G9a drives hypoxia-mediated gene repression for breast cancer cell survival and tumorigenesis

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G9a is an epigenetic regulator that methylates H3K9, generally causing repression of gene expression, and participates in diverse cellular functions. G9a is genetically deregulated in a variety of tumor types and can silence tumor suppressor genes and, therefore, is important for carcinogenesis. Although hypoxia is recognized to be an adverse factor in tumor growth and metastasis, the role of G9a in regulating gene expression in hypoxia has not been described extensively. Here, we show that G9a protein stability is increased in hypoxia via reduced proline hydroxylation and, hence, inefficient degradation by the proteasome. This inefficiency leads to an increase in H3K9me2 at its target promoters. Blocking the methyltransferase activity of G9a inhibited cellular proliferation and migration in vitro and tumor growth in vivo. Furthermore, an increased level of G9a is a crucial factor in mediating the hypoxic response by down-regulating the expression of specific genes, including *ARNTL*, *CEACAM7*, *GATA2*, *HHEX*, *KLRG1*, and *OGN*. This down-regulation can be rescued by a small molecule inhibitor of G9a. Based on the hypothesis that the changes in gene expression would influence patient outcomes, we have developed a prognostic G9a-suppressed gene signature that can stratify breast cancer patients. Together, our findings provide an insight into the role G9a plays as an epigenetic mediator of hypoxic response, which can be used as a diagnostic marker, and proposes G9a as a therapeutic target for solid cancers.

breast cancer | G9a | histone methylation | hypoxia | epigenetics

Hypoxia is an important factor recognized to influence tumor behavior. Hypoxia in solid tumors leads to a more aggressive phenotype by enhancing proliferative and metastatic potential (1, 2). Persistent hypoxia significantly reduces the efficacy of radiation and chemotherapy and leads to poor outcomes (3). This impact on tumor behavior is mainly due to the increase in prosurvival genes that suppress apoptosis (4) and enhance tumor angiogenesis (5) and the epithelial-to-mesenchymal transition (EMT) (6).

Much of the tumor hypoxia research has been centered on examining the transcriptional targets of hypoxia inducible factors (HIFs). HIF- α is a heterodimeric transcription factor that is comprised of an oxygen-regulated α subunit (HIF-1 α or HIF-2 α) and a constitutively expressed β subunit (HIF-1 β) (7). In normal oxygen tension, HIF-1 α is hydroxylated on at least one of the two proline residues by the prolyl hydroxylase domain (PHDs) containing enzymes (8, 9). Hydroxylated HIF-1 α is then recognized by the tumor suppressor von Hippel Lindau protein (pVHL) and subsequently ubiquitinated for degradation by the proteasome (10). Because PHDs require oxygen for their enzymatic activity, under low oxygen concentrations, PHD-mediated hydroxylation is inhibited and HIF-1 α can then heterodimerize with HIF-1 β in the nucleus, leading to specific target gene expression through binding to a hypoxia response element (HRE, recognized by the motif RCGTG in which R is either A or G).

G9a or euchromatic histone-lysine methyltransferase 2 (*EHMT2*) is one of a larger family of enzymes that can methylate histone

H3 lysine 9 (H3K9) from an unmodified state to a dimethylated state (H3K9me2). Dimethylation of H3K9 is correlated generally with gene repression and is used as a marker of epigenetically silenced genes (11). G9a is frequently overexpressed in several tumor types compared with matched normal controls, and its depletion by shRNA knockdown in cancer cells reduces tumor growth and metastasis, suggesting G9a as an oncogenic and metastatic factor (12, 13). It has been shown that G9a protein accumulation occurs in hypoxic conditions without altering the level of G9a transcript (14). However, a detailed molecular mechanism of this accumulation and the consequences has not been elucidated so far.

In the current study, we provide a detailed molecular mechanism by which G9a stabilization occurs in hypoxic conditions that leads to epigenetic silencing of specific genes. This increase in stabilization is mediated through a reduction in G9a prolyl hydroxylation. The loss of prolyl hydroxylation blocks pVHL interaction and subsequent proteasome-mediated degradation. G9a-mediated H3K9 methylation leads to a repression of a specific set of genes that is essential for tumor suppression in hypoxic condition. A subset of genes repressed as a result of increased H3K9 methylation by G9a is associated with poor prognosis in breast cancer patients. Our study not only identifies a posttranslational modification on G9a, but also links G9a as an epigenetic sensor of a hypoxic microenvironment, making G9a a promising therapeutic target for breast cancer.

