Tumor and Stem Cell Biology

Cancer Research

SMYD3 Promotes Cancer Invasion by Epigenetic Upregulation of the Metalloproteinase *MMP-9*

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Abstract

Upregulation of the matrix metalloproteinase (MMP)–9 plays a central role in tumor progression and metastasis by stimulating cell migration, tumor invasion, and angiogenesis. To gain insights into MMP-9 expression, we investigated its epigenetic control in a reversible model of cancer that is initiated by infection with intracellular *Theileria* parasites. Gene induction by parasite infection was associated with trimethylation of histone H3K4 (H3K4me3) at the *MMP-9* promoter. Notably, we found that the H3K4 methyltransferase SMYD3 was the only histone methyltransferase upregulated upon infection. SMYD3 is overexpressed in many types of cancer cells, but its contributions to malignant pathophysiology are unclear. We found that overexpression of *SMYD3* was sufficient to induce *MMP-9* expression in transformed leukocytes and fibrosarcoma cells and that proinflammatory phorbol esters further enhanced this effect. Furthermore, SMYD3 was sufficient to increase cell migration associated with *MMP-9* expression. In contrast, RNA interference–mediated knockdown of *SMYD3* decreased H3K4me3 modification of the *MMP-9* promoter, reduced MMP-9 expression, and reduced tumor cell proliferation. Furthermore, *SMYD3* knockdown also reduced cellular invasion in a zebrafish xenograft model of cancer. Together, our results define SMYD3 as an important new regulator of *MMP-9* transcription, and they provide a molecular link between SMYD3 overexpression and metastatic cancer progression. *Cancer Res; 72(3); 810–20. ©2011 AACR.*

Introduction

Cancer progression is a multistep process resulting from altered gene expression through genetic and epigenetic mechanisms (1, 2). As epigenetic events might be reversed using targeted therapies, there is intensive effort to understand the interplay between these mechanisms in the transcriptional control of key regulatory molecules and the altered epigenetic landscape of cancer cells (2). To investigate epigenetic events in cancer progression, we studied a transformation model offering several unique experimental features. Infection by intracellular *Theileria* parasites causes a lymphoproliferative disease in cows with clinical features similar to some human leukemia. *T. annulata* infects mainly B cells and macrophages, whereas *T. parva* infects B and T lymphocytes (3, 4). *Theileria*-infected cells are transformed and immortalized; displaying uncontrolled proliferation independent of exogenous growth factors *in vitro* and increased ability to migrate and form metastases in immunodeficient mice (5, 6, 7). Interestingly, *Theileria*-dependent transformation is reversible; animals treated with the theilericidal drug buparvaquone are cured in most cases (3, 8, 9). When *Theileria*-infected cells are treated *in vitro* with buparvaquone, the intracellular parasite disappears from the host leukocyte, which either enters apoptosis or stops proliferating, regaining growth factor dependence (8). Studies showed that *Theileria* alters host signal transduction pathways, but there is no evidence of permanent host genome changes (4). *Theileria* infection activates the c-Jun-NH_2-kinase (JNK) pathway and the activator protein (AP-1) transcription factor (10, 11), which are critical for both the survival and invasiveness of transformed B lymphocytes (5). *Theileria* also induces the IkappaB kinase (IKK) pathway leading to NF-κB activation (12) and the TGF-β pathway implicated in virulence (6). This system offers a unique model to address reversible, epigenetic changes involved in tumorigenesis.

Matrix metalloproteinase (MMP) proteins may function as mediators of metastasis of *Theileria*-infected cells (13–15). The large family of MMP proteases play critical functions in development and cancer progression (16). In particular, MMP-9 plays an important role in tumorigenesis and metastasis formation by regulating tumor growth, angiogenesis, cell migration, and invasion (16). MMP-9 is a collagenase that

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degrades extracellular matrix (ECM) components including collagens and laminin. Its role in cancer progression is related to ECM degradation and growth factor release (17). The *MMP-9* gene is mainly regulated at the transcriptional level by transactivators binding to the promoter, including AP-1 and NF-κB (18, 19). Recent studies provided insights into how *MMP-9* expression is regulated in different cancer types and the role of chromatin modifications (20). *MMP-9* transcription involves a stepwise, coordinated recruitment of activators, chromatin modifiers, coactivators, and general transcription factors to its promoter (21, 22).

Although *MMP-9* was linked to the invasive phenotype of *Theileria*-transformed cells (13–15), the involvement of epigenetic regulatory mechanisms (and their reversibility) has never been explored in *Theileria*-infected leukocytes. We studied *MMP-9* expression in *Theileria*-infected cells as a model for epigenetic regulation in leukocyte transformation. The invasive B-cell lymphosarcoma cell line TBL3 was generated by *in vitro* infection, with *T. annulata*, of immortalized BL3, a bovine B lymphosarcoma cell line (23). TBL3 cells have a higher invasive capacity than BL3 cells and elevated *MMP-9* expression (15). Here, we show that *Theileria* infection induces the reversible expression of the histone methyltransferase SMYD3 (SET and MYND domain containing 3). *SMYD3* encodes a histone H3K4 di- and trimethyltransferase involved in tumor proliferation in colorectal, hepatocellular, and breast carcinomas (24, 25). SMYD3 binds specific DNA sequences, 5′-CCCTCC-3′ or 5′-GGAGGG-3′, in the promoter region of target genes, leading to transcriptional activation (24). However, the role of SMYD3 in cancer progression is unclear and few direct target genes are known. We show that SMYD3 protein binds to the *MMP-9* promoter and regulates expression in *Theileria*-infected cells and human tumor models. Moreover, *SMYD3* knockdown led to decreased H3K4me3 marks on the *MMP-9* promoter and reduced *MMP-9*. Interestingly, SMYD3 levels impact the transformed phenotypes and tumor invasiveness. Thus, our work identifies SMYD3 as a novel regulator of the *MMP-9* metastatic gene affecting tumor migration and invasion *in vitro* and *in vivo*, thereby highlighting its potential as a therapeutic target to treat metastatic disease.

Materials and Methods

Cell culture and reagents

Parental BL3 and infected TBL3 cells, previously described (23), were cultured at 37°C in RPMI-1640 (Gibco) supplemented with 2 mmol/L L-glutamine, 10% fetal calf serum (FCS), 100 units/mL penicillin/streptomycin, 10 mmol/L HEPES, and 50 mmol/L 2-mercaptoethanol. HT1080-2.2 cells (with an integrated 2.2 Kb *MMP9* promoter-luciferase) obtained from Dr. C. Yan (Albany Medical College, Albany, NY; ref. 26) were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) with glutamax and high glucose, supplemented with antibiotics and 10% FCS. HT1080 shRNA cell lines were generated by transfecting the pSuper vector (Ctrl-sh) or the *SMYD3*-sh pSuper vector, and selecting with neomycin (300 μg/mL). Appropriate HT1080 clones were selected for further use following SMYD3-immunoblotting analysis. Cells were treated with buparvaquone (BW720c; Calbiochem) at 50 ng/mL for 64 hours or with phorbol 12-myristate 13-acetate 9 (PMA; Sigma p1585) 50 ng/mL for 8 hours. Compositions of all buffers are detailed in Supplementary Material.

