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Jmjd3 Activates *Mash1* Gene in RA-Induced Neuronal Differentiation of P19 Cells

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ABSTRACT

Covalent modifications of histone tails have fundamental roles in chromatin structure and function. Tri-methyl modification on lysine 27 of histone H3 (H3K27me3) usually correlates with gene repression that plays important roles in cell lineage commitment and development. *Mash1* is a basic helix-loop-helix regulatory protein that plays a critical role in neurogenesis, where it expresses as an early marker. In this study, we have shown a decreased H3K27me3 accompanying with an increased demethylase of H3K27me3 (Jmjd3) at the promoter of *Mash1* can elicit a dramatically efficient expression of *Mash1* in RA-treated P19 cells. Over-expression of Jmjd3 in P19 cells also significantly enhances the RA-induced expression and promoter activity of *Mash1*. By contrast, the mRNA expression and promoter activity of *Mash1* are significantly reduced, when Jmjd3 siRNA or dominant negative mutant of Jmjd3 is introduced into the P19 cells. Chromatin immunoprecipitation assays show that Jmjd3 is efficiently recruited to a proximal upstream region of *Mash1* promoter that is overlapped with the specific binding site of Hes1 in RA-induced cells. Moreover, the association between Jmjd3 and Hes1 is shown in a co-Immunoprecipitation assay. It is thus likely that Jmjd3 is recruited to the *Mash1* promoter via Hes1. Our results suggest that the demethylase activity of Jmjd3 and its mediator Hes1 for *Mash1* promoter binding are both required for Jmjd3 enhanced efficient expression of *Mash1* gene in the early stage of RA-induced neuronal differentiation of P19 cells. J. Cell. Biochem. 110: 1457–1463, 2010.

KEY WORDS: MASH1; JMJD3; NEURONAL DIFFERENTIATION; P19 CELLS

Neurogenesis is a multi-step process that is regulated by the activation and/or repression of the stage- or spatial-specific genes. In which, a family of conserved, basic helix-loop-helix (bHLH) transcription factors is critical in regulating the maintenance or differentiation of the neural stem cells [Kageyama et al., 2005]. Mash1 is one of the bHLH proteins that plays pivotal roles in the early development of neural and neuroendocrine progenitor cells in a number of tissues including the CNS, autonomic nervous system, adrenal medulla, thyroid, lung, and prostate among others [Akagi et al., 2004; Ball, 2004]. A recent study shows that Mash1 (also called Ascl1) plays a neuronal oriented role in converting fibroblast cells into induced neuronal cells [Vierbuchen et al., 2010].

Covalent modifications of the histones play critical roles in the higher order of chromatin structure and function in the eukaryotes [Martin and Zhang, 2005]. A striking feature of the core histones is that they can be covalently modified in a variety of ways as being acetylated, methylated, phosphorylated, or ubiquitylated [Kouzarides, 2007]. Among them, methylation of the lysine residues in the histones is specific for its dual functions as either an active mark at one site or a repressive one at the other. The trimethylation of the lysine residue at the 27th position from the N-terminus of histone H3 (H3K27me3) is associated with gene silencing in a number of developmental processes [Swigut and Wysocka, 2007]. Meanwhile, as an important member of polycomb repressive complex 2 in cell fate decision during development and differentiation [Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006], Ezh2 is pivotal in lineage commitment likely via its catalytic role on the methylation of H3K27 [Sparmann and van Lohuizen, 2006]. These and other reports showed that the methylation and demethylation of H3K27 played critical roles in the developmental process. However, despite that Jmjd3 and Utx have been identified as specific demethylases for H3K27me3 for years [Agger et al., 2007; De Santa et al., 2007; Jepsen et al., 2007; Lan et al., 2007; Lee et al., 2007], little is known on their functions in neuronal differentiation.

Here we first showed that while H3K27me3 expression decreased dramatically, histone demethylase Jmjd3 was induced in RA-treated

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P19 cells. Our data further revealed that Jmjd3 enhanced the expression of *Mash1* gene via its demethylase activity on H3K27me3 to release *Mash1* gene from silencing.

MATERIALS AND METHODS

CELL CULTURE AND RA TREATMENT

P19 cells were cultured in alpha minimum essential medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum. For neuronal differentiation, P19 cells were cultured in medium containing 0.5 M RA (all trans-retinoic acid, Sigma) for 4 days, and then the aggregates were plated as a monolayer and cultured for another 3 or 4 days in the absence of RA [Jones-Villeneuve et al., 1982].

