HMGA2/TET1/HOXA9 signaling pathway regulates breast cancer growth and metastasis

Miao Sun^{a,b}, Chun-Xiao Song^{c,1}, Hao Huang^{d,1}, Casey A. Frankenberger^{a,1}, Devipriya Sankarasharma^{e,1}, Suzana Gomes^a, Ping Chen^d, Jianjun Chen^d, Kiran K. Chada^e, Chuan He^c, and Marsha R. Rosner^{a,b,2}

^aBen May Department for Cancer Research, ^bCommittee on Genetics, Genomics, and Systems Biology, ^cDepartment of Chemistry and Institute for Biophysical Dynamics, and ^dDepartment of Medicine, University of Chicago, Chicago, IL 60637; and ^eDepartment of Biochemistry, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, NJ 08854

Edited* by Janet D. Rowley, University of Chicago, Chicago, IL, and approved May 6, 2013 (received for review March 21, 2013)

The ten–eleven translocation (TET) family of methylcytosine dioxygenases initiates demethylation of DNA and is associated with tumorigenesis in many cancers; however, the mechanism is mostly unknown. Here we identify upstream activators and downstream effectors of *TET1* in breast cancer using human breast cancer cells and a genetically engineered mouse model. We show that depleting the architectural transcription factor high mobility group AT-hook 2 (*HMGA2*) induces *TET1*. *TET1* binds and demethylates its own promoter and the promoter of homeobox A (*HOXA*) genes, enhancing its own expression and stimulating expression of *HOXA* genes including *HOXA7* and *HOXA9*. Both *TET1* and *HOXA9* suppress breast tumor growth and metastasis in mouse xenografts. The genes comprising the *HMGA2*–*TET1*–*HOXA9* pathway are coordinately regulated in breast cancer and together encompass a prognostic signature for patient survival. These results implicate the *HMGA2*–*TET1*–*HOX* signaling pathway in the epigenetic regulation of human breast cancer and highlight the importance of targeting methylation in specific subpopulations as a potential therapeutic strategy.

Epigenetic changes play an important role in cancer progression as well as development (1). Recent studies indicate that DNA demethylation can be catalyzed by a class of methylcytosine dioxygenases termed the ten–eleven translocation (TET) family (2–5). *TET1* promotes DNA demethylation by catalyzing conversion of 5-methylcytosine (5mC) primarily to 5-hydroxymethylcytosine (5hmC) as well as 5-formylcytosine or 5-carboxylcytosine (3, 5). The modified cytosines are then removed through active or passive mechanisms (2–6). While *TET1* is highly expressed in embryonic stem (ES) cells (5, 7–10), loss of *TET1* protein and decreased 5hmC levels have been recently shown in solid tumors relative to normal epithelial cells (2, 11–14). However, the mechanism by which *TET1* is suppressed in solid tumors has not been identified. Furthermore, the downstream targets by which *TET1* regulates growth and metastasis in cancer are largely unknown.

High mobility group AT-hook 2 (*HMGA2*), a chromatin-remodeling factor (15), binds to AT-rich regions in DNA, altering chromatin architecture to either promote or inhibit the action of transcriptional enhancers. *HMGA2* is highly expressed in ES cells but is generally low or lacking in normal somatic cells. Interestingly, *HMGA2* is highly expressed in most malignant epithelial tumors, including breast (16), pancreas (17), oral squamous cell carcinoma (18), and non-small-cell lung cancer (19). *HMGA2* overexpression in transgenic mice causes tumor formation, whereas *Hmga2*-knockout mice have a pygmy phenotype indicative of a growth defect (20). We have reported that *HMGA2* promotes tumor invasion and metastasis in breast cancer in part through regulation of prometastatic genes, including *Snail*, *osteopontin*, and *CXCR4* (21, 22).

To systematically identify critical downstream mediators of *HMGA2* that regulate invasion and metastasis, we performed gene expression array analysis by knocking down *HMGA2* in breast cancer cells. Here we show that *TET1* is an important effector of *HMGA2* in breast cancer. We further show that

TET1 regulates homeobox A (*HOXA*) genes, including *HOXA7* and *HOXA9*. Both *TET1* and *HOXA9* suppress breast tumor growth and metastasis. Our study reveals a regulatory pathway that stratifies breast patient survival.

