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The histone demethylase *Jmjd3* regulates zebrafish myeloid development by promoting *spi1* expression

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ABSTRACT

The histone demethylase *Jmjd3* plays a critical role in cell lineage specification and differentiation at various stages of development. However, its function during normal myeloid development remains poorly understood. Here, we carried out a systematic in vivo screen of epigenetic factors for their function in hematopoiesis and identified *Jmjd3* as a new epigenetic factor that regulates myelopoiesis in zebrafish. We demonstrated that *jmjd3* was essential for zebrafish primitive and definitive myelopoiesis, knockdown of *jmjd3* suppressed the myeloid commitment and enhanced the erythroid commitment. Only overexpression of *spi1* but not the other myeloid regulators rescued the myeloid development in *jmjd3* morphants. Furthermore, preliminary mechanistic studies demonstrated that *Jmjd3* could directly bind to the *spi1* regulatory region to alleviate the repressive H3K27me3 modification and activate *spi1* expression. Thus, our studies highlight that *Jmjd3* is indispensable for early zebrafish myeloid development by promoting *spi1* expression.

1. Introduction

Myelopoiesis is the process of producing all types of myeloid cells including granulocytes and monocytes/macrophages from stem and progenitor cells. Myeloid cells are critical effectors and regulators of tissue regeneration, innate and adaptive immune response [1]. Malfunction or dysregulation of myelopoiesis is tightly associated with various human blood diseases including acute myeloid leukemia (AML) [2]. Previous studies have illustrated the essential role of several transcription factors for normal vertebrate myelopoiesis, including *Spi1*, *C/ebpα*, *Gcsfr*, *Irf8*, and *Gfi1* [3–7]. However, the orchestrated regulatory programs, consisting of both transcriptional and epigenetic controls during normal myeloid development, remain unclear.

Recently, zebrafish has emerged as an excellent vertebrate animal model to study the development of myelopoiesis [8–10]. Similar to mammals, zebrafish has most of the myeloid cell types including neutrophil, monocyte, eosinophil, mast cell and dendritic cell [11–15]. In a developing vertebrate embryo, hematopoiesis consists of two successive waves, termed primitive hematopoiesis and definitive hematopoiesis [9]. In zebrafish, the primitive hematopoiesis takes place in the anterior lateral plate mesoderm (ALPM) and intermediate cell mass (ICM), producing primitive myeloid cells and erythrocytes, respectively [16]. On the other hand, definitive hematopoiesis occurs at the ventral wall of dorsal aorta at a site called aorta-gonad-mesonephros (AGM) around

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28 hours post-fertilization (hpf) [17]. By 48 hpf, the AGM-derived hematopoietic stem/progenitor cells (HSPCs) migrate to the caudal hematopoietic tissue (CHT, equivalent to the mammalian fetal liver) for rapid lineage expansion and differentiation [18,19]. Alternatively, the erythromyeloid progenitors (EMPs) that arise autonomously in the posterior blood island (PBI) between 24 and 36 hpf can also generate definitive erythroid and myeloid colonies [20].

The important role that histone methylation plays during transcriptional regulation of gene expression in cell differentiation and proliferation has been long recognized [21]. Trimethylated histone H3 at lysine 4 (H3K4me3) marks transcriptionally active chromatin states, whereas trimethylated histone H3 at lysine 27 (H3K27me3) marks transcriptionally repressive chromatin states. Jumonji domain-containing protein D3 (*Jmjd3*, also named Kdm6b) is a member of the H3K27me3/2-specific demethylase family that promotes gene transcription by acting as a rival of the Polycomb repressive complex 2 (PRC2) [22,23]. Studies using embryonic stem cells suggest that *Jmjd3* is required for the development of all three germ layers [24–26], *Jmjd3* accelerates the specification of pluripotent cells by removing H3K27me3 barriers [27]. It has also been shown that *Jmjd3* function is necessary for the differentiation and proliferation of cells in different tissues, such as neurons, epidermal cells, cardiac cells, M2 macrophages and T cells [23,25,28–30]. However, the in vivo function of *Jmjd3* in myeloid lineage development remains to be determined.

Here, we show that *Jmjd3*, a zebrafish JmjC domain-containing protein, possesses H3K27 histone demethylase activity in vivo, and down-regulation of *jmjd3* leads to significant reduction of primitive and definitive myelopoiesis. Importantly, we have demonstrated that *jmjd3* directly upregulates *spi1* expression to promote myeloid commitment in a histone demethylase-dependent manner. Our data, therefore, delineate the biology and the underline mechanism of *jmjd3* function during normal myelopoiesis.