Viability assays

A total of 1×10^4 cells were plated in 96-well plates in triplicate, and buparvaquone was added at different concentrations. After 48 hours, cell viability was measured by the Cell proliferation Kit II–XTT (Roche) and the GloMax-Multi Detection System (Promega).

Invasion assays

Bovine TBL3 and BL3 cells (5×10^4) were incubated with/without buparvaquone, resuspended in 1% FCS and seeded onto Matrigel (BD Biosciences; 70 μL) in Boyden chamber, BD Falcon Cell Culture Inserts (8-μm pores; BD Biosciences). Lower chambers contained 20% FCS medium as a chemoattractant. After 24 hours at 37°C, migrated cells were stained with crystal violet and counted under the microscope (40× objective, at least 10 fields per filter). To test migration of human HEK293 or HT1080 cells, cells were trypsinized and placed in the upper chamber (1×10^4 cells) coated with 10 μg/mL collagen I in media containing 0.3% and 20% FCS in the lower chamber. After 16 hours, cells were fixed with 4% formaldhehyde and migrated cells were stained and counted. All experiments were carried out in triplicate. Migration index is the number of migrated cells per filter (control set to 1).

Soft agar assay

Cells were suspended in 0.35% agarose containing 10% FCS and plated above 0.5% agarose at 1×10^4 cells per 6-well plate. After 10 to 14 days, colonies were stained with crystal violet and scored. Experiments were carried out in triplicate.

RNA extraction, reverse transcriptase PCR, and quantitative PCR

Total RNA was extracted by an RNeasy Plus Mini Kit (Qiagen). cDNA synthesis was carried out with Reverse Transcriptase (RT) Superscript III (Invitrogen) and semiquantitative PCR analysis with GoTaq polymerase (Promega). Quantitative PCR (qPCR) amplification was carried out in the ABI7500 machine (Applied Biosystems) using Sybr Green (Applied Biosystems). See Supplementary Table S1 for primer sequences. Relative quantities of mRNA were calculated with the ΔC_t method.

Western blotting

Proteins were extracted with Laemmli lysis buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membranes, before incubation overnight at 4°C with antibodies against MMP-9, SMYD3, or β-actin (see Supplementary data), followed by secondary antibodies. Membranes were developed by SuperSignal (Thermo Scientific).

Zymography

Supernatants from cells grown in serum-free media were mixed with lysis buffer and loaded into SDS-PAGE gels, copolymerized with 0.1% gelatin. Gels were washed twice

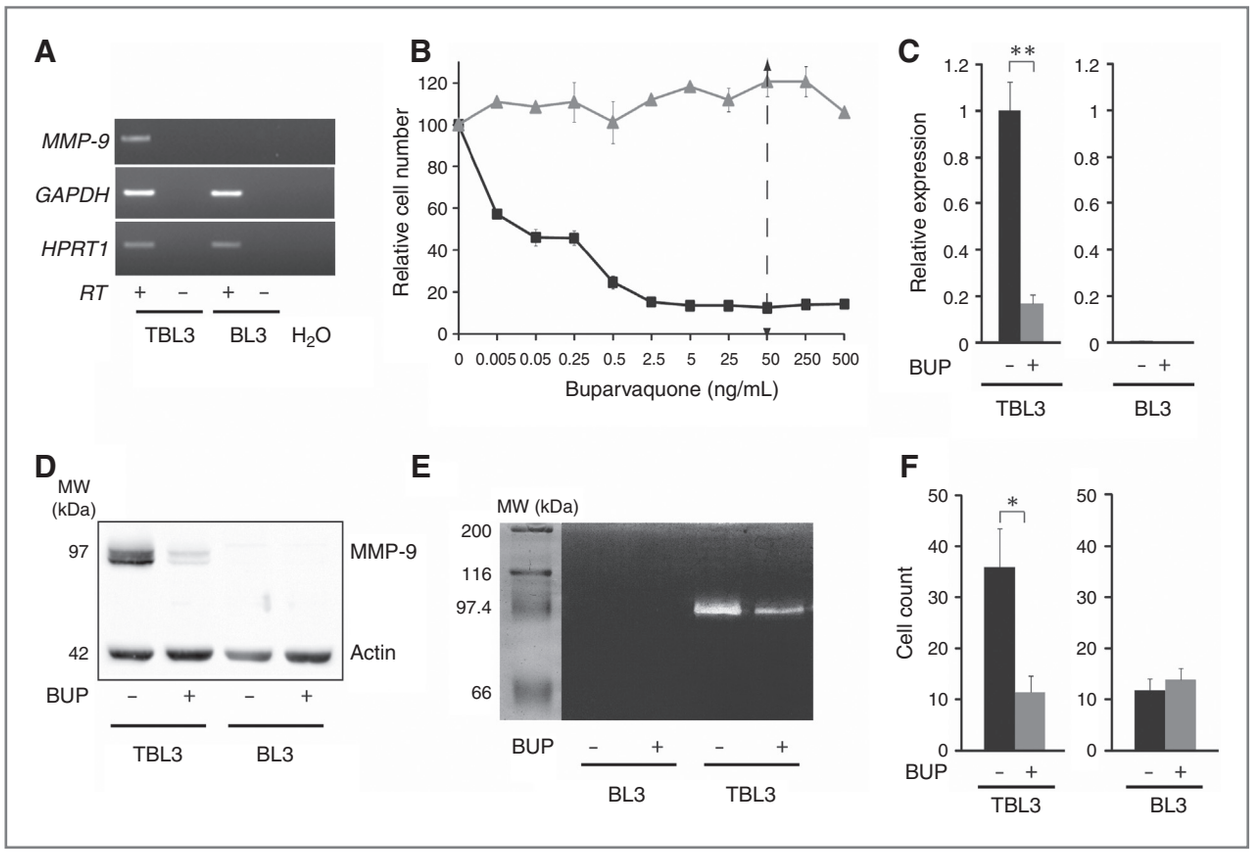

Figure 1. Inducible and reversible *MMP-9* expression upon *Theileria* transformation. A, *MMP-9* expression in infected (TBL3) and noninfected (BL3) cells by semiquantitative PCR analysis. mRNAs were incubated with or without reverse transcriptase (RT). Two housekeeping genes, *GAPDH* and hypoxanthine phosphoribosyltransferase (*HPRT1*), were used as controls. B, the relative number of TBL3 (squares) and BL3 (triangles) cells incubated with increasing concentrations of buparvaquone assessed by the 2,3-bis[2-methoxy-4-nitro-*S*-sulfophenynl]*H*-tetrazolium-5-carboxanilide inner salt (XTT) assay. The arrow represents the concentration used for all subsequent experiments. C, the reversibility of *MMP-9* expression was assessed by qPCR analysis. Cells were treated with the drug buparvaquone (BUP) for 64 hours. *β-Actin* mRNA was used for normalization. D, Western blot analysis of MMP-9 protein expression in TBL3/BL3 cells with/without buparvaquone treatment for 64 hours. Bovine β-actin was used as a control. MW is the molecular weight marker (kDa). E, gelatin zymography to analyze MMP-9 protein activity in supernatants from BL3 and infected TBL3 cells with/without buparvaquone treatment. F, invasion capacity tested by a modified Boyden chamber assay with/without buparvaquone (BUP). Results show the number of invasive cells counted under the microscope. All results represent the average of 3 independent experiments (mean ± SD) *, $P < 0.05$; **, $P < 0.01$.