Results

***TET1* and *HOX* Gene Expression Are Dramatically Induced upon Depletion of *HMGA2* in both Invasive Human Breast Cancer Cells and *MMTV-Wnt1* Mouse Breast Tumors.** Gene expression arrays were conducted using two invasive human breast cancer cell lines expressing either *HMGA2* shRNA or control scrambled shRNA (SCR sh): 1833, a bone-tropic derivative of the human breast cancer cell line MDA-MB-231 (23), and MDA-MB-436. We found a dramatic induction of *HOX* genes, particularly at the *HOXA* loci in the more invasive 1833 cells (Fig. 1A–C and Fig. S1). The *HOX* genes comprise four clusters (A, B, C, and D) located on different human chromosomes. This transcriptional factor family with 39 members controls posterior–anterior patterning during embryogenesis and the development of specific organs (reviewed in ref. 24). We validated the induction of *HOXA* gene expression including *HOXA4*, *HOXA5*, *HOXA6*, *HOXA7*, *HOXA9*, and *HOXA11* in *HMGA2*-depleted 1833 cells (Fig. 1D, F, and G).

Among the other genes induced by *HMGA2* depletion was *TET1* (Fig. 1A and Fig. S1). We confirmed that *HMGA2* is a negative regulator of *TET1* in 1833 cells by quantitative RT-PCR (qRT-PCR) and immunoblotting (Fig. 1E and G). Consistent with increased *TET1* expression, we observed elevated 5hmC levels in *HMGA2*-depleted 1833 cells (Fig. 1H). We also observed similar induction of *TET1* and *HOXA* gene expression following *HMGA2* depletion by shRNA in MDA-MB-436 cells, although the effects were not as robust, consistent with their relatively higher basal levels of *TET1* and *HOXA* protein and the less invasive phenotype of these cells (Fig. S2A–D). Consistent with these observations, analysis of gene expression in a cohort of 75 human breast tumors (25) showed a significantly negative correlation between *HMGA2* and *TET1* expression (Fig. S2E), and this relationship existed in both estrogen receptor (ER)-negative (Fig. S2F) and ER-positive (Fig. S2G) patient subpopulations.

To validate the regulation of *TET1* and *HOXA* genes by *HMGA2* in vivo, we used an *MMTV-Wnt1* transgenic mouse

Author contributions: M.S. and M.R.R. designed research; M.S., C.-X.S., H.H., C.A.F., D.S., S.G., and P.C. performed research; J.C., K.K.C., and C.H. contributed new reagents/analytic tools; M.S. analyzed data; and M.S. and M.R.R. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE43741).

¹C.-X.S., H.H., C.A.F., and D.S. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: m-rosner@uchicago.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1305172110/-DCSupplemental.

Fig. 1. Induction of *TET1* and *HOX* gene expression upon depletion of *HMGA2* in 1833 cells, a bone-tropic derivative of human breast cancer cell line MDA-MB-231, or in *MMTV-Wnt1* transgenic mouse breast tumors. (A, B, and D–H) We stably transduced 1833 cells with *HMGA2* shRNA (shHMGA2) or control SCR sh. (A) Gene expression array analysis showing up-regulation of *TET1* and 20 of 39 *HOX* genes in *HMGA2*-depleted cells. (B) The expression levels of *HOXA* genes are shown. *Fold change (fc) < 2; **fc > 2 based on the signal intensity of gene expression arrays. (C) Genomic transcription units of human *HOXA* genes on chromosome 7 viewed using the UCSC Genome Browser (40). *HOXA* genes are transcribed from right to left with the following order: 5'UTR (thin blue bar), Coding Sequence (thick blue bar), and 3'UTR (thin blue bar). Bar length is proportional to length of DNA sequence. (D–H) qRT-PCR and immunoblotting analyses validated induction of *TET1* and *HOXA* gene expression in *HMGA2*-depleted cells. (D–F) *HMGA2* (D), *TET1* (E), or *HOXA4/5/6/7/9/11* (F) mRNA analyzed by qRT-PCR (GAPDH as normalization control). (G) *HMGA2*, *TET1*, and *HOXA9/7* protein analyzed by immunoblotting (GAPDH as control). (H) Genome-wide 5hmC levels analyzed by dot blot assay. (I and J) Loss of *Hmga2* in *MMTV-Wnt1* transgenic mouse breast tumors induced *Tet1* and *Hoxa9/7* expression. *Wnt1* transgenic mice were crossed with *Hmga2*-specific knockout mice. Mouse primary breast tumors were obtained from *Hmga2* wild-type (*Hmga2*^{+/+}), heterozygous (*Hmga2*^{+/-}), or null (*Hmga2*^{-/-}) mice. (I) Murine *Hmga2*, *Tet1*, and *Hoxa9/7* mRNA analyzed by qRT-PCR (with mouse *Gapdh* as normalization control). (J) Murine *Tet1* and *Hoxa9* protein and 5hmC levels analyzed by immunostaining. (D–F, H, and I) Data are means ± SEM; *n* = 3. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

model for breast cancer. Deletion of *Hmga2* by crossing *MMTV-Wnt1* mice with *Hmga2*-specific knockout mice reduced tumor incidence (26) and decreased tumor cell proliferation as assessed by immunostaining of antigen Ki67 (Fig. S3). Analysis by qRT-PCR or immunohistochemistry showed a strong induction of *Tet1*, 5hmC, and *Hoxa* gene expression including *Hoxa9* and *Hoxa7* in tumors from the *MMTV-Wnt1/Hmga2*^{-/-} mice (Fig. 1 I and J). Moreover, expression of *HMGA2* in both 1833 and MDA-MB-436 cells inhibited *TET1* expression (Fig. S4). These results indicate that induction of *TET1* by depletion of *HMGA2* is not an off-target effect and raise the possibility that loss of *HMGA2* suppresses breast tumor growth by inducing *TET1* and *HOXA* genes.