CONSTRUCTS

Full length *Ezh2* was PCR amplified from P19 cells and cloned into pCDNA6-FLAG by *Bam*HI and *Xho*I. Primers used in PCR assays were as follows: forward primer, 5′-CGGGATCCGGCCAGACTGG-GAAGAAATC-3′; reverse primer, 5′-CCGCTCGAGAGCTAAGG-CAGCTGTTTCAG-3′. pCDNA3.1-Jmjd3-myc and pCDNA3.1-Jmjd3-mut-myc (H1390A, a demethylase-defective mutant) were from Chen Degui (Shanghai Institutes for Biological Sciences).

The shRNA construct against Jmjd3 gene was cloned into the pRS (Origene, Inc.) vector targeting the following sequences: shJmjd3, CAGTCTGATTACTGGAAGAATGCTGCCTT.

ANTIBODIES

Monoclonal antibody against Neuronal Class III β-tubulin (TuJ1) was from Covance, Inc. (MMS435P). Antibodies against GAPDH (MAB374) and Mash1 (AB15582) were from Chemicon; Ezh2 (07-689), trimethyl-Histone H3 (K27, 07-449) were from Upstate Biotech; antibody against Jmjd3 was from Abgent(AP1022a). Antibody against myc (sc-789) was from Santa Cruz. Anti-FLAG M2 affinity gel (F2426) was from Sigma.

QUANTITATIVE REAL-TIME RT-PCR ANALYSIS

Quantitative Real-time RT-PCR assays were carried out as previously described. The relative expression of *Mash1* was normalized against GAPDH, using the comparative CT method as the manufacturer's instructions (Rotor-Gene RG-3000A Real-time PCR System, Corbett Research, Australia). Primers used in PCR assays were as follows: *Mash1*, forward primer, 5′-TAACTCCCAAC-CACTAACAGGC-3′, reverse primer, 5′-TGAGGAAAGACATCAACC-CAGT- 3′; GAPDH, forward primer, 5′-GAAGGTGAAGGTCGGA-GTC-3′, reverse primer, 5′-GAAGATGGTGATGGGATTT-3′. Jmjd3, forward primer, 5′-TATTCCTGTTTACCGCTTCGTG-3′, reverse primer, 5′-TTGACGTTCTTCACCTCGTTCC-3′; Utx, forward primer, 5′-TATAGGTTTATTCAGCGACC-3′, reverse primer, 5′-GACATT-GCTTCAGCGTTC-3′.

Experiments were repeated at least three times with statistical analyses for each individual experimental set. All values in the experiments were expressed as mean ± SD.

IMMUNOBLOTTING

Whole cell extracts (WCE) were prepared as described [Wu et al., 2009]. Samples were separated in 8% SDS-polyacrylamide gels and analyzed by Western blotting.

CHROMATIN IMMUNOPRECIPITATION (ChIP)

ChIP assays were carried out with formaldehyde cross-linking as previously described [Li et al., 2007], and detected with PCR and gel electrophoresis. ChIPed DNA was subjected to PCR amplification to yield a positive fragment that was separated on 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Gel images were scanned with an AlphaImager 2000™. For quantitative assay, standard curve and ChIPed DNA samples were analyzed on a Rotor-Gene RG-3000A Real-time PCR System (Corbett Research) with PCR Master Mix for SYBR Green assays (TaKaRa, Biotech). Primer pairs used for amplification of *Mash1* gene were as follows:−1,580/−1,300, forward primer, 5′-GCTTAGGCTATG-CAATGAGA-3′, reverse primer, 5′-ACCCAGTCCAGCTTAGAAAA-3′; −690/−530, forward primer,5′-AAGGCTGCTCAGACAGGGTA-3′, reverse primer, 5′-TCCGGAGGATTCTTTGTGAC-3′; −265/−183, forward primer, 5′-TGTTTATTCAGCCGGGAGTC-3′, reverse primer, 5′-CTCCTTGCAAACTCTCCATTC-3′; −215/+16, forward primer, 5′-GCCCAGCAGTCTCTCACTTC-3′, reverse primer, 5′-ATTTGGTTG-AACCCCCTTTC-3′; +398/+499, forward primer, 5′-TGACAACTC-TGCCTCCTTCTG-3′, reverse primer, 5′-TGCGAAGCACGATCAAAG-3′. The cycle quantity required to reach a threshold in the linear range (Qt) was determined and compared with a standard curve for the primer set generated by five 10-fold dilutions of genomic DNA samples of known concentration.