(30 minutes) in renaturing buffer (2.5% Triton X-100) and incubated at 37°C, 18 hours in a gelatinase solution. Gels were stained with 0.5% Coomassie Blue and destained with methanol:acetic acid (50:10).

Transfection

HT1080 and HEK293T (human embryonic kidney transformed with the AgT) cells were transfected with Lipofectamine 2000 (Invitrogen). The pGL3-Nkx2.8–WT-SBE and pcDNA-SMYD3 vectors were obtained from Dr. Y. Furukawa (Tokyo University, Tokyo, Japan; ref. 24). The *MMP-9* promoter-luciferase construct was obtained from Dr. D. Boyd (MD Anderson Cancer Center, Houston, TX).

Luciferase assays

Dual luciferase assays (Promega) were conducted by the GloMax-Multi Detection System (Promega) to measure firefly and *Renilla* luciferase activity. The pGL4 *hRluc*/TK vector was used for luciferase assay normalization.

Gene silencing

For short-term depletion experiments, cells were transfected with siRNA oligonucleotides (60 nmol/L) Supplementary Table S1. The short hairpin RNA (shRNA) vector targeting human/bovine *SMYD3* was constructed by cloning double-stranded oligonucleotides into the pSuper-vector (Oligoengine), generating siRNA directed against the sequence AGCCTGATTGAAGATTTGA. *shCtrl* plasmid is an empty pSuper vector.

Chromatin immunoprecipitation

Chromatin was extracted from 5×10^6 to 20×10^6 cells and crosslinked with 1% formaldehyde (10 minutes, 37°C). After incubation in cell lysis buffer 30 minutes at 4°C, nuclei were resuspended in nuclei lysis buffer containing glass beads (400 μL per mL; Sigma) before sonication (4–5 cycles of 4 minutes) using the Bioruptor (Diagenode). The INPUT sample (5%) was removed before the immunoprecipitation. Chromatin aliquots (20 or 50 μg for transcription factors) were immunoprecipitated with specific antibodies overnight at 4°C and recovered with

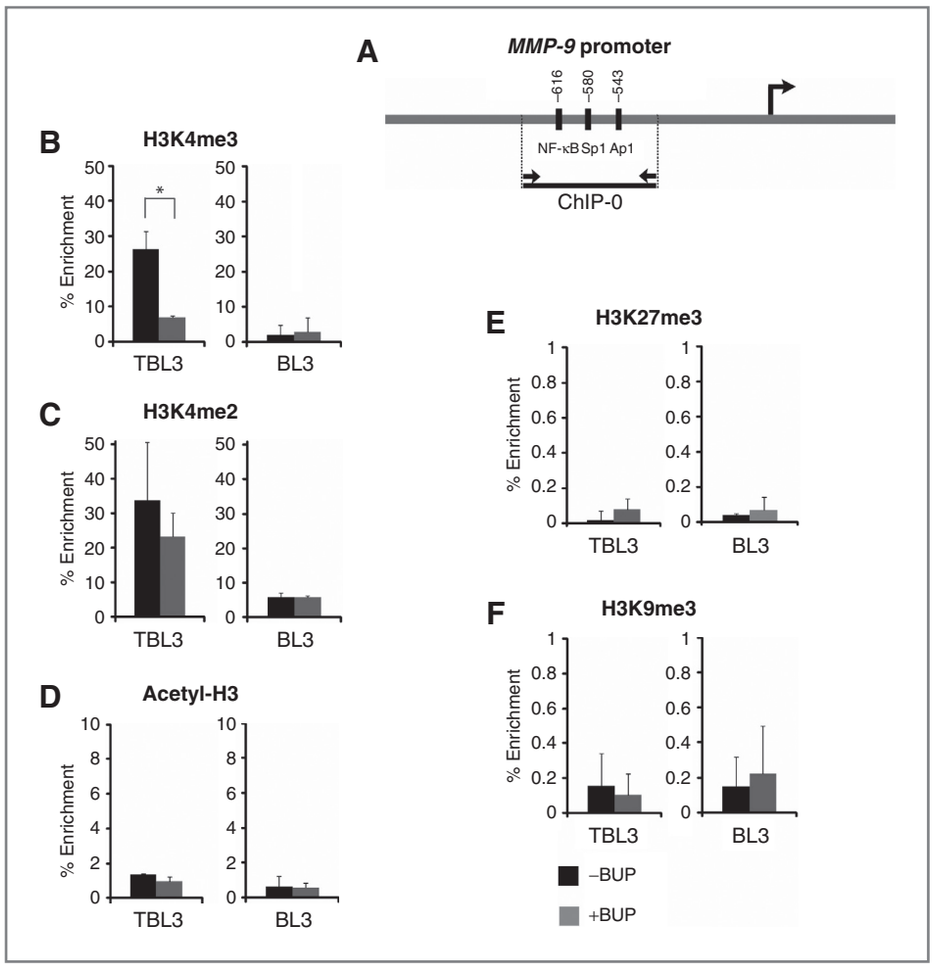

Figure 2. Chromatin analysis of bovine *MMP-9* promoter upon *Theileria* infection. A, primers (ChIP-0) were designed to amplify by qPCR a sequence surrounding the NF-κB and AP-1–binding sites in the distal bovine *MMP-9* promoter. The right arrow marks the TSS. B–F, BL3 and *Theileria*-infected TBL3 cells were incubated with/without buparvaquone (BUP) for 64 hours. ChIP analysis with antibodies recognizing (B) H3K4me3, (C) H3K4me2, (D) Acetyl-H3, (E) H3K27me3, and (F) H3K9me3 marks. Error bars are representative of 2 independent experiments.
*, $P < 0.05$.

preblocked Protein G/A sepharose beads (Sigma). Washes were as following: twice Tris/Sucrose/EDTA (TSE)-150 mmol/L NaCl buffer, once TSE-500 mmol/L NaCl buffer, once washing buffer, and twice Tris/EDTA (TE) buffer. Beads were diluted in 100 μL of dilution buffer plus 150 μL of TE/1% SDS. After decrosslinking, DNA was analyzed by qPCR. The immunoprecipitation enrichment was calculated with the ΔC_t method. Results were presented as "percentage of enrichment" of INPUT sample [100× (Output/Input)] or as "fold-enrichment" [100× (Output/Input) normalized to binding on *MMP-9* promoter in control cells].