***TET1* Is Involved in an Autoregulation in Human Breast Cancer Cells.**

Because the Tet1 protein may bind directly to its own promoter region as suggested by the ChIP-sequencing (ChIP-seq) data for *Tet1* in mouse ES cells (9, 10, 27, 28), we investigated whether *TET1* was also involved in regulating its own expression in human breast cancer cells. Conventional ChIP assays using 1833

cells expressing *TET1* or vector control showed that *TET1* bound to its own promoter region (Fig. 2A). Consistent with increased expression of *TET1*, ChIP assays also showed that 1833 cells expressing *TET1* exhibited increased binding of H3K4Me3, a histone marker for transcriptional activation, to the *TET1* promoter region (Fig. 2A). Furthermore, because TET proteins are typically involved in DNA demethylation pathways (2–5, 29, 30), we analyzed the effect of *TET1* on the methylation status of its own promoter regions. Our DNA methylation-specific digestion combined with qPCR showed that ~70% of the *TET1* promoter region within ± 1 kb from the transcription start site (TSS) in the parent 1833 cells contained methylated CpG islands, whereas the fraction decreased to 9% following *HMGA2* depletion (Fig. 2B). Similarly, bisulfite sequencing within the same region of the *TET1* promoter showed an increase in demethylated CpGs from 38% in 1833 cells to 86% in *HMGA2*-depleted cells (Fig. 2C). The 1833 cells treated with 5-azacytidine, a demethylation reagent that inhibits DNMTs, also showed an increase in *TET1* expression (Fig. 2D). Together, our results suggest that *HMGA2* depletion in 1833 cells causes extensive

Fig. 2. *TET1* is involved in an autoregulation in human breast cancer cells. (A) *TET1* binds to its own promoter. We analyzed 1833 cells expressing *TET1* or control by ChIP assay with anti-*TET1* or H3K4Me3 antibody followed by qPCR analysis: *TET1* and H3K4Me3 binding to the CpG island proximal to the TSS of *TET1* (see site-1 and -2 in *SI Materials and Methods*). Site-3 is a negative control. (B and C) *HMG2* depletion causes demethylation of CpG islands at the *TET1* promoter region. We analyzed 1833 cells stably expressing shHMG2 or SCR sh for CpG island methylation status by multiple approaches. (B) *TET1* promoter region was analyzed within ± 1 kb from the TSS. Methylation-specific digestions followed by qPCR distinguished between methylated CpGs vs. unmethylated or other modified (e.g., 5hmC) CpGs. The percentage of methylation vs. unmethylation (includes unmethylated or other modified C) is indicated. (C) Bisulfite sequencing of specific CpGs (see *SI Materials and Methods* for primers) at the *TET1* promoter proximal to the TSS. Results show unmethylated CpGs (open circles) vs. methylated or modified CpGs (filled circles) in 10 or more independent clones encompassing the region of interest. (D) We treated 1833 cells with 5-azacytidine followed by qRT-PCR analysis for *TET1* mRNA expression (*GAPDH* as normalization control). (A, B, and D) Data are means $\pm$ SEM; $n = 3$. * $P < 0.05$; ** $P < 0.01$.

demethylation of the *TET1* promoter and therefore results in a robust induction of *TET1* expression.

***TET1* Directly Induces *HOXA* Gene Expression in Breast Cancer Cells Through Binding to the Promoter Regions of *HOXA* Genes and Contributing to Local Demethylation.** Previous ChIP-seq analyses of Tet1 in mouse ES cells also implied that *Hoxa* genes might be downstream targets of Tet1 because their promoter regions are enriched with Tet1 protein binding (9, 10, 27, 28). To investigate whether *TET1* is an upstream regulator of the *HOXA* cluster in human breast cancer cells, we transfected *HMG2*-depleted 1833 cells with *TET1* siRNA and observed a significant decrease in *HOXA* gene expression and 5hmC levels along with decreased *TET1* expression (Fig. 3 A and B). Conversely, ectopic expression of *TET1* in the parent 1833 cells dramatically increased *HOXA9* expression and 5hmC levels (Fig. 3 C and D).

