Transcriptional repression of ANGPT1 by histone H3K9 demethylase KDM3B

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Here we report that the H3K9 demethylase KDM3B represses transcription of the angiogenesis regulatory gene, ANGPT1. Negative regulation of ANGPT1 by KDM3B is independent of its Jumonji (JmjC) domain-mediated H3K9 demethylase activity. We demonstrate that KDM3B downregulates ANGPT1 via interaction with SMRT, and suggest that the repressor complex is formed at the promoter area of ANGPT1. Using MTT and wound healing assays, depletion of KDM3B was found to increase cell proliferation and cell motility, indicating that KDM3B has a role in angiogenesis. [BMB Reports 2015; 48(7): 401-406]

INTRODUCTION

Chromatin is a highly common structure that contains DNA, histones, and other chromosomal proteins. The basic building block of chromatin is the nucleosome, which is made up of two copies of each histone: H2A, H2B, H3, and H4. The N-terminal tails of the histones are subject to posttranslational modifications (PTMs), which in turn affect numerous biological processes including transcription, replication, and chromosome maintenance. Methylation of the lysine residues within histones is regulated by methyltransferases (KMTs) and demethylases (KDMs) for the maintenance of cell fate and genomic stability. KDM3B, also known as JMID1B, is a histone H3K9-specific demethylase. Its gene is located in the 5q31 chromosomal region in humans, which has often been found to be deleted in breast cancer (1) and myeloid leukemia (2). KDM3B has been studied for regulation of the expression of the leukemic oncogene lmo2 (3).

Angiogenesis, the process by which blood capillaries grow from the pre-existing vascular tissue, is a key event in tumor growth and progression. Among the different growth factors which play roles in angiogenesis, VEGFA (vascular endothelial growth factor A) and its receptor are major mediators of tumor angiogenesis. Angiopoietin-1 (ANGPT1) is fundamental to physiological angiogenesis, including endothelial cell survival, vascular branching, and pericyte recruitment (4). ANGPT1 binds to and activates the TEK/TIE2 receptor by inducing its dimerization and tyrosine phosphorylation, playing an important role in the regulation of angiogenesis (5, 6). Overexpression of ANGPT1 has been proven to occur in malignant glioblastoma (7, 8), neuroblastoma (9), non-small cell lung cancer (10), and other tumors (4).

However, the role of ANGPT1 in tumor angiogenesis is less clear. Increasing amounts of experimental data have suggested that ANGPT1 contributes to the stabilization of newly organized blood vessels (11). In the present study, we report that KDM3B negatively regulates ANGPT1 transcription with its corepressor, SMRT, via recruitment to the ANGPT1 promoter. This activity is independent of its demethylase activity, suggesting that KDM3B has a mechanism by which to repress transcription of the target gene ANGPT1 to inhibit cellular proliferation and migration.

RESULTS

Negative regulation of ANGPT1 transcription by KDM3B

Previously, we conducted global gene expression profiling of human erythroleukemic K562 cells stably expressing KDM3B shRNA in the Tet-on inducible expression system (3). Among the differentially expressed genes, ANGPT1 was found to be up-regulated when KDM3B was knocked down in the K562 cell line (3). As a key regulator of angiogenesis, ANGPT1 binds to and activates the TEK/TIE2 receptor by inducing tyrosine phosphorylation. Since the transcriptional activation activity of KDM3B has been known to be conferred through H3K9 demethylation, we wanted to investigate the mechanism of the KDM3B-mediated negative regulation of ANGPT1. First, RT-PCR was performed on the K562 shKDM3B stable cell line to confirm the microarray data. As expected, the transcription of ANGPT1 was up-regulated by KDM3B knock-down (Fig. 1A). To further confirm the ANGPT1 up-regulation induced by the depletion of KDM3B, RT-PCR was also conducted on 293T cells. Again, ANGPT1 was increased in 293T cells when
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Fig. 1. KDM3B represses ANGPT1 expression. (A) K562 shKDM3B stable cells were analyzed by real time PCR to examine the mRNA expression level of ANGPT1. Results are shown as means ± SDs; n = 3. **P < 0.01. KDM3B knock-down was confirmed by western blot analysis. (B) shKDM3B transfected 293T cells were analyzed by real time PCR to examine the mRNA expression levels of ANGPT1. Results are shown as means ± SDs; n = 3. **P < 0.01. KDM3B knock-down was confirmed by western blot analysis. (C) GFP-KDM3B or GFP-KDM3B ΔC transfected 293T cells were analyzed by real time PCR to examine the mRNA expression levels of ANGPT1. Overexpression of KDM3B and KDM3B ΔC confirmed by western blot analysis. Results are shown as means ± SDs; n = 3. ***P < 0.001.

KDM3B was knocked down by transient transfection with KDM3B shRNA (Fig. 1B).

To investigate whether KDM3B regulates ANGPT1 through its demethylase activity, transfection was carried out with KDM3B or KDM3BΔC, in which demethylase activity of jmjC-domain was deleted. When KDM3B was overexpressed, significant decrease of ANGPT1 was observed. Notably, ANGPT1 was also decreased in cells overexpressing KDM3BΔC (Fig. 1C). These results indicated that the KDM3B-mediated down-regulation of ANGPT1 was independent of its demethylase activity.