Cell-cycle analysis

Cells were fixed in 70% ethanol and washed twice in PBS, incubated at room temperature for 20 minutes in propidium iodide buffer (50 μg/mL PI; Sigma) containing 100 μg/mL RNaseA. Cells were analyzed using the FACSCalibur flow cytometer (BD Biosciences), with data acquisition using CellQuest software.

Animal care and xenografts

Zebrafish (*Danio rerio*; wild-type AB strain, and Casper, generously given by GIS AMAGEN, Gif sur Yvette) were handled according to the European Union guidelines. Fish were kept at 28°C in aquaria with day/night light cycles. HT1080 cells were transfected with siRNA oligonucleotides. After 24 hours, cells were incubated with Dulbecco's PBS (DPBS) containing 10 μmol/L fluorescent CellTracker CM-Dil green (Invitrogen). A total of 20 to 30 cells were injected into the yolk of dechorionated and anesthetized (Tricaine methanesulfonate) 48 hours postfertilization embryos using a FemtoJet microinjector (Eppendorf). Embryos were incubated at 34°C for 3 days and analyzed by a microscope Station cell observer ZEISS and an Axio Vision software.

Results

Theileria infection induces MMP-9 expression and increased invasiveness

As *MMP-9* expression has been linked to *Theileria* transformation (7, 15, 27), we analyzed mRNA and protein in *Theileria*-infected TBL3 cells and the noninfected BL3 parental cell line. We observed high *MMP-9* mRNA levels in TBL3 cells but not in BL3 cells (Fig. 1A). To verify reversal by the theilericidal drug buparvaquone, we tested different concentrations of buparvaquone treatment on cell proliferation. The drug caused dramatic growth arrest in TBL3 cells, whereas BL3 growth was

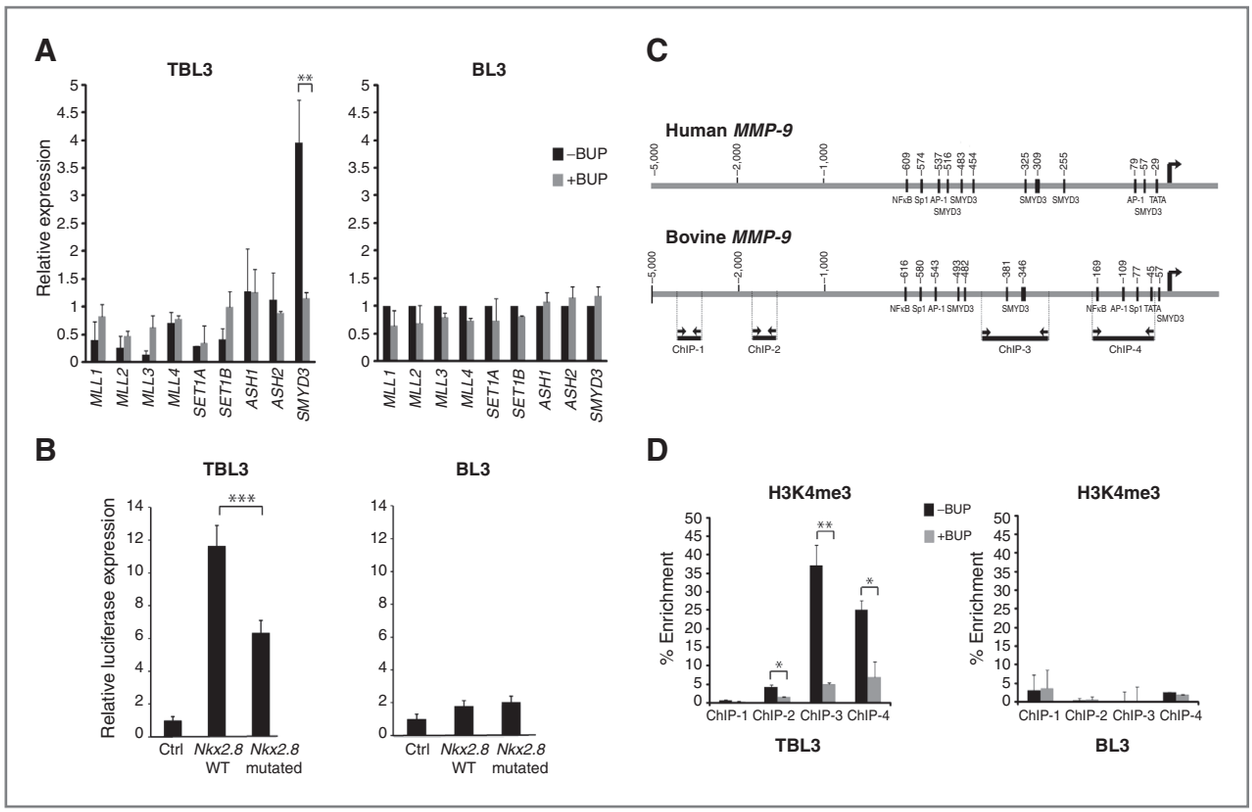

Figure 3. *SMYD3* methyltransferase is upregulated in *Theileria*-infected cells. A, qPCR analysis of the expression levels of H3K4 methyltransferases using RNA from *Theileria*-infected TBL3 or BL3 cells, with/without buparvaquone treatment (BUP) for 64 hours. *β-Actin* mRNA was used for normalization. B, SMYD3 activity measured by a dual-luciferase assay in TBL3/BL3 cells transfected with a SMYD3 reporter (wild-type *Nkx2.8* WT promoter-luciferase vector), compared with a control vector (Ctrl). Promoter activity was reduced when the SMYD3-binding site was mutated (*Nkx2.8* mutated). Transfection efficiency was normalized with cotransfection of a *Renilla*-encoding plasmid. C, schematic of the human and bovine *MMP-9* promoters highlighting potential SMYD3-binding sites. Oligonucleotides to amplify fragments of the bovine *MMP-9* promoter: ChIP-1 (−4.7 kb upstream), ChIP-2 (−1.7 Kb), ChIP-3 (3 putative SMYD3-binding sites), and ChIP-4 (proximal AP-1 site). The right arrows mark the TSS. D, TBL3/BL3 cells were treated with/without buparvaquone for 64 hours and ChIP was carried out with an antibody recognizing H3K4me3. qPCR analysis was conducted with the primer sets mentioned earlier. Error bars represent 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

unaffected (Fig. 1B). Using the standard dose concentration of 50 ng/mL for 64 hours (5), we observed a 5-fold reduction in *MMP-9* transcript levels in treated TBL3 cells but no effect in BL3 cells (Fig. 1C). Furthermore, we observed a similar *Theileria* induction and buparvaquone reversibility of MMP-9 protein and protease activity by gelatin zymography (Fig. 1D and E). Finally, we studied their invasive capacity by *in vitro* Matrigel invasion assays. TBL3 cells were more invasive than BL3 cells, and buparvaquone treatment reversed the phenotype (Fig. 1F). Thus, the *Theileria*-inducible and buparvaquone-reversible regulation of *MMP-9* expression offers a model system to explore epigenetic control of this metastasis gene.