To test whether *TET1* can regulate *HOXA* gene expression in vivo, we stably transduced 1833 cells with an inducible *Tet1* expression vector. Cells were orthotopically injected into the mammary fat pad of mice. qRT-PCR and immunohistochemistry analysis of the mouse xenografts after 6 wk of treatment with doxycycline showed a dramatic induction of Tet1 and *HOXA9*

expression (Fig. 3 E and F), as well as increased 5hmC levels (Fig. 3F). Gene expression analysis in a cohort of 54 human breast tumors (25) also showed a strong positive correlation between *TET1* and *HOXA* gene expression (Fig. 3G).

We next investigated whether the induction of *HOXA* gene expression could also be attributed to direct binding of *TET1* and subsequent demethylation of the *HOXA* promoter regions. Our ChIP-qPCR assays confirmed that *TET1* and H3K4Me3 also bound to the *HOXA* gene promoter regions (Fig. 4 A and B). Our DNA methylation-specific digestion combined with qPCR assay showed that, in the parent 1833 cells, only a small fraction of the promoter region of the *HOXA4–A11* genes lacked methylation (Fig. 4C), accounting for the low expression of *HOXA* transcripts; by contrast, *HMG2* depletion caused dramatic loss of methylation at the *HOXA* gene loci (Fig. 4C). Bisulfite sequencing of the 14 CpGs near the *HOXA7* TSS showed that only 4% were unmethylated, whereas *HMG2* depletion caused $>80\%$ demethylation (Fig. 4D). Similarly, bisulfite sequencing of the 15 CpGs in the upstream 1-kb locus of the *HOXA9* promoter showed that only 5% of the CpG sites were unmethylated, whereas unmethylated CpGs increased to 91% upon loss of *HMG2* in 1833 cells (Fig. 4E). The 1833 cells treated with 5-azacytidine to demethylate DNA also showed a dramatic increase in *HOXA7* and *HOXA9* expression (Fig. 4 F and G). These results suggest that *TET1* binds directly to the *HOXA* promoter regions and contributes to local demethylation, inducing activating histone binding and gene transcription in breast cancer cells.

***HMG2/TET1/HOXA* Pathway Regulates Breast Cancer Cell Invasion.** To assess the pathological significance of this *HMG2/TET1/HOXA* signaling cascade, we determined the effect of manipulating

Fig. 3. *TET1* induces *HOXA* gene expression. (A and B) Depletion of *TET1* by siRNA partially countered induction of *HOXA* genes. We transfected 1833 cells stably expressing *HMG2* shRNA with control or *TET1* siRNA. (A) Analysis of *TET1* and *HOXA* gene mRNA by qRT-PCR. (B, Upper) Analysis of *TET1* and *HOXA9/7* protein by immunoblotting. (Lower) Analysis of 5hmC levels by dot-blot assay. (C and D) Expression of *TET1* dramatically induced *HOXA9* expression. We analyzed 1833 cells expressing constitutive Tet1 (Flag-Tet1) by qRT-PCR for *HOXA9* mRNA (C) and by immunoblotting for Tet1 (Flag-M1) and *HOXA9* protein (D, Upper) and dot-blot assay for 5hmC levels (D, Lower). (E and F) Induced expression of *TET1* in breast xenograft tumors significantly induced *HOXA9* expression. The 1833 cells stably expressing an inducible *Tet1* expression vector were orthotopically injected into the mammary fat pad of nude mice. Tumor tissues were collected and analyzed after 6 wk with (+DOX) or without (–DOX) doxycycline treatment. (E) *Tet1* and *HOXA9* mRNA analyzed by qRT-PCR. (F) *Tet1* and *HOXA9* protein and 5hmC levels analyzed by immunostaining. (G) Significant positive correlation between *TET1* and *HOXA9/7* expression in breast cancer patients (see Table S3 for patient information). Correlations were determined by Pearson's correlation coefficient. P value was determined by Student t test. (A–E) *GAPDH* served as normalization control. Data are means $\pm$ SEM; $n = 3$. ** $P < 0.01$; *** $P < 0.001$.

Fig. 4. TET1 induces *HOXA* gene expression through binding to the promoter regions of *HOXA* genes and contributing to local demethylation in human breast cancer cells. (A and B) TET1 binds to the *HOXA* gene promoters. We analyzed 1833 cells expressing TET1 or control by ChIP assay with anti-TET1 or H3K4Me3 antibody followed by qPCR analysis: TET1 and H3K4Me3 binding to the CpG islands proximal to the TSS of *HOXA7* (see site-1 and -2 in *SI Materials and Methods*) where site-3 is a negative control (A); or *HOXA9* (see site-1 in *SI Materials and Methods*) where site-2 is a negative control (B). (C–E) *HMG2* depletion causes demethylation of CpG islands at *HOXA* gene promoter regions. We analyzed 1833 cells stably expressing shHMG2 or SCR sh for CpG island methylation status by multiple approaches (see Fig. 2 B and C for the specificity of each method). (C) *HOXA* promoter regions were analyzed within –5 to +3 kb from the TSS. The percentage of methylation vs. unmethylation is indicated. (D and E) Bisulfite sequencing of specific CpGs (see *SI Materials and Methods* for primers) at *HOXA7* (D) and *HOXA9* (E) promoters proximal to the TSS. Results show unmethylated CpGs (open circles) vs. methylated or modified CpGs (filled circles) in 10 independent clones encompassing the region of interest. (F and G) We treated 1833 cells with 5-azacytidine followed by qRT-PCR analysis for expression of *HOXA7* (F) or *HOXA9* (G) mRNA (*GAPDH* as normalization control). (A–C, F, and G) Data are means $\pm$ SEM; $n = 3$. ** $P < 0.01$; *** $P < 0.001$.