Significance

The hypoxic microenvironment in solid tumors is known to increase the aggressiveness of cancer cells by enhancing proliferative and metastatic potential and reduces the efficacy of radiation and chemotherapy. Previous studies have shown that G9a protein accumulates in hypoxic conditions. However, neither a detailed molecular mechanism nor its functional role has been elucidated so far. This study investigates the role G9a plays in gene expression in hypoxic conditions and its impact on tumor growth. G9a inhibition studies and the metaanalysis of G9a-suppressed gene signature using several breast cancer gene expression databases revealed that G9a is a potential therapeutic target in breast cancer.

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Results

Hypoxia Stabilizes G9a Methyltransferase. To determine the effect of hypoxia on the level of G9a protein expression, MCF7 and MDA-MB-231 (MDA231) breast cancer cells were exposed to hypoxic conditions (1%), and a significant increase in G9a protein level was observed (Fig. 1A). This increase in G9a protein levels corresponded to an elevation of the global level of H3K9me2 (Fig. 1B) without a significant change in two other methyltransferases that target H3K9 (GLP and SUV39h1; *SI Appendix, Fig. S14*). Quantitative PCR (qPCR) revealed that *G9a* transcription was not altered, suggesting that G9a is posttranscriptionally regulated (Fig. 1C). Indeed, treating cells with the proteasomal inhibitor, MG132, resulted in an increase in G9a protein level in normoxia, but not in hypoxia (Fig. 1D); suggesting that hypoxia-mediated regulation of G9a protein stability may involve perturbation of the proteasomal degradation pathway. To this end, we assessed the extent of G9a ubiquitination in normoxia and hypoxia and found that polyubiquitination of G9a was significantly reduced in hypoxia (Fig. 1E). Because G9a showed a similar stabilization dynamics to that of HIF-1 α protein in hypoxia (*SI Appendix, Fig. S1B*), we examined the effect of inhibiting PHDs on G9a protein stability. The level of G9a polyubiquitination was markedly reduced with treatment of the prolyl hydroxylase inhibitor dimethylxaloylglycine (DMOG) (Fig. 1F), consistent with an increase in G9a protein level in both MCF7 and MDA231 breast cancer cells treated with either DMOG or a hypoxia-mimicking agent deferoxamine (DFO) (Fig. 1G and *SI Appendix, Fig. S1C*). Together these data strongly suggest that hypoxic conditions lead to G9a protein stabilization by inhibiting the activities of PHDs, and

this process could facilitate changes in gene expression that are independent of HIFs.

Prolyl Hydroxylation-Mediated G9a Degradation. Because inhibition of PHD activity led to an increase in G9a protein stability, we examined the interaction between G9a and known PHD proteins by performing coexpression and coimmunoprecipitation assays. Both PHD1 and PHD3 were capable of physically interacting with G9a (Fig. 2A and *SI Appendix, Fig. S24*). However, hydroxylproline pull-down assays revealed that only PHD1 was able to hydroxylate G9a (*SI Appendix, Fig. S2B*). Moreover, G9a hydroxylation was readily detected in normoxic conditions, whereas it was almost completely absent in hypoxic condition (Fig. 2B). Because pVHL is known to interact with hydroxylated HIF-1 α (8, 9), we performed a coimmunoprecipitation assay between G9a and pVHL to determine whether G9a degradation was also regulated in a pVHL-dependent manner. G9a showed an interaction with pVHL in normoxia, but this interaction was significantly reduced in hypoxia (Fig. 2C). Moreover, the interaction between G9a and pVHL was significantly inhibited by DMOG treatment, suggesting that G9a hydroxylation is an essential modification required for G9a recognition by pVHL (*SI Appendix, Fig. S2C*). Moreover, hypoxia-dependent stabilization of G9a was absent in RCC4 cells in which pVHL was defective (*SI Appendix, Fig. S2D*); however, expression of wild-type pVHL restored G9a sensitivity to hypoxia (Fig. 2D). In addition, knockdown of PHD1 and pVHL, but not PHD3, led to an increase in G9a protein levels (Fig. 2E). Based on the known consensus hydroxylation motif "LXXLAP," we have identified three putative proline hydroxylation sites on G9a at amino acid residues P676, P1194, and P1207 (*SI Appendix, Fig. S2E*). These proline residues were mutated to alanine by site-directed mutagenesis and tested for their ability to interact with pVHL. P676A, and P1207A mutants showed reduced interaction with pVHL, whereas there was no effect for P1194 (*SI Appendix, Fig. S2F*). To confirm proline hydroxylation on P676 and P1207, we performed multiple reaction monitoring mass spectrometry on tryptic digests of G9a isolated from MCF7 cells. After establishing robust detection of tryptic peptides containing P676 or P1207 with at least three charges, primarily in the unmodified form (Dataset S1), we further examined G9a coexpressed with PHDs, and also purified by using anti-OH pull down. As shown in *SI Appendix, Table S1*, precursor and fragment ions consistent with proline hydroxylation at P676 could be detected when G9a was coexpressed with PHDs (Fig. 2F and *SI Appendix, Fig. S2G*). Peptides with proline hydroxylation at P1207 could only be detected following an additional enrichment step by using anti-OH antibody, suggesting lower stoichiometry at this site (Fig. 2G and *SI Appendix, Fig. S2H*). To further demonstrate the role of proline hydroxylation in G9a stability, we generated a double hydroxylation mutant G9a P2A and expressed it in G9a-deficient cells. The G9a P2A mutant failed to show hypoxia-dependent accumulation when expressed in G9a-deficient mouse embryonic fibroblasts (MEFs), but appeared to show a higher level of protein in normoxia compared with that of G9a WT (*SI Appendix, Fig. S2I*). We then performed hydroxylation assays and found that whereas a considerable amount of proline hydroxylation was detected for G9a WT, no detectable level of proline hydroxylation was observed for the G9a P2A mutant (*SI Appendix, Fig. S2J*). These data show that the role of PHD1 and pVHL play in regulating G9a protein stability.