Theileria induces chromatin marks on the bovine *MMP-9* promoter

To explore epigenetic changes linked to *MMP-9* regulation, we studied histone marks on the *MMP-9* promoter focusing on a regulatory region (0.5 kb upstream from the transcription start site (TSS) containing transcription factor binding sites AP-1, NF-κB, and Sp1) conserved between the bovine and human promoters (Fig. 2A and Supplementary Fig. S2). We conducted chromatin immunoprecipitation (ChIP) studies in TBL3 and BL3 cells, with and without buparvaquone treatment, using antibodies specific for activating histone marks (i.e., Acetyl-H3, H3K4me2, and H3K4me3) and repressive marks (i.e., H3K9me3 and H3K27me3; Fig. 2B–F). We observed enrichment of H3K4me2 and H3K4me3 marks in this region in TBL3 as compared with BL3 cells (Fig. 2B and C). Furthermore, buparvaquone treatment dramatically decreased H3K4me3 marks in TBL3 cells (but not H3K4me2), almost to the levels observed in BL3 cells (Fig. 2B). In contrast, we observed no significant changes for Acetyl-H3, nor the repressive H3K27me3 and H3K9me3 marks, upon *Theileria* infection or buparvaquone treatment (Figs. 2D–F). Thus, reversible *Theileria*-induced *MMP-9* transcription is associated with dramatic changes in H3K4 trimethylation on the *MMP-9* promoter.

SMYD3 expression is induced by *Theileria* infection

To identify enzymes responsible for the H3K4-methyl marks on the *MMP-9* promoter, we examined expression of a panel of H3K4 methyltransferases. Strikingly, *SMYD3* was the only methyltransferase gene clearly induced by *Theileria* in TBL3

cells (over 4-fold induction; Fig. 3A). Furthermore, buparvaquone treatment decreased *SMYD3* expression to BL3 levels (Fig. 3A). The bovine *SMYD3* gene encodes a protein very similar to the human enzyme, with almost complete conservation of key SET domain residues (Supplementary Fig. S1). To test SMYD3 activity in these 2 cell lines, we transfected cells with a SMYD3 reporter construct containing the *Nkx2.8* promoter, a known SMYD3 target gene, coupled to luciferase (24). This SMYD3 reporter was 6-fold more active in TBL3 cells (Fig. 3B). Furthermore, there was a significant reduction (50%) in the luciferase levels when the SMYD3-binding site in the promoter was mutated (Fig. 3B; ref. 24). Thus, SMYD3 was a promising candidate as the methyltransferase responsible for elevated H3K4me2/me3 marks on the *MMP-9* promoter.

SMYD3 binds to DNA via specific binding motifs (24) and bioinformatic analyses identified putative SMYD3-binding motifs in both the human and bovine *MMP-9* promoters (Fig. 3C, Supplementary Fig. S2). ChIP-qPCR analysis of the bovine *MMP-9* promoter, using oligonucleotides that amplify a cluster of putative SMYD3-binding sites, revealed a marked enrichment for H3K4me3 modifications in this region in *Theileria*-infected cells (Fig. 3D). Enrichment was reversed by buparvaquone treatment and absent in BL3 cells (Fig. 3D). These results suggested that SMYD3 binds to sites in the *MMP-9* promoter to drive *MMP-9* expression. Deeper investigation in TBL3/BL3 cells was hindered by the poor cross-reactivity of commercially available antibodies with bovine SMYD3.

SMYD3 binds to the *MMP-9* promoter and regulates transcription in human cancer cells

The putative SMYD3-binding motifs are conserved in the human *MMP-9* promoter (Fig. 3C, Supplementary Fig. S2), so we investigated the SMYD3-*MMP-9* link further in human cancer cells. We studied a fibrosarcoma cell line, HT1080, which expresses *MMP-9* and displays high invasive properties *in vitro* and *in vivo* (28, 29). We used an HT1080 derivative containing a 2.2 Kb *MMP-9-Luc* reporter stably integrated into the genome (26) to test SMYD3 effects on promoter activity in a chromosomal context (Fig. 4A). We also tested SMYD3 function by transient cotransfection with a *MMP-9-Luc* reporter plasmid (Fig. 4B). In both cases, *SMYD3* transfection caused a modest, but statistically significant, increase in *MMP-9* promoter activity (1.5–2.5 fold), which was enhanced by PMA treatment (2–5 fold; Fig. 4A and B). We observed similar synergistic effects on MMP-9 protein levels when *SMYD3* was transfected with PMA stimulation (Fig. 4C). We carried out knockdown experiments using 2 different siRNA oligonucleotides that led to marked reduction in *SMYD3* mRNA and protein (Fig. 5A–C). *SMYD3* knockdown caused a significant decrease in *MMP-9* mRNA and protein levels (Fig. 5B and C). Furthermore, siRNA *SMYD3* caused a reduction in the activity of the *MMP-9-Luc* reporter integrated into the HT1080 genome (Fig. 5D). These results support the hypothesis that SMYD3 regulates *MMP-9* expression in human cancer cells.

We generated stable HT1080 cell lines expressing shRNA against *SMYD3* and control. *SMYD3* reduction caused a decrease in *MMP-9* mRNA and protein levels (Supplementary Fig. S3A and S3B). We carried out ChIP and observed *SMYD3*