these genes on cell invasion. *HMG2* depletion in 1833 cells decreased cell invasion (Fig. 5A); this effect was reversed in part by siRNA depletion of *TET1*, *HOXA9*, or *HOXA7* (Fig. 5B

and C and Fig. S5 I–K). The 1833 cells treated by demethylation reagent 5-azacytidine or decitabine showed a similar decreased cell invasion and a partial rescue in invasion followed by siRNA

Fig. 5. Both TET1 and its target, *HOXA9*, suppress breast tumor growth, invasion, and metastasis. (A–D) *HMG2*/TET1/*HOXA* pathway regulates breast cancer cell invasion. (A) Inhibition of cell invasion in 1833 cells with *HMG2* depletion. (B) Transfection of *TET1* siRNA into *HMG2*-depleted 1833 cells increases invasion. (C) Transfection of *HOXA7* or *HOXA9* siRNA into *HMG2*-depleted 1833 cells increases invasion. (D) Decitabine (5-aza-dC) treatment of 1833 cells decreases cell invasion, and transfection of *HOXA9* siRNA into treated cells partially reversed cell invasion. (A–D) Data are means $\pm$ SEM; $n = 3$. (E–K) We orthotopically injected 1833 cells stably expressing an inducible control, *Tet1*, or *HOXA9* expression vector into the mammary fat pad of nude mice. Mice were administered drinking water with (+DOX) or without (–DOX) addition of doxycycline. (E–G) Both TET1 and *HOXA9* suppress xenograft breast tumor growth. (E) Representative bioluminescence images of mice bearing 1833 cells treated as indicated. (F) Photograph of representative xenograft breast tumors of 1833 cells treated as indicated. (G) Xenograft breast tumors of 1833 cells treated as indicated and analyzed for tumor weight. (F and G) Tumors were dissected at 6 wk after implantation. (H and I) Both TET1 and *HOXA9* suppress the proliferation in xenograft breast tumors: immunostaining showing Ki67-positive cells in tumor sample of 1833 cells with induced (+DOX) vs. noninduced (–DOX) expression of *Tet1* (H) or *HOXA9* (I). (J and K) Both TET1 and *HOXA9* inhibit intravasation of 1833 cells. Cells isolated from the blood after 6 wk were analyzed for *GAPDH*/*Gapdh* transcripts derived from human (tumor) or mouse (control) by qRT-PCR: intravasation of 1833 cells with induced (+DOX) vs. non-induced (–DOX) expression of *Tet1* (J) or *HOXA9* (K). Data are means $\pm$ SEM; $n = 8$ per group. (L–N) Both TET1 and *HOXA9* suppress bone metastasis of 1833 cells. We injected 1833 cells stably expressing an inducible *Tet1* or *HOXA9* expression vector into the left ventricle of mice. Mice were administered drinking water with (+DOX) or without (–DOX) addition of doxycycline and imaged for luciferase activity after 3 wk. (L) Representative bioluminescence images of mice with bone metastasis. (M) Quantification of bone colonization by 1833 cells with induced (+DOX) vs. noninduced (–DOX) expression of *Tet1* or *HOXA9*. Data are means $\pm$ SEM; $n = 7$ –9 per group. (N) Kaplan–Meier survival analysis of mice over 8 wk after injection of the tumor cells.

depletion of *HOXA9* (Fig. 5D and Fig. S5 A–C and E–G). These data are consistent with a previous study showing that *HOXA9* is a breast cancer inhibitor (31). Together, our results reveal a signaling cascade whereby *HMGA2* promotes breast cancer cell invasion in part through inhibition of *TET1*-mediated demethylation and *HOXA* gene expression.

