Hypoxia Drives Breast Tumor Malignancy through a TET–TNFα–p38–MAPK Signaling Axis

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Abstract

Hypoxia is a hallmark of solid tumors that drives malignant progression by altering epigenetic controls. In breast tumors, aberrant DNA methylation is a prevalent epigenetic feature associated with increased risk of metastasis and poor prognosis. However, the mechanism by which hypoxia alters DNA methylation or other epigenetic controls that promote breast malignancy remains poorly understood. We discovered that hypoxia deregulates TET1 and TET3, the enzymes that catalyze conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), thereby leading to breast tumor-initiating cell (BTIC) properties. TET1/3 and 5hmC levels were closely associated with tumor hypoxia, tumor malignancy, and poor prognosis in breast cancer patients. Mechanistic investigations showed that hypoxia leads to genome-wide changes in DNA hydroxymethylation associated with upregulation of TNFα expression and activation of its downstream p38–MAPK effector pathway. Coordinate functions of TET1 and TET3 were also required to activate TNFα–p38–MAPK signaling as a response to hypoxia. Our results reveal how signal transduction through the TET–TNFα–p38–MAPK signaling axis is required for the acquisition of BTIC characteristics and tumorigenicity in vitro and in vivo, with potential implications for how to eradicate BTIC as a therapeutic strategy. Cancer Res; 75(18); 3912–24. ©2015 AACR.

Introduction

Breast cancer is the most common malignancy among women and exhibits a high rate of heterogeneity (1). Although mortality of patients with breast cancer in recent years has significantly decreased, tumor malignancy of breast cancer still leads to a poor prognosis and limits therapeutic options. It was proposed that tumor-initiating cells (TICs), defined by their abilities for tumor-forming and self-renewing, account for tumor malignancy (2). Recently, the population of TICs has been identified in numerous tumors, including those of brain, head and neck, and lung cancer, through various molecular markers, side population, and enzyme activity (3–5). Likewise, breast tumor–initiating cells (BTIC) have been isolated by sorting for CD44high/CD24low cells, high aldehyde dehydrogenase1 (ALDH1) activity, or enriched in anchor-independent conditions (6–8). They were found to share a high degree of similarity of characteristics with stem cells as well as tumorigenic capacity (9). High content of BTICs are enriched with high grade of breast tumor and associated with enhanced invasiveness, chemoresistance, and metastasis (10), supporting the concept that BTICs account for tumor malignancy of breast cancer (11).

Hypoxia, a microenvironment stress existing in various solid tumors, has been recognized as an important factor promoting tumor malignancy (12). Recent reports demonstrating the association of hypoxia with high-grade breast tumor and poor prognosis of patients with breast cancer have suggested an important role for hypoxia in breast cancer (13). Noticeably, hypoxia has been demonstrated to be associated with a stem-like phenotype in breast cancer and provides a breeding ground for BTICs (14). This is consistent with the fact that TICs are supported by their niche where they are regulated by complex interactions with multiple factors derived from the tumor microenvironment (15).

One key mechanism by which hypoxia regulates tumor malignancy is alteration of the cancer epigenome, which provides selective advantages for cancer cells during tumorigenesis (16, 17). Aberrations in DNA methylation and proteins involved in controlling DNA methylation are associated with tumor malignancy and prognosis of patients (18). However, the mechanism by which hypoxia regulates DNA methylation and key enzymes in regulating tumor malignancy remains largely unknown. The
Ten-Eleven Translocation (TET) family of enzymes convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) for the demethylation of mammalian DNA (19). The TET family comprises three members: TET1, TET2, and TET3. The TET1 gene was firstly identified as a fusion partner of MLL in acute myeloid leukemia associated with a chromosome translocation. The expression of TET1 is required for ESC pluripotency and normal differentiation during ESC lineage specification (20). TET2 plays an important role in hematopoiesis. Loss-of-function of TET2 has been reported as one of the most frequent genetic defects in myeloid malignancies (21). In addition, TET3, but not TET1 or TET2, highly expressed in oocytes and zygotites, is essential for epigenetic reprogramming of the zygotic paternal DNA (22). Despite these findings suggesting critical roles that TET family proteins play during biologic processes, it is unclear whether TET proteins act as key factors in regulating hypoxia-enhanced tumor malignancy and tumor-initiating capabilities.

In this study, we reveal an epigenetic mechanism centered on hypoxia-induced activation of the TNFα-p38–MAPK signaling axis leading to breast tumor malignancy, and highlight that TET1/3 and 5hmC might serve as prognostic biomarkers for breast cancer. The inhibitory effect of blocking the TET–TNFα-p38–MAPK signaling pathway on BTIC characteristics and tumorigenicity indicates a potential strategy to improve targeted therapy in breast cancer.