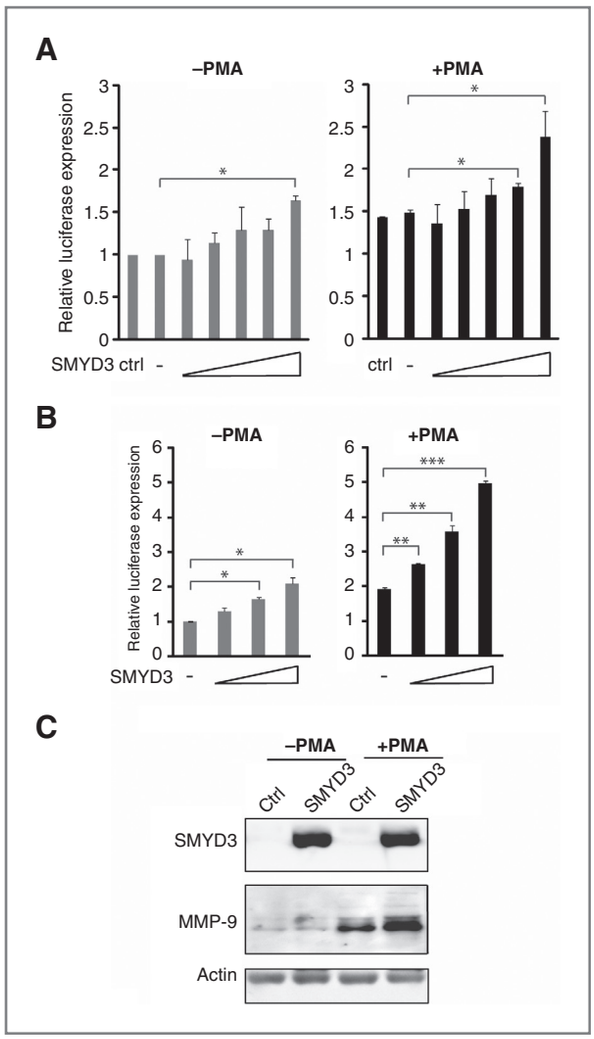

Figure 4. SMYD3 induces *MMP-9* expression in human fibrosarcoma cells. A, the luciferase activity of an integrated *MMP-9* reporter construct assessed in HT1080-2.2 cells, with increasing amounts of exogenous *SMYD3* expression, with/without PMA treatment. The effects of *SMYD3* transfection were compared with nontransfected controls (ctrl) and vector-transfected mock (–) cells. B, luciferase assays showing expression of an *MMP-9*-luciferase construct transfected into HT1080 cells, with increasing amounts of ectopic *SMYD3* expression, with/without PMA treatment compared with vector-transfected mock controls (–). C, Western blot analysis showing the expression of exogenous-transfected SMYD3 and upregulation of endogenous MMP-9 protein in HT1080 cells transfected with *SMYD3* or control vectors (Ctrl), with/without PMA treatment. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

binding to the *MMP-9* promoter in control cells but not in knockdown cells (Fig. 5F). Moreover, *SMYD3* inhibition caused a significant decrease in the H3K4me3 marks on the *MMP-9* promoter but not on the control glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) promoter (Fig. 5F). Also, *SMYD3* inhibition did not affect Acetyl-H3 marks on the *MMP-9* promoter (Fig. 5F). We excluded the possibility of an indirect effect of *SMYD3* knockdown on *MMP-9* transcription via the modulation of chromatin modifiers known to participate in the

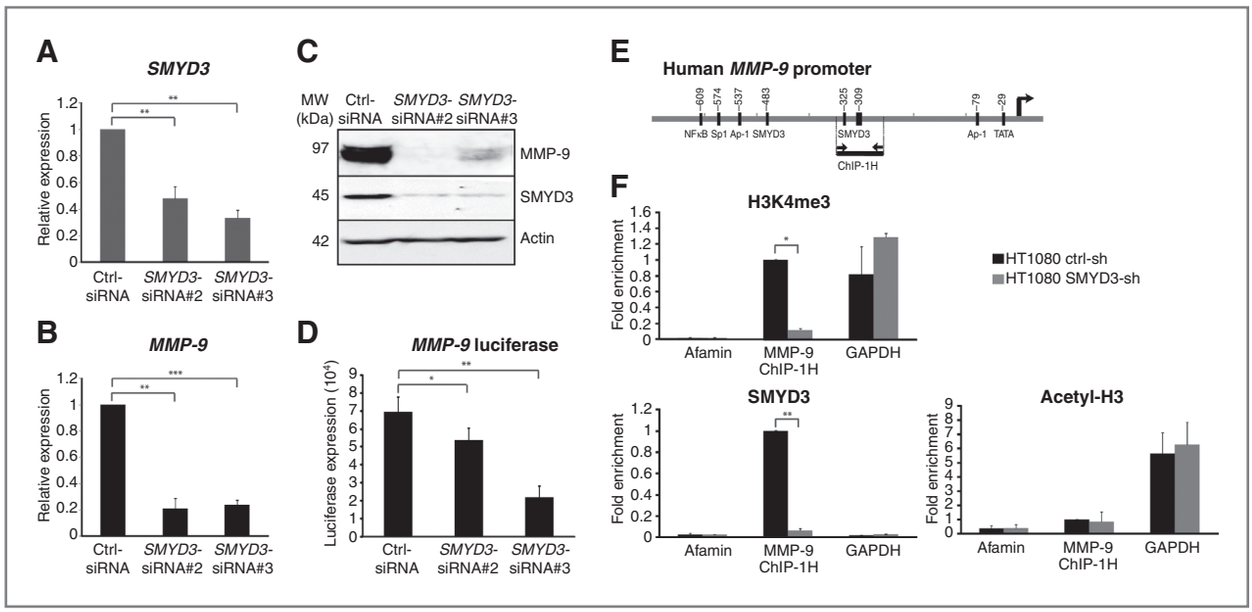

Figure 5. *SMYD3* knockdown causes epigenetic repression of *MMP-9* in human fibrosarcoma cells. A and B, HT1080 cells were transfected with 2 different siRNA oligonucleotides against *SMYD3* or control followed by qPCR analysis of *SMYD3* (A) and *MMP-9* expression (B), 48 hours after transfection. *GAPDH* mRNA was used for normalization. C, Western blot analysis in HT1080 cells transfected with 2 different siRNA oligonucleotides against *SMYD3* or control siRNA, showing SMYD3 and MMP-9 proteins. β-Actin was used as control. MW, molecular weight marker. D, luciferase activity in HT1080-2.2 cells with an integrated *MMP-9* promoter-luciferase reporter, following transfection with siRNA oligonucleotides against *SMYD3* or control siRNA (Ctrl). E, amplicon surrounding putative SMYD3-binding sites (ChIP-1H) in the human *MMP-9* promoter. The right arrow marks the TSS. F, ChIP was carried out with antibodies recognizing SMYD3, H3K4me3, or Acetyl-H3. *GAPDH* promoter is a positive control for the histone marks, and the *Afamin* promoter a negative control. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

regulation of *MMP-9* (22). We found no evidence for altered expression of these regulators upon *SMYD3* knockdown (Supplementary Fig. S4). Thus, SMYD3 binds directly to the human *MMP-9* promoter and appears responsible for H3K4 methylation and transcriptional activation.

SMYD3 drives the invasiveness and proliferation of human cancer cells

To test whether SMYD3 regulation of *MMP-9* contributes to tumor phenotypes, we examined the effect of *SMYD3* knockdown by *in vitro* migration assays with collagen-coated filters. The downregulation of *SMYD3* using 2 independent siRNAs caused a significant reduction in the migratory capacity of HT1080 cells, similar to knocking down *MMP-9* alone (Fig. 6A and B). Conversely, we also tested the effect of overexpressing *SMYD3* on cell migration; we transfected *SMYD3* into human transformed HEK293T cells, which express low levels of endogenous SMYD3 (Fig. 6C) and observed an upregulation of *MMP-9* mRNA (Fig. 6D) and a consequent increase in invasiveness (Fig. 6E).