Transcriptional regulation of ANGPT1 by KDM3B
In order to determine the mechanism of ANGPT1 down-regulation by KDM3B more precisely, a reporter assay was performed using the ANGPT1 promoter-driven luciferase (luc) reporter system. Consistent with the results of microarray analysis, the transcriptional activity of ANGPT1 was increased in a dose-dependent manner with the knockdown of KDM3B in 293T cells (Fig. 2A). In addition, ANGPT1 was decreased when KDM3B was overexpressed (Fig. 2B). As mentioned above, KDM3B activates gene expression through the demethylation of H3K9me1/2. However, the regulation of ANGPT1 indicated the opposite results. Thus, we hypothesized that other transcription factors were involved in the regulation of ANGPT1.

Having established that KDM3B negatively regulates the transcription of ANGPT1, we next searched for any transcription factor which is involved in the KDM3B-mediated transcriptional regulation. Transcription factors known for their roles in angiogenesis were screened, such as JunB, and GATA1. Each transcription factor was then tested in ANGPT1 transcriptional activity reporter analysis.

As shown in Fig. 2C, KDM3B knockdown increased ANGPT1 transcription, which was further increased by the addition of JunB (Fig. 2C). Similar results were obtained when another transcription factor, GATA1, was added (Fig. 2D). These results indicated that JunB and GATA1 are involved in the transcriptional activation of ANGPT1. However, further activation of the ANGPT1 promoter activity despite KDM3B-mediated transcriptional repression suggests that JunB and GATA1 regulate ANGPT1 via a different pathway from KDM3B.

KDM3B interacts with corepressor SMRT
Since our results indicated that KDM3B functions as a transcriptional repressor for ANGPT1, we decided to investigate whether KDM3B works in concert with corepressors such as HDAC, NcoR, or SMRT to repress ANGPT1. To accomplish...
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Fig. 2. KDM3B regulates \( \text{ANGPT1} \) transcription. (A and B) shKDM3B (A) or KDM3B (B) were co-transfected with the \( \text{ANGPT1} \) promoter-luc construct in 293T cell line. Cell extracts were then assayed for luciferase activity, which was normalized to \( \beta \)-galactosidase. Results are shown as means ± SDs; \( n = 3 \). ***\( \text{P} < 0.001; **\( \text{P} < 0.01; *\( \text{P} < 0.05. \)

(C and D) 293T cells were co-transfected with the indicated plasmids. Cell extracts were then assayed for luciferase activity, which was normalized to \( \beta \)-galactosidase. Results are shown as means ± SDs; \( n = 3 \). ***\( \text{P} < 0.001; *\( \text{P} < 0.05. \)

 transient transfection and Immunoprecipitations assays were conducted. These assays clearly showed that SMRT, but not HDAC or NcoR1 (data not shown), strongly interacted with KDM3B, suggesting that KDM3B and SMRT form a repressor complex during \( \text{ANGPT1} \) transcription (Fig. 3A). To further test the SMRT-mediated KDM3B activity, reporter assays were carried out to investigate whether KDM3B works together with SMRT. As expected, overexpression of both wild type KDM3B and that with the jmjC-domain deleted repressed \( \text{ANGPT1} \) transcription (Fig. 3B). When cells were cotransfected with KDM3B and SMRT, \( \text{ANGPT1} \) transcription was further downregulated (Fig. 3B). However, when cells were co-transfected with KDM3B and shSMRT, downregulation of \( \text{ANGPT1} \) was recovered (Fig. 3B). Next, we knocked down KDM3B with shRNA and \( \text{ANGPT1} \) transcription was upregulated (Fig. 3C). Furthermore, when cells were cotransfected with shKDM3B and shSMRT, \( \text{ANGPT1} \) transcription was further upregulated (Fig. 3C). These results suggest that KDM3B and SMRT form a corepressor complex and negatively regulate \( \text{ANGPT1} \) transcription.

Increase of cell motility by depletion of KDM3B

Previous studies reported induction of \( \text{ANGPT1} \) increase cell proliferation, wound healing, and invasion (6, 12-14). To further investigate the physiological effects of the KDM3B-mediated transcriptional regulation of \( \text{ANGPT1} \), we first conducted the cell proliferation assay (Fig. 4A). When KDM3B was overexpressed, a decrease in cell proliferation was observed. However, cellular proliferation was significantly increased when KDM3B was knocked down.