Identification of G9a Target Genes and its Use as a Prognostic Indicator in Breast Cancer. To determine the functional consequence of G9a accumulation in hypoxic conditions, we performed a microarray analysis from RNA isolated from MCF7 cells, expressing either nonsilencing control shRNA (shNS) or G9a shRNA (shG9a), and exposed to normoxic and hypoxic conditions. The global analysis investigated the general impact of hypoxia and G9a knockdown on gene expression (Fig. 3A). Given the strong association between methyltransferase activity of G9a toward H3K9 methylation in

Fig. 1. G9a nuclear accumulation in hypoxic conditions. (A) Immunoblotting analysis of G9a in nuclear extracts from MCF7 and MDA-MB-231 (MDA231) breast cancer cells exposed to normoxic and hypoxic conditions as indicated. (B) Immunoblotting analysis of G9a, H3K9me2 in MCF7, and MDA231 cells cultured at hypoxic (1% O₂) conditions as indicated. (C) G9a and VEGFA transcript levels analyzed by quantitative RT-PCR from RNA isolated from MCF7 cells. Results are expressed as relative mRNA levels compared with 0 h (normoxia). (D) G9a immunoblotting analysis on MCF7 cells exposed to normoxic or hypoxic conditions (9 h) in the presence or absence of MG132 (20 μ M). (E and F) Ubiquitination of G9a from MCF7 cells transfected with His-ubiquitin, exposed to normoxia or hypoxia (E) or DMOG (1 mM) (F). (G) G9a immunoblotting on MCF7 and MDA231 cells treated with DMOG (1 mM) for indicated times.

Fig. 2. Mechanism of G9a stabilization under hypoxic stress. (A) MCF7 cells transfected with the indicated expression plasmids in the presence of MG132 (20 μ M), and immunoprecipitated by using anti-GFP antibody and immunoblotted by using antibodies indicated. EV, empty vector. (B) Immunoprecipitation of proline hydroxylated G9a with anti-hydroxylproline antibody from MCF7 cells overexpressing Flag-tagged G9a either exposed to normoxia or hypoxia in the presence of MG132 (20 μ M). (C) Interaction assay between G9a and pVHL in MCF7 cells transfected with the indicated expression plasmids in normoxic or hypoxic conditions by using anti-Flag antibody immunoprecipitation and immunoblotted by using antibodies indicated. (D) Immunoblotting analysis of G9a in RCC4 renal cell carcinoma cell line or overexpressing wild-type pVHL exposed to normoxic and hypoxic conditions. (E) Immunoblotting analysis of G9a in whole cell lysates from MCF7 cells transfected with either shNS or shRNA constructs targeting pVHL, PHD1, or PHD3. (F and G) Multiple reaction monitoring mass spectrometric analysis of proline hydroxylation on G9a tryptic peptides containing P676 and P1207. Shown are retention time and peak area (%) for individual fragment ions.