Both *TET1* and Its Target, *HOXA9*, Suppress Breast Tumor Growth, Intravasation, and Metastasis. To determine whether *TET1* or *HOXA9* can reverse the tumorigenic phenotype in breast cancer cells transformed by *HMGA2*, we injected 1833 cells expressing inducible *Tet1* or *HOXA9* into the mammary fat pad of mice followed by doxycycline treatment and tested their effect on xenograft tumor growth. Consistent with our in vitro observation (Figs. S5D and S6 A–C), induced expression of *Tet1* (Fig. 3 E and F) or *HOXA9* (Fig. S6 D and E) significantly suppressed xenograft tumor growth (Fig. 5 E–G) and tumor cell proliferation (Fig. 5 H and I).

To test *TET1* or *HOXA9* regulation of invasion in vivo, we determined their effect on tumor cell intravasation from a primary site in a murine orthotopic model. The 1833 cells expressing inducible *Tet1* or *HOXA9* were injected into the mammary fat pad of mice. After 6 wk of treatment with doxycycline, cells isolated from the blood were lysed and analyzed for human (tumor) or mouse (control) *GAPDH* transcripts. qRT-PCR analysis showed that both *TET1* and *HOXA9* significantly inhibited tumor cell intravasation (Fig. 5 J and K).

Because *HMGA2* depletion suppresses breast tumor cell bone metastasis (21, 22), we determined whether its downstream effectors *TET1* and *HOXA9* similarly inhibit tumor metastasis. Luciferase-labeled 1833 cells expressing inducible *Tet1* or *HOXA9* were injected into the left ventricle of mice that were subsequently treated with doxycycline. After 3 wk, mice were imaged for luciferase activity. *TET1* or *HOXA9* expression caused a dramatic decrease in bone metastasis (Fig. 5 L and M) and a significant increase in overall survival rate (Fig. 5N and Fig. S6 F–H).

***HMGA2/TET1/HOXA9* Regulate a Common Set of Important Genes and Encompass a Prognostic Signature for Patient Survival.** To identify and compare target genes of *HMGA2*, *TET1*, and *HOXA9*, we performed additional microarray assays for cells expressing induced *TET1* and *HOXA9*. Compared with the parental 1833 cells, there were 1,012; 7,220; and 7,132 genes differentially expressed [$P < 0.05$; false discovery rate (FDR) < 0.05 ; and fold change > 1.25] upon *HMGA2* depletion, *TET1* induction, or *HOXA9* induction, respectively (Fig. 6A). Interestingly, $>60\%$ of the genes differentially regulated by *TET1* or *HOXA9* were the same (4,510 genes; Fig. 6A), indicating that *HOXA9* is a major downstream effector of *TET1*. There were 214 genes that overlapped among all three sets, including 144 up-regulated and 70 down-regulated genes (Fig. 6A and Table S1). Gene annotation enrichment analysis (Database for Annotation, Visualization and Integrated Discovery) (32) indicated that the 144-gene up-regulated set was enriched in genes that have functions such as binding, catalytic activity, transcription regulator activity, and developmental processes, whereas the 70-gene down-regulated set was enriched in genes related to epithelial cell proliferation ($P = 0.041$) and the extracellular matrix ($P = 0.0077$) (Table S2). Gene set enrichment analysis (33) indicated that the down-regulated set was also enriched in genes that promote tumor growth and comprise metastasis signatures, such as *CCL2*, *EFEMP1*, *IL7R*, *PPAP2B*, and *STX3* (34). This pattern of gene regulation is consistent with a role for *TET1* through its effector *HOXA9* in the suppression of breast tumor growth and metastasis.

These data illustrate a signaling cascade in human breast cancer progression, by which expression of the oncogene *HMGA2* leads to *TET1* suppression. Because *TET1* binds and demethylates itself as well as *HOXA* genes, including *HOXA7*

Fig. 6. The *HMGA2/TET1/HOXA* pathway regulates breast tumorigenesis. (A) Comparison of the genes regulated by *HMGA2*, *TET1*, or *HOXA9* in 1833 cells (human breast cancer cells; hBrCa). (B) Scheme illustrating *HMGA2/TET1/HOXA* signaling pathway in breast tumorigenesis. (C) Kaplan–Meier analysis of gene expression data from 101 breast tumor patients (see Table S3 for patient information). Patients were stratified for survival using *HMGA2*, *TET1*, *HOXA9*, *HOXA7*, or the complete pathway as indicated. (Right) Red line, high *HMGA2* and low *TET1/HOXA9* ($n = 34$); blue line, low *HMGA2* and high *TET1/HOXA9* ($n = 35$); P , χ^2 P value.

and *HOXA9*, decreased *TET1* causes further inhibition of *TET1* in addition to loss of *HOXA* gene expression. Suppression of *TET1* and *HOXA9* then enables expression of genes that promote breast tumor growth and metastasis (Fig. 6B). When considered individually, gene expression of *HMGA2*, *TET1*, *HOXA7*, or *HOXA9* does not significantly predict survival in a heterogeneous group of breast cancer patients (Fig. 6C, Left and I, Left). By contrast, Kaplan–Meier analysis using the complete *HMGA2–TET1–HOXA* pathway (*HMGA2* high and *TET1/HOXA9/7* low vs. *HMGA2* low and *TET1/HOXA9/7* high) or a combination of *HMGA2* and *HOXA* genes was able to stratify patients and predict survival (Fig. 6C, Right and I, Right). There are no significant differences in the composition of cancer subtypes between the two stratified groups of patients (Table S3), suggesting that this regulatory mechanism exists in a variety of breast cancer subtypes.