SMYD3 has also been implicated in tumor cell growth (24, 25). We found that knocking down *SMYD3* (using siRNA or shRNA) HT1080 cells provoked a decrease in proliferation (Supplementary Fig. S3C), associated with an increased G_1 phase and reduced S-phase (Supplementary Fig. S3D). Strikingly, *SMYD3* knockdown also reduced colony formation in soft-agar growth assays, a standard *in vitro* test for tumorigenic potential (Fig. 7A–D). The few colonies that formed were small, with few dispersed cells (Fig. 7C). Moreover, this effect could be phenocopied by depleting *MMP-9* (Fig. 7A and B), suggesting that these transformed phenotypes are linked to the regulation of *MMP-9*.

Finally, we tested *SMYD3* knockdown *in vivo* using a zebrafish xenotransplantation model (30) as a simple and effective system for testing cell invasion and metastasis formation of HT1080 cells. The embryos that developed tumors 72 hours postinjection were scored for the dissemination of tumor cells (Fig. 7E, Table 1). We detected widely dispersed fluorescent spots in the yolk and in several cases in the tail region of zebrafish embryos in control xenografts, whereas the tumor mass detected in almost all of the embryos injected with *SMYD3*-siRNA or *MMP9*-siRNA–treated cells exhibited no dissemination (Fig. 7E, Table 1). Thus, *SMYD3* expression levels affect the invasiveness and metastatic behavior of human tumor cells *in vitro* and *in vivo*.

Discussion

The discovery of a large number of histone lysine methyltransferases and demethylases has revealed that histone methylation is a dynamic process directing the fine regulation of chromatin structure and gene expression (2, 31, 32). Yet, it remains a significant challenge to dissect the precise series of epigenetic events in tumor progression, which is critical to develop targeted therapeutic strategies. It is particularly difficult to reconcile the global changes in histone modifications and enzymes, with localized effects on specific gene promoters (33, 34, 35). H3K4 methylation remains particularly intriguing,

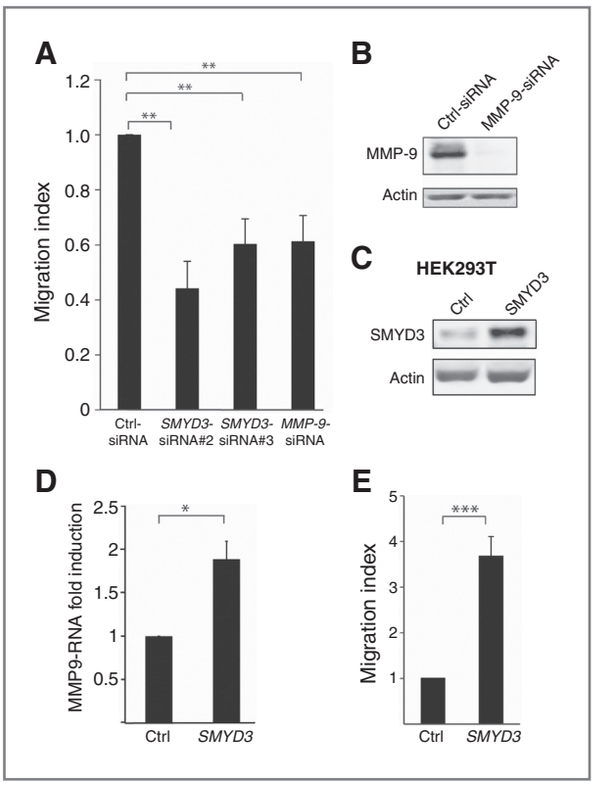

Figure 6. SMYD3 modulation affects cell migration of human cell lines. A, HT1080 cells were transfected with 2 different siRNA oligonucleotides against *SMYD3* or against *MMP-9* or control siRNA (Ctrl), followed by analysis of cell migration on collagen-coated filters. Results are shown with respect to controls, and error bars represent 3 independent experiments. B, Western blot analysis showing the efficiency of siRNA *MMP-9* knockdown in HT1080 cells. β-Actin was used as control. C, HEK293T cells were transfected with *SMYD3*-expressing vector or control vector (Ctrl). Exogenous SMYD3 protein levels were analyzed by Western blot analysis, and β-actin was used as control. D, *SMYD3* transfection into HEK293T cells caused an upregulation of endogenous *MMP-9* levels as assessed by qPCR analysis. E, *SMYD3* overexpression in HEK293T cells caused a significant increase in cell migration compared with control (Ctrl) transfected cells. Error bars represent 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

with examples of deregulated H3K4 methyltransferases (e.g., SMYD3; ref. 24) and demethylases (e.g., PLU-1/JARID1B; refs. 36–38). SMYD3 is interesting in this respect, as it binds to a specific DNA sequence, allowing it to target particular promoters (24). Here, we present evidence that SMYD3 is specifically upregulated upon *Theileria* transformation and targets the promoter of the *MMP-9* gene. This study offers insight into how *Theileria* parasites might mold the host cell epigenome and provides the first direct link between SMYD3 and a specific target gene in invasion and metastasis.

We focused on the *MMP-9* gene as a paradigm to explore parasite-induced chromatin changes associated with host cell gene expression and invasion. *MMP-9* is regulated extensively at the transcriptional level by classical transcription factors such as AP-1 and NF-κB (19). An elegant study by Ma and colleagues showed that these transcription factors lie downstream of signal transduction pathways and are recruited to the *MMP-9* promoter in a stepwise process coordinated by chromatin-modifying complexes (21). Although AP-1 activation is important for *MMP-9* regulation upon *Theileria* infection, it is not sufficient, as some *Theileria*-transformed cell lines express AP-1 without activating *MMP-9* (data not shown; ref. 14). This drove us to search for epigenetic modulators that contribute to host gene regulation. We found that H3K4me3 was specifically associated with inducible and reversible *MMP-9* expression (Fig. 2). Surprisingly, we saw no changes in Acetyl-H3, previously linked to *MMP-9* regulation (21), nor in any repressive histone marks. *SMYD3* was the only H3K4 methyltransferase whose expression correlated with the marks observed on the *MMP-9* promoter. Our gain-of-function and loss-of-function studies showed that SMYD3 plays a direct role in *MMP-9* regulation. This is likely via binding to putative SMYD3 recognition motifs in the *MMP-9* promoter and a multiple repeat (5′-CCCTCCCTCCC-3′) that is conserved between the bovine and human *MMP-9* promoters (Supplementary Fig. S2). SMYD3 binding to this region seems essential for *MMP-9* gene regulation. The effects of *SMYD3* knockdown appear to be independent of the expression of other chromatin modifiers (21, 39; Supplementary Fig. S4). But it is formally possible that SMYD3 and associated H3K4 methylation recruit additional regulatory proteins required for full *MMP-9* induction (22). Another possibility is that promoter-bound SMYD3 contributes to gene expression by methylating nonhistone proteins locally, such as the AP-1 transcription factor. There are precedents for transcription factor regulation by methylation and this merits further investigation (40). Our study highlights the dynamic nature of H3K4 methylation in transcriptional regulation and how methyltransferase binding to select promoters can control gene expression.