gene silencing (15), we investigated G9a as a potential mediator of hypoxia-dependent gene repression (Fig. 3A). Among genes down-regulated by hypoxia, 36% of genes (212) appeared to be G9a-dependent because these genes were no longer down-regulated by hypoxia with G9a knock-down (Fig. 3A and *SI Appendix*, Fig. S3B). The remaining 64% of genes (385) were down-regulated by hypoxia in a G9a-independent manner (Fig. 3B and *SI Appendix*, Fig. S3C). Given that tumor hypoxia is correlated with treatment resistance (3) and metastatic transformation of

cancer cells (16), we asked whether genes repressed by hypoxia in a G9a-dependent manner correlate with patient outcome. We have divided our patient survival analysis into estrogen receptor-positive (ER-positive) and estrogen receptor-negative (ER-negative). The analysis was performed on these two groups in three large gene expression datasets for breast cancer: KM Plotter, ROCK, and TCGA (*SI Appendix*, Fig. S4). Because these datasets contained gene expression profiles of tumors that are hypoxic and those that harbor G9a amplification, we used the previously published 13 genes (17) as a marker for hypoxia in the tumors and G9a expression as initial filtering criteria. We identified those genes whose expression was inversely correlated to both G9a expression and hypoxia (determined by the 13-gene hypoxic marker). These genes were then analyzed for commonality between the three datasets (*SI Appendix*, Fig. S4A). We found 10 genes that associated with relapse-free survival (Fig. 3C and *SI Appendix*, Fig. S4A) and notably, 6 of these genes (*ARNTL*, *CEACAM7*, *GATA2*, *HHEX*, *KLRG1*, and *OGN*) have been reported to possess tumor suppressor activity (18–23). qPCR analysis of the 10 genes in three additional breast cancer cell lines revealed that their expression was down-regulated in hypoxia in a similar manner to that observed in MCF7 breast cancer cells (*SI Appendix*, Fig. S4B).

We then analyzed the expression of these genes for their association with relapse-free survival as a gene signature. Breast cancer cases in each of the three datasets (KM Plotter, ROCK, and TCGA) were allocated to one of four quartiles based on the

Fig. 3. Expression and prognostication of the G9a-suppressed genes across breast cancer subtypes. (A) Diagram showing the strategy of cDNA microarray analysis and G9a-dependent gene selection process. (B) Gene expression heatmap of hypoxia responsive genes from MCF7 cells expressing shNS and shG9a. Heatmap values represent log twofold change of intensity values relative to the values in the control cells in normoxia. Up-regulated and down-regulated gene clusters are represented as red and green, respectively. (C) G9a-suppressed gene signature. List of the 10 genes associated with relapse-free survival identified with a heatmap representing relative expression from the microarray analysis. Genes in bold are known to have tumor suppressor functions. * indicate genes overlapping in both ER-positive and ER-negative groups. (D) Kaplan-Meier curve of breast cancer cases: Kaplan-Meier Plotter database using the G9a-suppressed gene signature. The hazard ratio (HR) and the log-rank P values for survival comparison between the quartile 1 group (bottom 25%) and the other groups is also shown.

average expression of the G9a-suppressed genes (G9a-suppressed gene signature), and relapse-free survival was compared. KM Plotter breast cancer gene expression database contained the largest patient number analyzed ($n = 3,524$). Patients with the lowest expression of the G9a-suppressed signature (quartile 1; Q1 in black) had a poorer relapse-free survival with ~50% of patients free from relapse after 10 y, whereas the patients with the highest G9a-suppressed gene signature (quartile 4; Q4 in red) had significantly better survival with close to 80% of patients free from relapse (Fig. 3D). The ROCK and TCGA gene expression datasets also showed similar association between the G9a-suppressed gene signature and relapse-free survival, further strengthening the prognostic power and clinical relevance of the G9a-suppressed gene signature (*SI Appendix, Fig. S4C*).

Stratification of patients in the KM Plotter dataset was also analyzed in ER-positive and ER-negative groups (*SI Appendix, Fig. S4D*). The patient group with the lowest expression of the G9a-suppressed gene signature (first quartile in black) in the ER-positive group had poorer survival compared with the rest of the group (rest in red). Significant prognostic effect of the G9a-suppressed gene signature is also observed in the ER-negative group and when the patients were divided into molecular breast cancer subtypes (Luminal A, Luminal B, HER2-enriched, and Basal-like) (*SI Appendix, Fig. S4E*). Multivariate survival analysis of the G9a-suppressed gene signature in three datasets also revealed that the signature was significantly associated with survival in a multivariate Cox proportional hazards model in every dataset tested (*SI Appendix, Table S2*). Collectively, we have identified the G9a-suppressed gene signature that associated with poor prognosis and can potentially be used for the stratification of breast cancer patients.