Discussion

We identify here not only an important upstream regulator (*HMGA2*) of *TET1*, but also a previously undescribed downstream regulatory pathway for *TET1* involving *HOXA* genes; in addition, we provide compelling evidence that *TET1* and *HOXA9* play an important role not only in breast tumor invasion and growth, but also in metastasis through commonly regulated genes. Because *HMGA2* is a genomic architectural factor, we do not exclude the possibility that *HMGA2* might be able to regulate *TET1* expression by direct binding to the *TET1* promoter or by alteration of its chromatin structure. Nonetheless, a recent investigation of *HMGA2* binding sites in HCT116 cells that highly express *HMGA2* protein showed only 49 binding sites (35). None of them are even close to the genomic locus of *TET1* gene. Thus, it is very likely that *HMGA2* regulates *TET1* expression indirectly via other mediators.

While this work was under completion, it was reported that TET1 inhibits growth and metastasis in prostate and breast cancer (36). In that report, TET1 was shown to inhibit invasion in culture in part through tissue inhibitors of metalloproteinases (TIMPs) (36). By contrast, we did not observe significant induction of *TIMP* expression by TET1. Instead, we identify a group of genes commonly altered by *HMG2* depletion or induction of either *TET1* or *HOXA9*, including a subset of induced genes that promote development and a subset of inhibited genes that promote cell proliferation, consistent with a role for TET1/*HOXA9* in suppression of breast tumor growth and metastasis.

The TET1/*HOXA9* signaling pathway that we identify here also highlights the importance of cell context in determining the pathological function of TET1. In contrast to our results for breast cancer, the mixed-lineage leukemia–TET1 fusion protein and the *HOXA9* protein both promote leukemogenesis (37, 38). Recently, *HOX* family members were reported to play key roles in regulating tumorigenesis, including the epithelial/mesenchymal transition, invasion, and apoptosis (24). Highly methylated *HOXA* gene loci have been reported in human breast cancer (39), although mutations in these genes are not common. Whether these

genes function in similar ways or promote different phenotypes is an interesting question that requires further investigation.

Finally, this report identifies a gene signature comprising three mechanistically linked genes (*HMG2*, *TET1*, and *HOXA9*) that is prognostic for breast cancer survival. This signature has the potential to identify patients harboring breast or other tumors with suppressed TET1/*HOXA9* signaling who might benefit from DNA demethylation agents currently used in the clinic.

Materials and Methods

HMG2-depleted cell lines were generated by lentiviral transduction with *HMG2* shRNA. *TET1* and *HOXA* gene knockdown cell lines were generated by transfection with relative siRNA. *Tet1*- and *HOXA9*-inducible expression cells were generated by lentiviral transduction with relative inducible expression vector. Detailed descriptions are in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Alex Rothenberg and Eva Eves for helpful discussions; Huiping Liu for sharing her invasion assay; and Xi Jiang for sharing plasmids. This work was supported by National Institutes of Health (NIH) Grant GM087630 (to M.R.R.), NIH Grant GM071440 (to C.H.), NIH Specialized Programs of Research Excellence Grant P50 CA125183-05 (Developmental Research Program) (to M.R.R. and C.H.), and NIH Grant CA127277 (to J.C.). M.S. was supported by Department of Defense Predoctoral Traineeship Award W81XWH-10-1-0396.