Materials and Methods

Cells, plasmids, stable transfection, and oxygen deprivation

Primary breast cancer cells were obtained from surgical specimens of patients with breast cancer. This study was approved by the Institutional Review Board of the Tri-Service General Hospital (Taipei, Taiwan). The primary cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. MCF7 and MDA-MB-231 cell lines were cultured in the DMEM supplemented with 10% FBS at 37°C in the presence of 5% CO2. A pBabe-HIF-1α P402A/P564A (Addgene) plasmid was used in generating HIF-1α overexpression clones. The plasmids for gene knockdown experiments were generated by construction of an oligonucleotide targeting a specific gene sequence into pSUPER vector (oligoengine), which was used to establish stable transfectants. The target sequences in the experiments are listed in Supplementary Table S2. Oxygen deprivation was conducted in the hypoxic incubator with 1% O2, 5% CO2, and 94% N2 for 24 hours.

Patient selection

This research followed the tenets of the Declaration of Helsinki and was approved by the Cardinal Tien Hospital institutional review board. All samples were obtained from patients after informed consent. Tissue specimens from 183 patients were obliquity as well as tumor-initiating capacities. A retrospective study of patients selected from a clinical database from the archives of the Department of Pathology, Cardinal Tien Hospital between 1998 and 2005. Eighteen patients either without follow-up or insufficient clinicopathologic data for analysis were excluded. Therefore, only 165 cases of human breast carcinoma were included in this study.

Immunohistochemistry

Immunohistochemistry was performed on 165 archived, formalin-fixed, paraffin-embedded (FFPE) blocks of breast invasive ductal carcinoma (IDC). Tissue sections were dewaxed in xylene and rehydrated in alcohol, followed by incubation in 0.01 mol/L citrate buffer pH 6.0 at 95°C for 40 minutes in a water bath. They were then treated with 0.3% H2O2 for 30 minutes to block endogenous peroxidase, and incubated with block nonspecific antibody reactions. All the slides were counterstained with hematoxylin (Merck). Antibodies used in the experiments are listed in Supplementary Table S3.

Immunohistochemical staining evaluation

The histopathologic slides of all the specimens were reviewed concurrently and independently by two expert pathologists using the same type of microscope without prior knowledge of each patient’s clinical details. To evaluate precisely and objectively the intensity of 5hmC, TET1, TET3, and HIF1α staining, we used a Zeiss AxioImager-Z1 microscope to capture images of all sections, and measured the intensity of staining for the four markers using Program Metamorph software. Intensity was classified into four categories: no staining scored 0, fewer staining scored 1, less than average intensity (low intensity) scored 2, and higher than average (high intensity) scored 3. The mean immunoreactivity score of each marker was selected as the cut-off point to separate tumors showing negative (<mean) and positive (>mean) expression. The correlation between expression levels of specific factor and tumor grade was calculated as described previously (23).

Western blot analysis, RNA extraction, quantitative real-time PCR

Western blot was performed following a standard protocol as described previously (16). Briefly, cells were harvested and lysed by RIPA buffer (50 mmol/L Tris, 150 mmol/L NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40). Protein extracts were subjected to SDS-PAGE analysis. The membranes were blocked with 5% nonfat milk followed by antibody hybridization and the signals were visualized on X-ray film. For RNA extraction, total RNAs from cultured cells were extracted using the TRizol reagent (Invitrogen, Life Technologies), and 1 μg of RNA was used for cDNA synthesis. Quantitative real-time PCR was carried out to quantify gene expression level by using the CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). The primers and antibodies used in the experiments are listed in Supplementary Tables S2 and S3, respectively.

Luciferase reporter assays

Cells were seeded onto 6-well plates and transfected with the following: reporter plasmid containing TET1 or TET3 gene promoter with wild-type or mutated hypoxia response element, internal control plasmid encoding β-gal, and expression vector encoding wild-type HIF1α. Transfected cells were exposed to 20% or 1% O2 for 24 hours. Firefly luciferase activities in cell lysates were measured using Luciferase Assay System (Promega) and β-gal activities were used as an internal control for data normalization.

ELISA assay

Cells were seeded on 6-well plates at an equal density and incubated for 24 hours. Then, cells were further incubated for 24 hours with serum-free DMEM in normoxia or hypoxia. The collected supernatants were subjected to ELISA assay following the standard protocol as described in the instruction of the ELISA
Kit (eBioscience). ELISA assays were performed in duplicate three separate times, and the data are expressed as mean ± SD.