LUCIFERASE REPORTER ASSAYS

The firefly luciferase reporter plasmid pGL3-Mash1-luc was constructed as follows: *Mash1* fragment of −1,580 to +491 bp relative to transcription start site, was amplified by PCR from the genome DNA of P19 cells and cloned into pGL3-Basic vector (Promega). Primers were as follows: forward, 5′-CCGCTCGA-GAGGCCAGCTTAGGCTATGCA-3′; reverse, 5′-CCCAAGCTTCTC-CACTCTCCATCTTGCCA-3′. P19 cells were transfected with VigoFect Reagent (Vigorous Biotech) according to the manufacturer's instructions. To normalize firefly luciferase activity of the reporter construct (pGL3-Mash1-luc), 1/20 (mol ratio) of internal control plasmid expressing Renilla luciferase (pRL-TK vector, Promega) was co-transfected into the cells. Six hours after the transfection, the medium was replaced with fresh α-MEM (RA−) or (RA+). Promoter activity was measured 48 h later. The activities of both luciferases were determined by means of a Dual-Luciferase Reporter System (Promega) according to the manufacturer's instructions. All of these relative luciferase activities, as measured in relative light units, were compared to a co-transfected internal control (pRL-TK). Assays were performed 3 times each in triplicate, and all results were shown as means ± SD.

STATISTICAL ANALYSIS

Statistical analysis was performed using two-tailed Student's *t*-test. All data were shown as mean with standard deviations (SD).

Probabilities of $P < 0.05$ were considered significant (*) and $P < 0.01$ as highly significant (**).

RESULTS

Mash1 ELEVATION IS REVERSELY CORRELATED WITH THE REDUCED RECRUITMENT OF H3K27ME3 ON *Mash1* PROMOTER IN RA-TREATED P19 CELLS

We have shown elsewhere that P19 cells can be induced by RA to aggregate on the 4th day of treatment, these cells were then cultured without RA and gradually turned into neuron-like phenotype in another 4 days [Wu et al., 2009]. Mash1 is a neuronal development marker in vivo. How it expresses and is it regulated in RA-induced neuronal differentiation of P19 cells remains unclear. Here, we showed that the mRNA and protein expression of Mash1 both increased dramatically after RA treatment (Fig. 1A,B), suggesting that *Mash1* gene is activated during RA-induced neuronal differentiation. We then applied ChIP assays to explore whether this efficiently induced expression is regulated by changes in the histone modification that elicit an open chromatin conformation at the *Mash1* gene. In which, five sets of primer pairs were used to amplify genome fragments either flanked or within the *Mash1* gene between −1,580 and +491 bp (Fig. 1C). ChIP data revealed that the recruiting of H3K27me3 decreased gradually and became more obvious after the 3rd day of RA treatment on the promoter of *Mash1* (Fig. 1D), which is reversely correlated with the hundreds increase of *Mash1* induction. Because H3K27me3 is a specific substrate of Jmjd3, the reduced H3K27me3 recruiting is likely via the activation of its specific demethylase Jmjd3 and is reversely correlated with the activation of *Mash1* under RA induction.

Jmjd3 IS ACTIVATED IN RA-INDUCED P19 CELLS

Because both Jmjd3 and Utx are H3K27 demethylases, we next examined their expression in RA-treated P19 cells by Real-time RT-PCR and Western blot, which showed a significant increase in Jmjd3 but not Utx expression (Fig. 2A,B). These results suggest that Jmjd3 dominates over Utx in depleting H3K27me3 from *Mash1* promoter and thereby enhances Mash1 expression after RA induction.

Jmjd3 IS REQUIRED FOR *Mash1* ACTIVATION IN RA-INDUCED P19 CELLS

To further examine the impact of Jmjd3 on the activation of *Mash1*, we examined the mRNA expression of *Mash1* with Real-time RT-PCR assays, and the reporter activity assays with firefly luciferase reporter driven by a fragment of *Mash1* designated as pGL3-Mash1-luc. With over-expression and knockdown of Jmjd3, we showed that the former significantly enhanced both the mRNA expression and promoter activity of *Mash1* under RA treatment (Fig. 3A,C). By contrast, the Jmjd3 siRNA significantly repressed the RA-induced mRNA and promoter activity of *Mash1* (Fig. 3B,E). As a control, the promoter of *Ngn1*, another important bHLH protein in neurogenesis process [Wu et al., 2009] showed no obvious change no matter Jmjd3 was over-expressed or knocked-down in the cells (Fig. 3D,F). These results suggest that Jmjd3 is pivotal in *Mash1* activation during RA induction.