We provide the first evidence that *Theileria* parasites influence host cell gene expression by modulating epigenetic regulators. The inducible and reversible nature of *MMP-9* expression provided a model gene to focus on chromatin events induced by the parasite, leading to the discovery of SMYD3 as a promising candidate. It is likely that other chromatin-modifying enzymes contribute to *Theileria*-induced transformation, just as multiple modifiers cooperate in human tumors (2). The *Theileria* system offers an attractive model to search for novel SMYD3 target genes, by comparing infected and buparvaquone-treated transcriptomes accompanied by genome-wide ChIP analysis. One intriguing aspect is the striking upregulation of solely *SMYD3* and not other H3K4 methyltransferases, such as mixed-lineage leukemia (MLL) members that are deregulated in human cancers and regulate *MMP-9* (41). *SMYD3* is overexpressed in human colorectal and hepatocellular carcinomas (24) and breast cancer cells (25). Elevated *SMYD3* expression was recently observed in chronic lymphocytic leukemia (CLL) and in highly metastatic pancreatic cancers (42). However, little is known about *SMYD3* transcriptional control (43) and our model provides a tool for studying the inducible and reversible regulation of *SMYD3* in cancer cells. Our results show that the SMYD3-MMP9 circuit is functional in human cells, thereby demonstrating that the *Theileria*

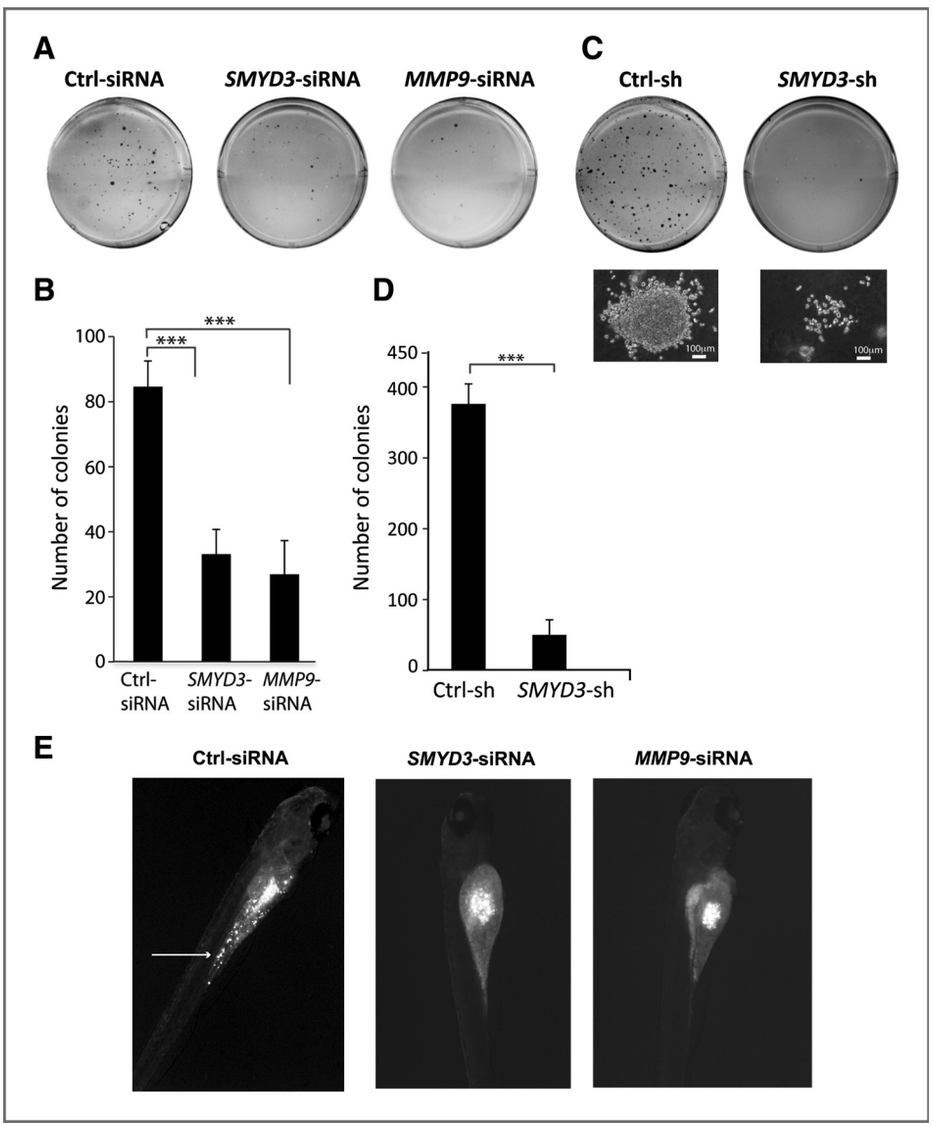

Figure 7. *SMYD3* knockdown reduces tumor cell growth *in vitro* and invasion *in vivo*. A, HT1080 cells were transfected with siRNA oligonucleotides against *SMYD3* or *MMP-9* or control siRNA (Ctrl) followed by soft-agar colony formation assay. Representative wells for each condition are shown. B, average colony numbers for 3 independent experiments comparing control, *SMYD3*, or *MMP-9*-siRNA-transfected cells. C and D, colony formation assays (plates, photomicrographs of individual colonies, and colony numbers) as above were conducted using stable clones using shRNA against *SMYD3* or control (Ctrl-sh). E, tumor dissemination in zebrafish embryos: HT1080 cells transfected with control (Ctrl-siRNA), *SMYD3*-siRNA, or *MMP9*-siRNA were injected into the embryos. Images show representative examples of xenografted embryos. Disseminated tumor cells in the yolk are marked with an arrow. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

model can be effectively used to generate mechanistic hypotheses to be tested in human cancers.

Our study directly links the SMYD3 methyltransferase to the *MMP-9* gene, which is important in angiogenesis and metastasis. *MMP-9* expression is linked to lymphoma, leukemia, and metastasis (44, 45). There are few characterized targets for SMYD3 in tumorigenesis, except for the *hTERT* telomerase and *WNT10B* genes (25, 39). Previous studies highlighted the role of SMYD3 in cancer proliferation, but those linking SMYD3 to invasion have not identified a direct molecular target (24, 25, 46, 47). *SMYD3* was also identified in gene signatures in metastatic pancreatic tumors (42). The urgent need to develop

Table 1. Development of micrometastases after xenotransplantation of HT1080 fibrosarcoma cells in the zebrafish model

	siRNA control	*SMYD3*-siRNA	*MMP-9*-siRNA
Total number of embryos with tumors	74	32	29
Dissemination in yolk	9	0	1
Dissemination in tail	5	1	0
Dissemination in heart	1	0	0
Total dissemination (%)	20.3	3.80	3.40

better therapies for metastatic disease makes it important to identify targets of regulators induced in metastatic cells. Clinical trials using MMP inhibitors have been hampered by problems with selectivity (48). Thus, our finding that a histone methyltransferase, which is induced in metastatic tumors, specifically regulates *MMP-9* expression and tumor invasion *in vivo*, offers a target to develop therapeutic strategies aimed at preventing cancer progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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