Functional Role of G9a Accumulation in Regulating Gene Expression.

Upon verifying the prognostic value of the G9a-suppressed gene signature, we tested the ability of a G9a inhibitor to reverse G9a-mediated suppression. qPCR analysis revealed that the expression of all 10 genes was significantly down-regulated by hypoxia in control cells, whereas this repression was abolished by either treating cells with UNC0642 (24), a small molecule inhibitor of G9a methyltransferase activity, or by knockdown of the enzyme, demonstrating that their expression is controlled by G9a (Fig. 4A and *SI Appendix, Fig. S5*). To understand the molecular mechanism by which hypoxia-mediated accumulation of G9a results in the repression of specific genes, chromatin immunoprecipitation (ChIP) was performed on promoters of the 10 G9a-repressed genes (Fig. 4B and *SI Appendix, Fig. S6A*). Hypoxia significantly increased H3K9me2 at the promoters of these genes and correlated with transcriptional silencing (Fig. 4B). This increase in H3K9me2 was almost completely abrogated with UNC0642 treatment or with G9a knockdown (Fig. 4B and C and *SI Appendix, Fig. S6A*). It appears that these genes are directly regulated by changes in histone methylation and not by a transcriptional repressor such as DEC1 (25) because we were not able to see any physical interaction between G9a and DEC1 in neither normoxic nor hypoxic conditions (*SI Appendix, Fig. S6B*). Together, these results demonstrate the role of G9a in repressing specific genes via methylating H3K9, and that this modification can be abrogated by the use of a small molecule inhibitor of G9a.

G9a Inhibitor Suppresses Breast Cancer Cell Growth and Survival in Vitro. To gain insight on the molecular function of G9a, we performed a functional annotation network analysis by using Ingenuity Pathway Analysis (QIAGEN Redwood City). Several molecular and cellular pathways related to cellular growth and development and survival were predicted to be affected (*SI Appendix, Fig. S7*). We then performed Western immunoblotting analysis to determine G9a protein levels in 14 different breast epithelial cells including normal-like (MCF10A and BRE80), four ER-positive (ZR751, BT474, T47D, and MCF7) and eight

Fig. 4. Molecular analysis of G9a inhibition in gene expression. (A) Quantitative RT-PCR analysis of the G9a-suppressed genes following treatment with UNC0642 (3 μ M, 9 h). Results are expressed as relative mRNA levels compared with vehicle treatment under normoxic or hypoxic conditions. (B and C) ChIP analysis of G9a, H3K9me2, and Pol II on *ARNTL* and *HEX* promoter in MCF7 cells treated with UNC0642 (3 μ M, 9 h) (B) or G9a knockdown (C). Values are expressed as mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$, $n = 3$ (unpaired t test).

ER-negative subtypes (HCC1937, HS578T, BT549, SKBR3, MDA231, MDA157, MDA436, and MDA468) (Fig. 5A). Most breast cancer cell lines expressed a higher level of G9a protein in normoxia compared with that observed in normal-like breast epithelial cells. Upon evaluating the therapeutic potential of UNC0642 on these breast cancer cell lines, we found that cell survival was unaffected by UNC0642 in normal-like BRE80 and MCF10A breast epithelial cells, whereas significant reduction was observed in cancer cell lines. Survival analysis and Western immunoblotting performed in hypoxia showed similar results to that observed in normoxia (*SI Appendix, Fig. S8A and B*). Further characterization of MCF7 and MDA231 cells revealed that inhibiting G9a resulted in a dose-dependent reduction in proliferation compared with vehicle control. The confluency of UNC0642 treated cells (2–3 μ M) was 2- to 2.5-fold less compared with vehicle (Fig. 5B and *SI Appendix, Fig. S8C*). Similar inhibitory effect on proliferation was observed with G9a knockdown, which associated with a decrease in H3K9me2 levels (Fig. 5C and D). The inhibitory effect of UNC0642 on cellular proliferation and survival in both normoxic and hypoxic conditions was confirmed by performing sulforhodamine B assay in MCF7 and MDA231 breast cancer cells (Fig. 5E and *SI Appendix, Fig. S8D*). A significant reduction in global H3K9me2 was observed with UNC0642 treatment (*SI Appendix, Fig. S8E*), suggesting that the dose used was effective for inhibiting G9a methyltransferase activity. Together, these results demonstrate the oncogenic function of G9a can be abrogated by blocking the methyltransferase activity of G9a using a small molecule inhibitor.