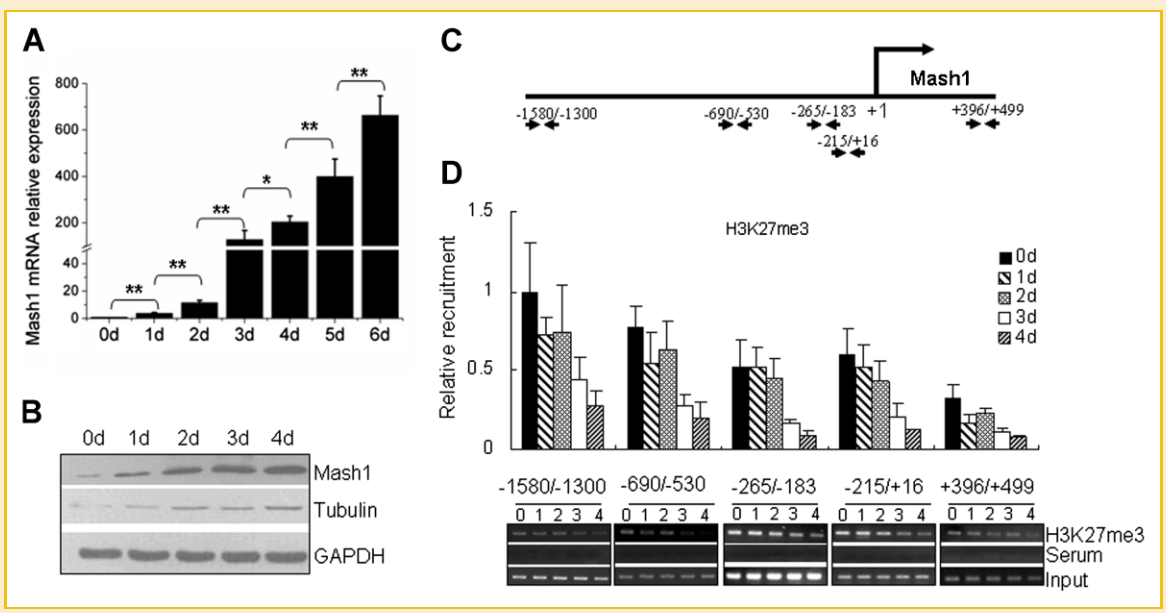

Fig. 1. Mash1 elevation is reversely correlated with the reduced recruitment of H3K27me3 on *Mash1* promoter in RA-treated P19 cells. A: Real-time RT-PCR analysis on *Mash1* mRNA expression in RA-treated P19 cells. Each bar represents a normalized mean value from at least three independent experiments at each time point of RA treatment and was shown as mean ± SD. *Significant, $P < 0.05$. **Highly significant, $P < 0.01$. B: Western blot analysis for the expression of Mash1 and β-Tubulin. Whole cell lysates were separated on a 10% SDS-PAGE and blotted with antibodies against Mash1, β-Tubulin and GAPDH respectively. C: Schematic diagram shows the positions of the five primer pairs (as arrows) on the promoter of *Mash1* used for PCR in ChIP assay. Digits represent the most upstream nucleotide of the 5′ primer and the down-most one of the 3′ primers relative to the transcription start site as +1. D: The recruitment of H3K27me3 on *Mash1* gene relative to the "Input" in RA-treated P19 cells by ChIP assays. Top panel: ChIP-qPCR assay. Bottom panel: ChIP-Gel electrophoresis. Input and pre-immune serum are detected as positive and negative control.