2. Materials and methods

2.1. Zebrafish maintenance and breeding

All experimental procedures followed the rules of the Committee on Animal Care of Shanghai, China. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University. Zebrafish were raised and maintained at 28.5 °C in an aquatic system equipped with continuously filtration, UV treatment and aeration of fish water in circulation. The embryos were collected in dishes and reared in an incubator at 28.5 °C. The transgenic line Tg (*gata1*: EGFP) [31], Tg (*lyz*: EGFP) [32] and Tg (*spi1*: EGFP) [33] were used in this study.

2.2. Plasmids construction, morpholino and mRNA injections

The zebrafish WT cDNA of the *jmjd3*, *spi1*, *c/ebpα*, and *gcsfr* genes were amplified from reverse transcription products and cloned into the pCS2+ vector. Zebrafish morpholino oligonucleotides (MOs) were purchased from Gene Tools. The sequences of MOs used in this studies are following; *Jmjd3* translation-blocking MO: 5′-CCCATCTCGCTGTT ACTGTGTTTTC-3′; *Jmjd3* splice-blocking MO: 5′-ATATTTTGTGTGTGT GGACTGACCT-3′; *Spi1* MO: 5′-GATATACTGATACTCCATTGGTGGT-3′; control MO: 5′-CCTCTTACCTCAGTTACAATTTATA-3′. Capped mRNAs were transcribed with mMESSAGE mMACHINE Kit (Ambion). All MOs and mRNAs were injected into embryos at the one-cell stage.

2.3. Wish

Antisense RNA probes were transcribed using linearized constructs with T3 or T7 polymerase (Ambion) in the presence of digoxigenin (DIG, Roche)-labeled UTP using the DIG-RNA Labeling Kit (Roche). DIG-labeled antisense probes for these genes were synthesized with T3 or T7 polymerase (Ambion). WISH was performed as described previously, using 5-nitro-blue tetrazoliumchloride/bromo-4-chloro-3′-indolyl phosphate *p*-toluidine salt (NBT/BCIP, Vector Laboratories)as substrates [34]. Embryos were mounted in 4% methylcellulose and captured under a Nikon SMZ1500 microscope equipped with a Nikon DXM1200F digital camera and ACT-1 software.

2.4. Real-time qPCR

Total RNAs from embryos or cells were extracted using TRIzol reagent (Ambion). RNA was reverse transcribed using random hexamers and oligodT primers (Invitrogen). A 2XPCR Mix (Toyobo) was used for the real-time qPCR analysis, with an ABIPrism 7900HT Sequence Detector (Applied Biosystems). The relative expression values were normalized against the internal control gapdh. Primer sequences are available in Table S2.

2.5. Western blotting

Cells were homogenized in lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 10% glycerol, and 0.1% Triton X-100) containing protease inhibitor cocktail and phosphatase inhibitor (Roche Diagnostics). Proteins were separated on a 10% to 12% SDS–polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked and then incubated with the indicated antibodies followed by incubation with an appropriate horseradish peroxidase–conjugated secondary antibody (1:10,000). Band densities were quantified using the Image-Pro Plus software.

2.6. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed according to the standard cross-linking ChIP protocol (Abcam) with minor modifications. For ChIP using H3K27me3 antibody, 200 each wild-type embryos; control morphants; *jmjd3* morphants and HA-*jmjd3*-overexpression embryos at 22 hpf were used. For ChIP using H3K4me3 antibody, 200 each control morphants and *jmjd3* morphants at 22 hpf were used. For ChIP using HA antibody, 200 each wild-type embryos and HA-*jmjd3*-overexpression embryos at 22 hpf were used. Zebrafish embros were enzymatically dechorionated, and cells were harvested and crosslinked with 1% formaldehyde for 10 min at room temperature. After sonication, the soluble chromatins were incubated with 5 μg antibodies specific for H3K27me3 (ab85392 Abcam) or HA (ab9110, Abcam). Chromatin immunocomplexes were then precipitated with Protein A (Millipore, 16–661) or Protein G (Millipore, 16–662). The immunoprecipitated complex was washed, and DNA was extracted and purified by QIAquick PCR Purification Kit (Qiagen). ChIP DNA was analyzed by qPCR using specific primers against genomic regions of interest, and the data were normalized by input DNA. The results were derived from three independent experiments. The primer sequences are available in Table S2.

2.7. TUNEL assay and pH3 (phospho-histone 3) staining

TUNEL assays were performed using the In Situ Cell Death Detection Kit and TMR Red (Roche Diagnostics) according to the manufacturer's recommendations. PH3 labeling of fixed embryos was performed by an overnight incubation with a rabbit anti-phospho-histone H3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C, followed by an incubation with the Alexa Fluor 594 donkey anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA, USA). The embryos were mounted in 4% methylcellulose and images captured under a 270 Nikon SMZ1500 microscope equipped with a Nikon DXM1200F digital camera and ACT-1 software.