G9a Inhibition Reduces Tumor Growth in Vivo. To determine the effect of inhibiting G9a methyltransferase activity on tumor growth, syngeneic AT3 luminal mammary tumor cells were s.c. injected into C57BL/6 mice and allowed to form palpable tumors (2 wk) before administering G9a inhibitor. Consistent with our in vitro data where UNC0642 reduced cell viability of AT3 cells (*SI Appendix, Fig. S9*), administration of UNC0642 significantly reduced tumor growth in vivo (Fig. 6A and *SI Appendix, Fig. S10A*) with a greater than twofold reduction in mean tumor volume in the UNC0642-treated group compared with that of the vehicle-treated group (Fig. 6B

Fig. 5. UNC0642 inhibits cell proliferation and survival in breast cancer cell lines. (A) Cell survival was analyzed by performing MTT assay on cells following vehicle or UNC0642 (96 h). Immunoblotting analysis of G9a in nuclear extracts from various breast epithelial cells. (B) IncuCyte ZOOM time-lapse imaging analysis for MCF7 treated with various concentrations of UNC0642 as indicated. (C) Cell survival analysis of MCF7 cells following G9a knockdown. (D) Immunoblotting analysis of G9a following G9a knockdown. (E) Cell survival assay on MCF7 cells following vehicle or UNC0642 treatment in normoxia. Data are presented as the mean $\pm$ SEM, * P < 0.05, ** P < 0.01, *** P < 0.0005, **** P < 0.0001, n = 3 (unpaired t test).

and *SI Appendix, Fig. S10B*). Immunohistochemical analysis of two G9a-suppressed genes, ARNTL and HHEX revealed that UNC0642 treatment increased the level of both proteins compared with the vehicle (Fig. 6C). Together, our results demonstrate that the methyltransferase activity of G9a is critical for tumor growth, and a small molecule inhibitor of G9a effectively blocks tumor growth in vivo.

Discussion

In this study, we identified G9a as an epigenetic sensor of the hypoxic environment that links gene repression with increased survival and cellular motility of breast cancer cells. In a hypoxic microenvironment, G9a protein accumulates as a result of increased protein stability and contributes to the repression of specific genes. When G9a is hydroxylated on specific proline residues (P676 and P1207), it is targeted by pVHL for degradation, thereby maintaining a homeostatic expression of genes in normoxic conditions. However, in hypoxia, this balance is perturbed by a shortage of oxygen required for the hydroxylase activity of PHD enzymes. We found that the regulation of G9a protein stability is similar to that of HIF-1 α protein in the sense that G9a is hydroxylated on proline residues. However, this regulation of G9a protein stability in normoxic conditions is somewhat different to that of HIF proteins, since a variable but considerable level of G9a protein is maintained in normoxic conditions. This difference is presumably due to the inefficient degradation of G9a via hydroxylation because G9a can only be targeted by PHD1, in contrast to HIF-1 α being targeted by all PHDs. Therefore, this inefficiency in proline hydroxylation and subsequent degradation may allow G9a protein levels to be maintained in normoxia. Maintaining the appropriate level of G9a protein in normoxia is crucial as G9a participates in the methylation of histones that are necessary for homeostasis (26).

Our work emphasizes the role G9a plays as an epigenetic oxygen sensor that it can modulate the expression of a specific set of genes when its level is increased under hypoxic conditions. Although the increase in G9a stability in hypoxia appears to be as rapid and sensitive as HIF-1 α stabilization, G9a is able to repress its target gene expression by increasing H3K9 methylation. We have shown that G9a is able to modulate gene expression through nonhistone methylation in hypoxic conditions (27). However, genes identified in the current study are distinct from those that are regulated through nonhistone methylation. We have observed a hypoxia-dependent increase in G9a recruitment at its target promoters inducing H3K9me2 and this increase led to silencing of several genes while pharmacological inhibition of G9a by UNC0642 blocked this silencing (*SI Appendix, Fig. S11*). Together our data suggests the link between epigenetic regulation and tumor microenvironment where G9a can act as a key epigenetic enzyme for transcriptional repression in hypoxic conditions.