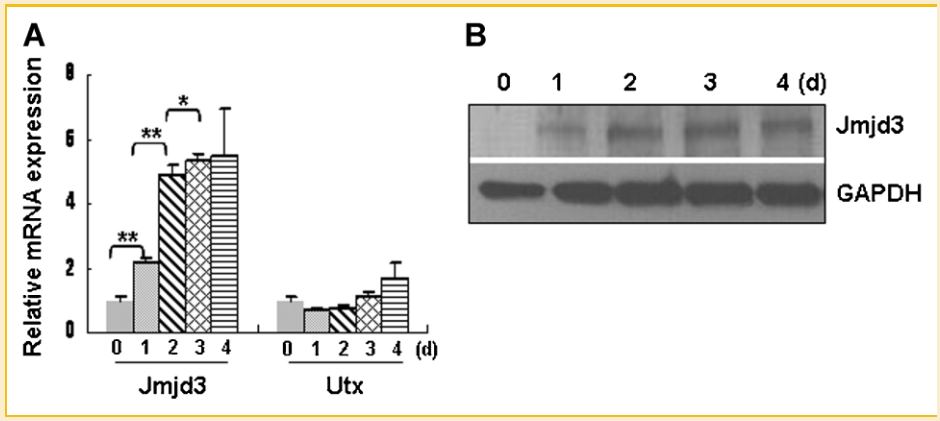

Fig. 2. Elevated expression of Jmjd3 in RA-treated P19 cells. A: Real-time RT-PCR analysis of Jmjd3 and Utx mRNA expression in RA-treated P19 cells. B: Western blot for Jmjd3 expression. GAPDH served as a loading control. Annotations are described as in Figure 1A,B respectively.

DEMETHYLASE ACTIVITY OF Jmjd3 IS PREREQUISITE IN RA INDUCED Mash1 EXPRESSION

To examine whether the demethylase activity of Jmjd3 or the Jmjd3 protein by itself played a critical role in Mash1 enhancement, we showed a threefold higher mRNA expression of *Mash1* in RA-treated cells transfected with wild-type Jmjd3 than that of the dominant negative mutant of Jmjd3 without enzyme activity (Fig. 4A). Meanwhile, the promoter activity of *Mash1* gene also