Previous studies suggested that \( \text{ANGPT1} \) contributes to angiogenesis by stabilizing blood vessel maturation (11). A scratch-motility assay was then performed using 293T cells transiently expressing KDM3B or shKDM3B with either control pCMV-3X-flag or shCTL. The closure of wounded areas was observed to be significantly accelerated in KDM3B knock-down
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Fig. 3. KDM3B interacts with corepressor SMRT. (A) 293T cells were co-transfected with Flag-KDM3B and pCMX-SMRT prior to immunoprecipitation with anti-Flag antibodies. Immunoprecipitated and associated proteins were eluted, resolved by SDS-PAGE, and immunoblotted with anti-Flag and anti-SMRT antibodies. (B and C) 293T cells were co-transfected with the indicated plasmids. Cell extracts were then assayed for luciferase activity, which was normalized to β-galactosidase. Results are shown as means ± SDs; n = 3. ***P < 0.001; *P < 0.05.

cells suggesting that KDM3B plays a role in the repression of cell motility, probably via regulation of ANGPT1 (Fig. 4B). As expected, the closure of wounds was decelerated when KDM3B was overexpressed. These results indicated that increase of ANGPT1 induced by knock-down of KDM3B may have regulated cell motility in our assay. Collectively, our findings indicate that angiogenesis-mediated wound healing is regulated through the involvement of a subset of KDM3B target genes exemplified by ANGPT1, and thereby affects cell proliferation and cell motility properties.

DISCUSSION

KDM3B is a H3K9me1/2 demethylase, and is highly expressed in hematopoietic cells (15). H3K9 methylation status is linked to heterochromatin formation and the silencing of target genes (16, 17). Herein, we attempted to elucidate the negative regulation of ANGPT1 transcription by KDM3B. Consistent with our previous microarray analysis, depletion of KDM3B increased the expression of ANGPT1. By reporter assay using the ANGPT1 promoter, it was found that ANGPT1 promoter activity was up-regulated when KDM3B was knocked down. The negative regulation of ANGPT1 by KDM3B was demonstrated to be independent of the H3K9 demethylase activity of KDM3B. There have been reports that G9a positively regulates the transcription mediated by various nuclear receptors through an HMTase-independent mechanism (18, 19). Previously, we reported that H3K9 HMTase G9a activates the transcription of p21 in p53-null H1299 cells independent of its HMTase activity (20). These reports, including the current study, indicate that there are different layers of regulatory networks in epigenetic modifiers, including HMTases and histone demethylases. It is also interesting that up-regulation of the ANGPT1 transcription level was independent of angiogenesis-related transcription factors such as JunB and GATA1, tested herein. Further studies will be needed to elucidate the fine mechanism of transcriptional regulation by KDM3B, including identification of the responsible transcription factors involved.

Furthermore, we found that wound healing and invasion were increased by the knock-down of KDM3B. These results
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Fig. 4. Depletion of KDM3B induces cell proliferation and motility. (A) Proliferation was monitored over 72 h in 293T cells expressing Flag-KDM3B or shKDM3B. (B) Photomicrographs from a scratch-motility assay of MCF7 expressing Flag-KDM3B or shKDM3B for 24 h (Left panel). Percent of the gap distance closed between the two migrating form fronts was measured and shown as a graph (Right panel). Results are shown as means ± SDs; n = 3. *P < 0.05; **P < 0.01.

indirectly suggest that KDM3B plays a role in cell migration and proliferation. In conclusion, depletion of KDM3B increased the transcription of ANGPT1. Down-regulation of ANGPT1 was independent of the demethylase activity of KDM3B, and the SMRT corepressor plays role in the transcriptional repression of ANGPT1 by KDM3B.

MATERIALS AND METHODS

Cell culture and transient transfection, RNA isolation and real-time PCR, Western blot analysis, Immunoprecipitation, and Statistical analysis are described in the online data supplement, available at http://www.bmbreports.org/.
Luciferase assay
293T and HCT116 cells were seeded at the density of $6 \times 10^4$ cells in 48-well plates and transfected with pGL3-ANGPT1 promoter (100 ng), shKDM3B (200 ng or 400 ng), shSMRT (300 ng), GFP-KDM3B (100 ng or 200 ng), pCMV10-Flag-Imo2 (100 ng), and pCMX-PL1-JunB (50 ng) using PEI. After 48 h of transfection, the cells were collected and lysed in cell culture lysis reagent. Luciferase activities were then determined by adding 10 µl of luciferase assay substrate into 70 µl of cell lysates, then measuring using a Glomax luminometer (Promega). The results were confirmed by performing each experiment at least in triplicate.

MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay
293T cells were seeded in 48-well plates (8 x 10<sup>4</sup> cells/well) and transiently transfected with pCMV10-Flag-KDM3B and shKDM3B. After 0-4 days of incubation at 37°C, 20 µl of MTT (1 mg/ml) was added to the each well and incubated for an additional 4 h at 37°C, followed by aspiration of the medium and addition of 200 µl of dimethyl sulfoxide (DMSO). OD values were determined with an ELISA reader (Biochrom) at the wavelength of 570 nm. DMSO alone was measured as the blank and subtracted from all values to correct for background in the measurements.

Wound healing assay
Cells were seeded in 24-well plates and transfected with pLKO.1 and shKDM3B. Twenty four hours after transfection, the cells were located in monolayers, and were scratched once with a pipette tip to obtain a "scratch" in each well. Cells were then washed once with medium. Migration was monitored every 12 h, and photomicrographs were taken at each time point.

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