We developed a gene signature based on G9a-suppressed genes as a measure of G9a activity for patient stratification and G9a-based therapy by using the integration of in silico data available through breast cancer gene expression databases, combined with the use of small molecule inhibitors as a potential therapeutic agent. Using the G9a-suppressed gene signature, we identified a cohort of breast cancer patients with poor survival independent of other clinicopathological indicators in multiple datasets. The patient group with poor survival was associated with lower expression of the G9a-suppressed signature genes. The potential that G9a and the G9a-suppressed gene signature may have clinical utility in breast cancer is supported by the ability of G9a inhibitor to elevate the expression of the G9a-suppressed signature genes, inhibiting

Fig. 6. Effect of G9a inhibition on tumor growth in vivo. (A) Growth curve of AT3 tumors in B6 WT mice treated with vehicle (DMSO) or UNC0642 (5 mg/kg) i.p. every 2 d from day 16. (* P < 0.05, n = 9 (unpaired t test)). (B) Tumor volume at end-point shown for vehicle and UNC0642-treated mice represented as mean $\pm$ SEM. (C) Immunohistochemical analysis of ARNTL and HHEX protein expression in AT3 tumors at day 28. Scale bars represent 60 μ m. Results are summarized in bar graphs and are shown as the mean $\pm$ SEM, * P < 0.05, n = 5 (unpaired t test).

breast cancer cell proliferation in vitro as well as reducing tumor growth in vivo. Together, our findings suggest not only the feasibility and therapeutic potential of targeting G9a in solid tumors, but also that the G9a-suppressed gene signature can be used to stratify patients who will most likely to respond to therapy targeting G9a using a small molecule inhibitor that may slow or prevent recurrence.

Materials and Methods

Antibodies. The following commercially available antibodies were used as follows: Anti-GFP (sc-8334) was purchased from Santa Cruz Biotechnology. Anti-HA (MMS-101P-500) was from Jomar Biosciences. Anti-H3 (ab1791), anti-H3K9me1 (ab9045), anti-H3K9me2 (ab1220), anti-H3K9me3 (ab8898), anti-RNA Polymerase II (ab817), anti-tubulin (ab6046), anti-pontin (ab51500), anti-LC3 (ab38394), and anti-hydroxyproline (ab37067) were from Abcam. Anti-G9a (07-551), anti-GLP (B0422), anti-Lamin A/C (05-714), and anti-SUV39h1 (S8316) were from EMD Millipore. Anti-Flag (F3165) was from Sigma Aldrich. Anti-HIF-1 α (NB100-479), anti-PHD1 (NB100-310), anti-PHD2 (NB1-30328), and anti-PHD3 (NB100-303) were from Novus Biologicals.

Cell Culture and Drug Treatments. Human breast cancer cell lines were obtained from American Type Culture Collection (ATCC) and cultured as per ATCC instructions. C57BL/6 mouse AT3 mammary adenocarcinoma was maintained as described (28). All human cell lines were regularly tested for mycoplasma and authenticated by using short tandem repeat profiling. UNC0642 was purchased from Sigma-Aldrich and MG132 (M-1157) from A.G. Scientific.

shRNA Knockdown. shRNA-mediated knockdown cells were generated as described (27). To generate knockdown cells, retroviral shRNA constructs (shNS and shG9a) with viral packaging plasmids (pMD2.G and pMD-MLV) were transfected into HEK293T cells. Viral supernatant was collected 3 d after for target cell infection.

In Vivo Tumor Studies. C57BL/6 mice were purchased from the Animal Resources Centre or Walter Eliza Hall Institute (WEHI). Groups of 6–10 mice per experiment were used for experimental tumor assays to ensure adequate power to detect biologic differences. All experiments were approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee. AT3 tumor cells (1×10^6) were injected s.c. into mice in 100 μ L of volume (day 0), and treatments were given as indicated in the figure legends. Tumor volumes ($\text{width}^2 \times \text{length}/2$) were measured by using a digital caliper and presented as mean $\pm$ SEM.