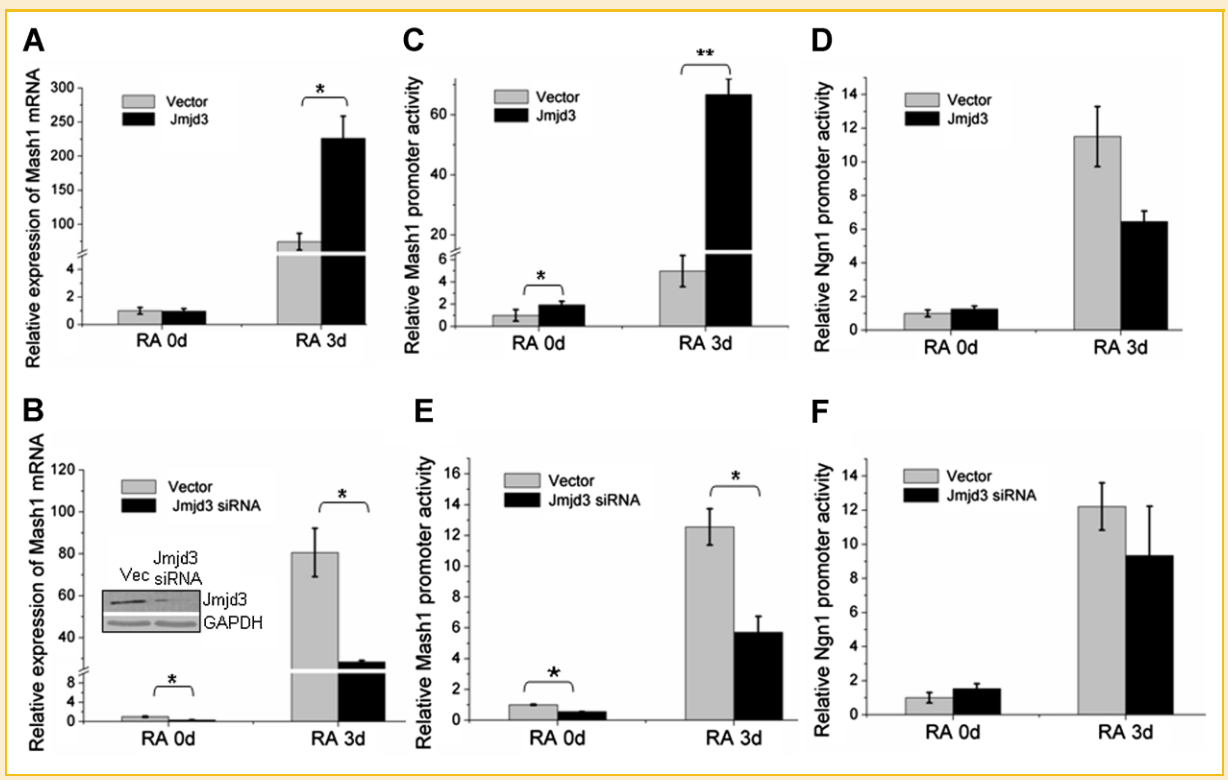

Fig. 3. Jmjd3 enhances the activation of *Mash1* gene. A: Effect of over-expressed Jmjd3 on mRNA level of *Mash1* in P19 cells by Real-time RT-PCR. Two aliquots of P19 cells were transfected respectively with Jmjd3 expression plasmid (Jmjd3, black) and empty vector as control (vector, gray). Twelve-hour later, each group of cells were treated with or without RA for 3 days and shown in the bottom as RA 3d and RA 0d respectively. B: Impact of Jmjd3 siRNA on the mRNA expression of *Mash1* in P19 cells. Transfection of Jmjd3 siRNA (black) or vector (gray) were the same as described in (A). Inner panel: The efficacy of Jmjd3 knockdown in P19 cells with Jmjd3 siRNA transfected. C,D: Impact of over-expression of Jmjd3 on the promoter activity of *Mash1* gene (C) or *Ngn1* gene (D) in P19 cells. pGL3-Mash1-luc reporter plasmid and pRL-TK were co-transfected with either expression vector of Jmjd3 or empty vector as control. The relative luciferase activities, as measured in relative light units, were compared to pRL-TK. E,F: Impact of Jmjd3 knockdown on the promoter activity of *Mash1* gene (E) or *Ngn1* gene (F) in P19 cells. pGL3-Mash1-luc reporter plasmid and pRL-TK were co-transfected with either Jmjd3-siRNA or vector as control. Reporter assay is performed as described in (C,D). All the bars in histograms of (A–E) are described as in Figure 1A.

