: anti-OCT4 (Santa Cruz, #sc-5279), anti-MAP2 (Santa Cruz, #sc-20172), anti-β3 tubulin (Santa Cruz, #sc-51670), anti-BRPF2 (Abcam, #ab181060), anti-MORF (Abcam, #ab191994), anti-H3 (Abcam, #ab1791), anti-FLAG (Sigma-Aldrich, #F3165), anti-alpha tubulin (Ab Frontier, #LF-PA0146), anti-acetyl-H3K9 (Upstate, #07-352), anti-acetyl-H3K14 (Upstate, #06-911), and anti-MOZ (Biorbyt, UK, #orb30176).

2.7. EB formation

EBs were formed by hanging drop culture as previously described [9]. Briefly, approximately 200 drops, each consisting of 200 cells per 20 μl, were plated on the lids of 150-mm bacterial culture petri dishes. After 2 days, EBs were collected in 2 ml of culture media. The EB suspension was then transferred into 6-well culture plates prior to imaging. Circularity of EB was measured by Image J software (Java image processing program inspired by NIH Image) using the formula [Circularity=4pi (area/perimeter^2)].

2.8. Co-immunoprecipitation

As previously described [18], cells were lysed by IP lysis buffer (1% Triton X-100 and 10% glycerol in 20 mM Tris–HCl [pH 8.0] containing 137 mM NaCl, 2 mM EDTA with protease inhibitor, NAF, and PMSF). Cell lysates were incubated with anti-FLAG affinity gel (Sigma-Aldrich) overnight at 4 °C. The next day, beads were washed five times and then boiled with 2 × Laemmle's SDS sample buffer (GenDEPOT, Katy, TX). Eluted protein complexes were analyzed by western blotting.

2.9. Chromatin immunoprecipitation (ChIP)

Cells were fixed and cross-linked with 1% formaldehyde and quenched by 1 × Glycine. ChIP experiments were performed using SimpleChIP Enzymatic chromatin IP Kit (Cell signaling), according to the manufacturer's instruction. Primer sequences are shown in Supplementary Table S2.

2.10. Establishment of knock-out cell lines using CRISPR-CAS9

For construction of BRPF2 knock out cell lines, we used CRISPR-CAS9 system as previously reported [3]. We selected two sets of sgRNA sequence targeting BRPF2 genomic DNA by http://www.genome-engineering.org/crispr/. Two oligonucleotides were cloned into pX459 (Addgene plasmid 62988) and co-transfected into E14Tg2A mESCs using DNA-In stem transfection reagent (Global Stem). Transfected mESCs were selected by puromycin (2 μg/ml) for one day and after selection, cells were trypsinized and about 100 cells were cultured in 100 mm culture dish. After about 10 days, single colonies were picked and genomic DNA of each clones was extracted using QuickExtract™ DNA Extraction Solution (Epicentre) for screening by PCR. Then, PCR products were purified and analyzed by Sanger sequencing to confirm double-strand break (DSB) caused by CAS9. sgRNA sequence are shown in Supplementary Table S3.

2.11. Statistical analysis

Data are expressed as standard error of the mean (SEM). Pairwise comparisons were performed using two-tailed Student's *t*-