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- Harris AL (2002) Hypoxia—a key regulatory factor in tumour growth. *Nat Rev Cancer* 2: 38–47.
- Semenza GL (2003) Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 3:721–732.
- Vaupel P (2004) Tumor microenvironmental physiology and its implications for radiation oncology. *Semin Radiat Oncol* 14:198–206.
- Erler JT, et al. (2004) Hypoxia-mediated down-regulation of Bid and Bax in tumors occurs via hypoxia-inducible factor 1-dependent and -independent mechanisms and contributes to drug resistance. *Mol Cell Biol* 24:2875–2889.
- Semenza GL (2000) Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. *Crit Rev Biochem Mol Biol* 35:71–103.
- Hill RP, Marie-Egyptienne DT, Hedley DW (2009) Cancer stem cells, hypoxia and metastasis. *Semin Radiat Oncol* 19:106–111.
- Semenza GL, Wang GL (1992) A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol* 12:5447–5454.
- Ivan M, et al. (2001) HIF1 α targeted for VHL-mediated destruction by proline hydroxylation: Implications for O₂ sensing. *Science* 292:464–468.
- Jaakkola P, et al. (2001) Targeting of HIF-1 α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science* 292:468–472.
- Maxwell PH, et al. (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 399:271–275.
- Tachibana M, et al. (2005) Histone methyltransferases G9a and GLP form heteromeric complexes and are both crucial for methylation of euchromatin at H3-K9. *Genes Dev* 19:815–826.
- Dong C, et al. (2012) G9a interacts with Snail and is critical for Snail-mediated E-cadherin repression in human breast cancer. *J Clin Invest* 122:1469–1486.
- Liu S, et al. (2015) G9a is essential for EMT-mediated metastasis and maintenance of cancer stem cell-like characters in head and neck squamous cell carcinoma. *Oncotarget* 6:6887–6901.
- Chen H, Yan Y, Davidson TL, Shinkai Y, Costa M (2006) Hypoxic stress induces dimethylated histone H3 lysine 9 through histone methyltransferase G9a in mammalian cells. *Cancer Res* 66:9009–9016.
- Chen MW, et al. (2010) H3K9 histone methyltransferase G9a promotes lung cancer invasion and metastasis by silencing the cell adhesion molecule Ep-CAM. *Cancer Res* 70:7830–7840.
- Joyce JA, Pollard JW (2009) Microenvironmental regulation of metastasis. *Nat Rev Cancer* 9:239–252.
- Hu Z, et al. (2009) A compact VEGF signature associated with distant metastases and poor outcomes. *BMC Med* 7:9.
- Li L, et al. (2014) Quantitative proteomics approach to screening of potential diagnostic and therapeutic targets for laryngeal carcinoma. *PLoS One* 9:e90181.
- Hu QP, Kuang JY, Yang QK, Bian XW, Yu SC (2016) Beyond a tumor suppressor: Soluble E-cadherin promotes the progression of cancer. *Int J Cancer* 138:2804–2812.
- Li YW, et al. (2014) Decreased expression of GATA2 promoted proliferation, migration and invasion of HepG2 in vitro and correlated with poor prognosis of hepatocellular carcinoma. *PLoS One* 9:e87505.
- Kershaw RM, Siddiqui YH, Roberts D, Jayaraman PS, Gaston K (2014) PRH/HHex inhibits the migration of breast and prostate epithelial cells through direct transcriptional regulation of Endoglin. *Oncogene* 33:5592–5600.
- Schölzel S, et al. (2000) Carcinoembryonic antigen family members CEACAM6 and CEACAM7 are differentially expressed in normal tissues and oppositely deregulated in hyperplastic colorectal polyps and early adenomas. *Am J Pathol* 156:595–605.
- Yeh CM, et al. (2014) Epigenetic silencing of ARNTL, a circadian gene and potential tumor suppressor in ovarian cancer. *Int J Oncol* 45:2101–2107.
- Liu F, et al. (2013) Discovery of an in vivo chemical probe of the lysine methyltransferases G9a and GLP. *J Med Chem* 56:8931–8942.
- Chakrabarti J, et al. (2004) The transcription factor DEC1 (stra13, SHARP2) is associated with the hypoxic response and high tumour grade in human breast cancers. *Br J Cancer* 91:954–958.
- Tachibana M, Matsumura Y, Fukuda M, Kimura H, Shinkai Y (2008) G9a/GLP complexes independently mediate H3K9 and DNA methylation to silence transcription. *EMBO J* 27:2681–2690.
- Lee JS, et al. (2010) Negative regulation of hypoxic responses via induced Reptin methylation. *Mol Cell* 39:71–85.
- Ngiow SF, et al. (2015) A threshold level of intratumor CD8⁺ T-cell PD1 expression dictates therapeutic response to anti-PD1. *Cancer Res* 75:3800–3811.