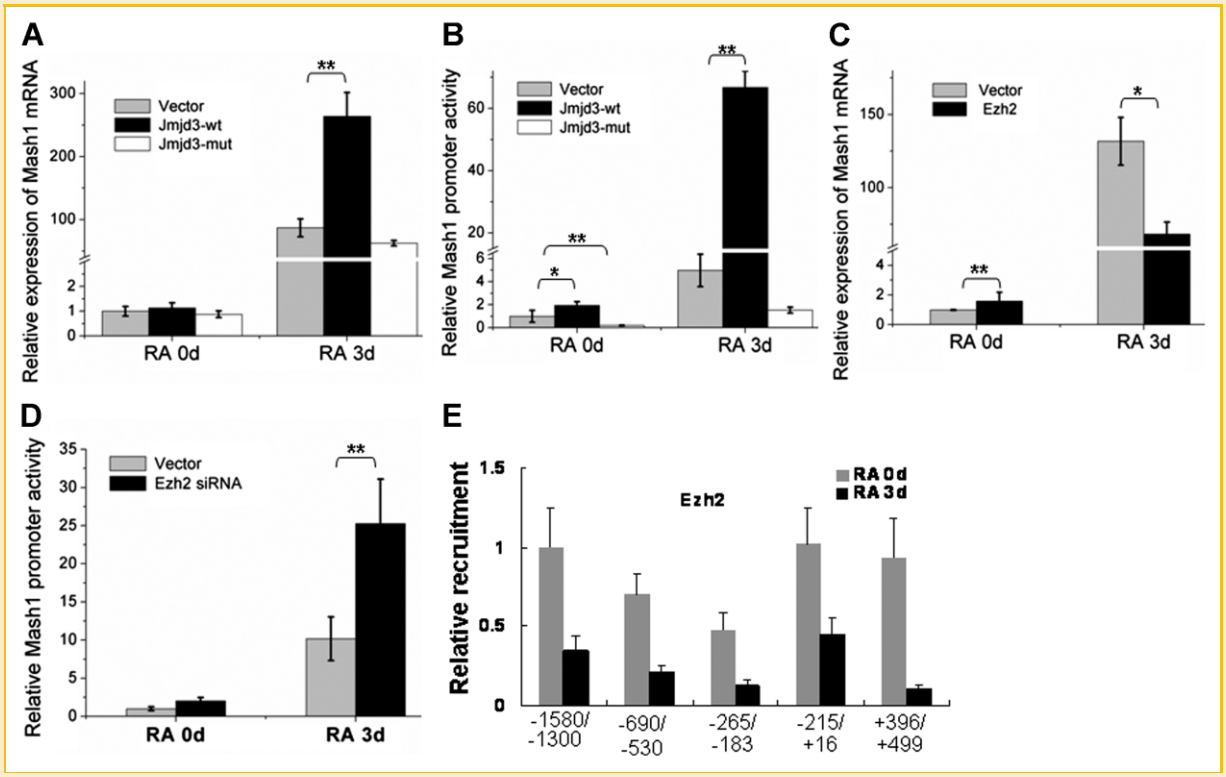

Fig. 4. The demethylase activity of Jmjd3 is required for Mash1 expression. A,B: Expression vectors of the wild-type (Jmjd3-wt) or dominant negative mutant Jmjd3 (Jmjd3-mut) were transfected to detect *Mash1* mRNA expression. Three aliquots of P19 cells were transfected respectively with Jmjd3-wt (black), Jmjd3-mut (open) and vector as control (gray) for mRNA expression of *Mash1* (A), and co-transfected with pGL3-Mash1-luc reporter plasmid and pRL-TK for promoter activity of *Mash1* (B) into P19 cells with (RA 3d) or without RA (RA 0d). Annotations are described as in Figures 3D and 1A. C: Impact of over-expressed Ezh2 on mRNA expression of *Mash1*. Ezh2 was transfected into P19 cells, other annotations are as described in Figure 3A. D: Impact of Ezh2-siRNA on *Mash1* promoter activity. Ezh2 siRNA was co-transfected with the pGL3-Mash1-luc reporter plasmid and pRL-TK for promoter activity of *Mash1* as described in (B). E: Recruiting of Ezh2 on *Mash1* promoter with RA treated (RA 3d) or without (RA 0d) in P19 cells by ChIP assays. Annotations are as described in Figure 1A.

responded similarly to the two distinct Jmjd3 constructs shown above (Fig. 4B). To confirm that the demethylation of H3K27me3 is an indispensable event ahead of the activation of *Mash1* in the RA-treated cells, Ezh2, a H3K27 methyltransferase is applied to counteract the functions of Jmjd3. We showed that while Ezh2 inhibited the mRNA expression of *Mash1* (Fig. 4C), its knockdown elevated the promoter activity of *Mash1* gene (Fig. 4D). ChIP assay accordingly showed that the recruiting of Ezh2 at *Mash1* promoter reduced remarkably on the 3rd day of RA treatment (Fig. 4E). These results suggest that the demethylase activity of Jmjd3 is the prerequisite for Mash1 expression in the early stage of RA-induced neuronal differentiation.

RECRUITING OF Jmjd3 AND HES1 AT *Mash1* PROMOTER AND THEIR INTERACTIONS

To detect if Jmjd3 is associated with the *Mash1* promoter in P19 cells, ChIP assay with Real-time PCR was carried out with antibody specific for Jmjd3. ChIP data revealed a remarkable elevation of Jmjd3 at the proximal promoter of *Mash1* on the third day of RA treatment (Fig. 5A). This event was comparable with the significantly increased promoter activity of *Mash1* under the same treatment (Fig. 1A). Because another bHLH protein Hes1 binds to *Mash1* promoter via a specific CACGCG site at −243/−225 [Meredith and Johnson, 2000], which is overlapped within the Jmjd3 fragment of −265/−183 on the *Mash1* as shown in Figure 5A,C. To examine the correlations between Jmjd3 and Hes1, we co-transfected HEK293T cells with the Jmjd3 tagged with Myc and the FLAG tagged Hes1, and showed in co-Immunoprecipitation assay that Hes1 did interact with Jmjd3 (Fig. 5B). ChIP assay showed a remarkably elevated recruiting of Hes1 on the proximal promoter of *Mash1*, in particular, at the −265/−183 site on the 3rd of RA treatment. These results suggest that Hes1 is responsible for recruiting Jmjd3 to the promoter of *Mash1* gene.

DISCUSSION

Mash1 is a bHLH transcription factor that is essential for the survival and differentiation of neuronal progenitor cells and is one of the earliest markers expressed in committed neural progenitor cells [Guillemot et al., 1993; Pattyn et al., 2004]. It has been reported recently that Mash1 (also called Ascl1) participates in the converting fibroblasts into induced neuronal cells [Vierbuchen et al., 2010]. Mash1 expression is tightly up-regulated by RA and neurotrophins whereas down-regulated by Notch signaling [Sriuranpong et al., 2002; Williams et al., 2006].