- Hansen KD, et al. (2011) Increased methylation variation in epigenetic domains across cancer types. *Nat Genet* 43(8):768–775.
- Guo JU, Su Y, Zhong C, Ming GL, Song H (2011) Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. *Cell* 145(3):423–434.
- Ito S, et al. (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* 333(6047):1300–1303.
- He YF, et al. (2011) Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science* 333(6047):1303–1307.
- Tahiliani M, et al. (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 324(5929):930–935.
- Inoue A, Zhang Y (2011) Replication-dependent loss of 5-hydroxymethylcytosine in mouse preimplantation embryos. *Science* 334(6053):194.
- Ito S, et al. (2010) Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature* 466(7310):1129–1133.
- Koh KP, et al. (2011) Tet1 and Tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. *Cell Stem Cell* 8(2):200–213.
- Xu Y, et al. (2011) Genome-wide regulation of 5hmC, 5mC, and gene expression by Tet1 hydroxylase in mouse embryonic stem cells. *Mol Cell* 42(4):451–464.
- Williams K, et al. (2011) TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. *Nature* 473(7347):343–348.
- Haffner MC, et al. (2011) Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. *Oncotarget* 2(8):627–637.
- Kudo Y, et al. (2012) Loss of 5-hydroxymethylcytosine is accompanied with malignant cellular transformation. *Cancer Sci* 103(4):670–676.
- Yang H, et al. (2013) Tumor development is associated with decrease of TET gene expression and 5-methylcytosine hydroxylation. *Oncogene* 32(5):663–669.
- Lian CG, et al. (2012) Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. *Cell* 150(6):1135–1146.
- Fusco A, Fedele M (2007) Roles of HMG proteins in cancer. *Nat Rev Cancer* 7(12):899–910.
- Rogalla P, et al. (1997) Expression of HMGI-C, a member of the high mobility group protein family, in a subset of breast cancers: Relationship to histologic grade. *Mol Carcinog* 19(3):153–156.
- Abe N, et al. (2003) An increased high-mobility group A2 expression level is associated with malignant phenotype in pancreatic exocrine tissue. *Br J Cancer* 89(11):2104–2109.
- Miyazawa J, Mito A, Kawashiri S, Chada KK, Imai K (2004) Expression of mesenchyme-specific gene *HMG2* in squamous cell carcinomas of the oral cavity. *Cancer Res* 64(6):2024–2029.
- Meyer B, et al. (2007) *HMG2* overexpression in non-small cell lung cancer. *Mol Carcinog* 46(7):503–511.
- Benson KF, Chada K (1994) Mini-mouse: phenotypic characterization of a transgenic insertional mutant allelic to pygmy. *Genet Res* 64(1):27–33.
- Yun J, et al. (2011) Signalling pathway for RKIP and Let-7 regulates and predicts metastatic breast cancer. *EMBO J* 30(21):4500–4514.
- Dangi-Garimella S, et al. (2009) Raf kinase inhibitory protein suppresses a metastasis signalling cascade involving LIN28 and let-7. *EMBO J* 28(4):347–358.
- Kang Y, et al. (2003) A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* 3(6):537–549.
- Shah N, Sukumar S (2010) The Hox genes and their roles in oncogenesis. *Nat Rev Cancer* 10(5):361–371.
- Enerly E, et al. (2011) miRNA-mRNA integrated analysis reveals roles for miRNAs in primary breast tumors. *PLoS ONE* 6(2):e16915.
- Morishita A, et al. (2013) *HMG2* is an in vivo driver of tumor metastasis. *Cancer Res*, in press.
- Wu H, et al. (2011) Dual functions of Tet1 in transcriptional regulation in mouse embryonic stem cells. *Nature* 473(7347):389–393.
- Wu H, Zhang Y (2011) Tet1 and 5-hydroxymethylation: A genome-wide view in mouse embryonic stem cells. *Cell Cycle* 10(15):2428–2436.
- Bhutani N, Burns DM, Blau HM (2011) DNA demethylation dynamics. *Cell* 146(6):866–872.
- Cortellino S, et al. (2011) Thymine DNA glycosylase is essential for active DNA demethylation by linked deamination-base excision repair. *Cell* 146(1):67–79.
- Gilbert PM, et al. (2010) *HOXA9* regulates *BRCA1* expression to modulate human breast tumor phenotype. *J Clin Invest* 120(5):1535–1550.
- Huang W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4(1):44–57.
- Subramanian A, et al. (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 102(43):15545–15550.
- Han HJ, Russo J, Kohwi Y, Kohwi-Shigematsu T (2008) SATB1 reprogrammes gene expression to promote breast tumour growth and metastasis. *Nature* 452(7184):187–193.
- Winter N, Nimzyk R, Bösch C, Meyer A, Bullerdiek J (2011) Chromatin immunoprecipitation to analyze DNA binding sites of *HMG2*. *PLoS ONE* 6(4):e18837.
- Hsu CH, et al. (2012) TET1 suppresses cancer invasion by activating the tissue inhibitors of metalloproteinases. *Cell Rep* 2(3):568–579.
- Ono R, et al. (2002) LCX, leukemia-associated protein with a CXXC domain, is fused to MLL in acute myeloid leukemia with trilineage dysplasia having t(10;11)(q22;q23). *Cancer Res* 62(14):4075–4080.
- Faber J, et al. (2009) *HOXA9* is required for survival in human MLL-rearranged acute leukemias. *Blood* 113(11):2375–2385.
- Novak P, et al. (2006) Epigenetic inactivation of the *HOXA* gene cluster in breast cancer. *Cancer Res* 66(22):10664–10670.
- Dreszer TR, et al. (2012) The UCSC Genome Browser database: Extensions and updates 2011. *Nucleic Acids Res* 40(Database issue):D918–D923.