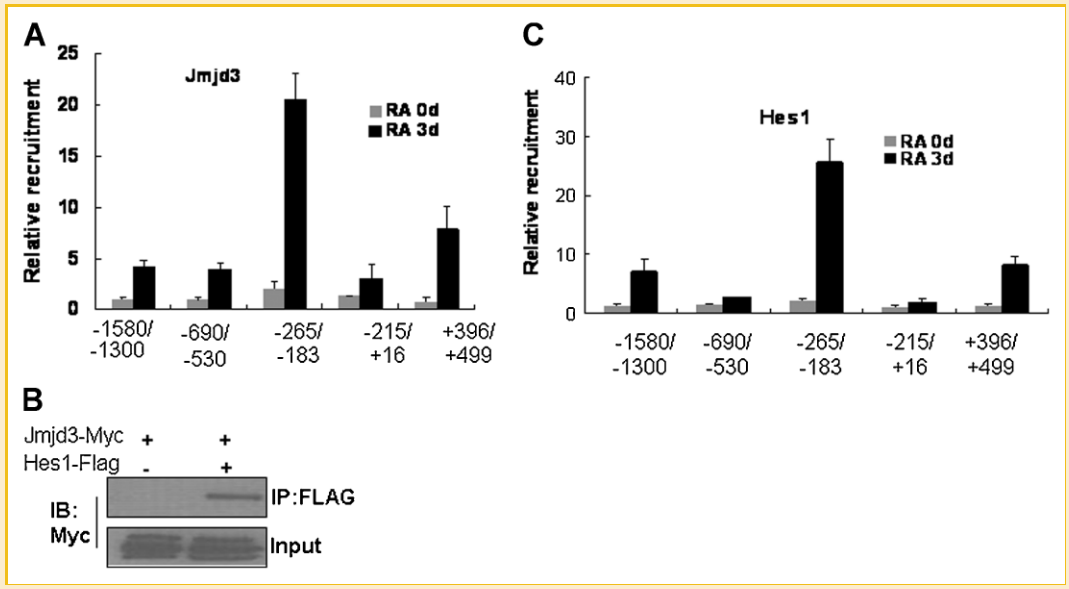

Fig. 5. Recruiting of Jmjd3 and Hes1 at *Mash1* promoter and their interaction. A: Recruiting of Jmjd3 at *Mash1* promoter with (RA 3d) or without (RA 0d) RA in P19 cells in ChIP assay. B: Association of Jmjd3 with Hes1. HEK293T cells were transfected "+" or not transfected "−" with the indicated plasmid on the left. Immunoprecipitated (IP) with anti-FLAG. Immunoblotted (IB) with anti-Myc. C: Recruiting of Hes1 at *Mash1* promoter in P19 cells by ChIP assays. All the bars in histograms in this figure are as described in Figure 1A.

Although H3K4me3 and H3K27me3 have opposing roles in gene regulation, they may also coexist in a number of genes that are silenced in stem cells but are poised for activation during development [Bernstein et al., 2006; Roh et al., 2006; Mikkelsen et al., 2007; Pan et al., 2007; Lim et al., 2009]. Here, *Mash1* is activated immediately to enhance early neuronal differentiation with the H3K27me3 decreased and the H3K4me3 increased (data not shown) on its promoter in RA-treated P19 cells. In this study, Jmjd3 expression increased and its association with the *Mash1* promoter enhanced, which ensure Jmjd3 could demethylate H3K27me3 at the *Mash1* promoter and play important roles in neuronal differentiation.

Several lines of evidence showed that Jmjd3 expressed at a significantly lower level in various types of tumor [Agger et al., 2009; Barradas et al., 2009], and that the inhibition of H3K27 methylation has a negative impact on tumor cells [Tan et al., 2007]. In context with our results, Jmjd3 can be a potential target in cancer gene therapy, in particular, for neuronal cancers.

However, how Jmjd3 is recruited to the promoter of *Mash1* remains unclear. We were inspired by an example of Sirt1 that functions in the inhibition of *Mash1* activation in response to oxidative stress [Prozorovski et al., 2008]. As this effect was mediated by the interaction of Sirt1 with another bHLH protein Hes1, and was thus recruited to the promoter of the *Mash1* gene to bind to a CACGCG consensus (C-site) at the −243/−225 in the upstream promoter of *Mash1* [Meredith and Johnson, 2000]. Similar to the case of Sirt1, in this study, an elevated co-recruitment of Jmjd3 and Hes1 at the −265/−183 region of *Mash1* promoter where the Hes1 binds (Fig. 5A,C), and that Jmjd3 can interact with Hes1 (Fig. 5B) becomes one of the most exciting findings in RA-treated P19 cells. It is thus suggested that Jmjd3 is recruited by Hes1 to *Mash1* promoter and functions as an H3K27me3 specific demethylase to activate the *Mash 1* gene.

Overall, our data show that the elevation of Jmjd3 expression and its demethylase activity are required to enhance the expression and promoter activity of *Mash1* in RA-induced neuronal differentiation of P19 cells.

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