**ChIP-qPCR, hMeDIP, and hMeDIP-seq**

Chromatin immunoprecipitation quantitative real-time PCR (ChIP-qPCR) assay was performed following a standard protocol as described previously (16). Briefly, crosslinked cell lysate was sonicated and subjected to immunoprecipitation reaction. The immunoprecipitated DNA was purified through a phenol-chloroform DNA extraction protocol and then subjected to qPCR analysis. The hydroxymethyl-DNA immunoprecipitation (hMeDIP) assay was performed as described previously. Briefly, genomic DNA was prepared with genomic DNA extraction kit (Promega) and sonicated into fragments ranging approximately 500 bp. The fragmented DNA was denatured for 10 minutes at 95°C and immunoprecipitated overnight at 4°C with anti-5hmC antibody (Active Motif) in 500 μl IP buffer (10 mmol/L sodium phosphate, 140 mmol/L NaCl, 0.05% Triton X-100). DNA was eluted from the beads followed by purification with chromatin IP DNA purification kit (Active Motif). For hMeDIP followed by deep sequencing (hMeDIP-seq), genomic DNA was purified and sonicated. Illumina barcode adapters were ligated before hMeDIP. Adaptor-ligated DNA was denatured, followed by incubation with 5-hmC antibody (Active Motif). The immunoprecipitated DNA was purified and sequenced followed by standard Illumina protocols. hMeDIP reads were mapped to the human genome (hg19, GRCh37) using bowtie2 (24). Only reads that map uniquely to the genome were used for downstream analysis (MAPQ > 10). HOMER was used to generate normalized bedGraphs for the UCSC Genome Browser (25). Both hMeDIP-seq and transcription start site (TSS)-Seq read depths at promoter regions were calculated using HOMER, and significant differentially regulated promoters were determined using EdgeR (26). Hi-C analysis and calculation of PC1 values were carried out by HOMER as previously described (27). Gene ontology term analysis was performed by database for annotation, visualization, and integrated discovery (DAVID) programs.

**Orthotopic transplantation assays**

Animal studies were approved by the Institutional Review Board of the Salk Institute for Biological Studies (La Jolla, CA). Cells were trypsinized and resuspended in PBS/Matrigel (1:1; BD Biosciences). Suspended cells were then transplanted into the mammary fat pad of a young (3–4 weeks) nude female mouse. Before injection of suspended cells, the abdominal skin of mice was opened by a Y-shaped incision. The incision was made around the fourth nipple. After incision, the skin was folded back to expose the mammary fat pad where suspended cells were injected. Tumor incidence was monitored by using an in vivo imaging system. After 10 weeks, all of the tumors were collected and animals were sacrificed.

**Statistical analysis**

The χ2 tests (with Fisher exact test when expected value <5 in >20% table cells) were used to measure the significance between nominalized 5hmC, TET1, TET3, and HIF1α intensity of immunoreactivity and distribution percentages as well as clinicopathologic characteristics. The associations between 5hmC, TET1, TET3, and HIF1α scores and clinicopathologic multivariables were calculated by the Kruskal–Wallis H test with Bonferroni multiple comparison procedure. The Kaplan–Meier method was used to obtain survival curves. SPSS software v. 15.0 (SPSS UK Ltd) was used for the analysis. All data were reported as mean ± SD. Statistical analysis was performed by using the Student t test, and P < 0.05 was considered statistically significant.

**Accession number**

Sequencing data have been deposited to the Gene Expression Omnibus (GEO) under accession number GSE60434.

**Results**

**Hypoxia induces tumor-initiating characteristics in breast cancer cells**

Recent findings suggest that hypoxia results in the acquisition of stem cell–like tumor-initiating phenotypes, which are associated with tumor malignancy (14, 28). Thus, we first investigated whether similar phenotypes could be recapitulated by hypoxia in breast cancer. We found that hypoxia resulted in the acquisition of putative breast tumor-initiating cell phenotypes reported previously (7, 8, 29), including the appearance of a CD44+/CD24low population, an increased ALDEFLUOR-positive (ALDH+;) population, and the upregulation of genes related to stemness in primary cultures from patients with breast cancer as well as breast cancer cell lines, MCF7 and MDA-MB-231 cells (Fig. 1A–F). Likewise, increased mammosphere forming capacity, a phenotypic characterization of BTICs, was observed in hypoxic primary cells and breast cancer cell lines (Fig. 1G). As expected, stable overexpression of a constitutively active form of HIF1α consistently recapitulated these phenotypes in breast cancer cell lines (Supplementary Fig. S1). As putative BTICs have been found to reside within a CD44high/CD24low population (7), we thus sorted CD44+/CD24− subpopulations from cells in which HIF1α was stably overexpressed (Supplementary Fig. S2A). Upon characterization of the sorted cells (hereafter referred to as isolated BTICs), we found that these cells expressed canonical BTIC phenotypes (Supplementary Fig. S1D and S2B–S2D, see also in Fig. 4H). Next, we examined whether these isolated BTICs undergo the epithelial–mesenchymal transition (EMT) process, given that it has been identified as a feature of BTICs (30). Western blot analysis demonstrated that the EMT process was induced in isolated BTICs, as shown by downregulation of an epithelial marker (E-cadherin) and upregulation of mesenchymal markers (N-cadherin and vimentin) as well as an EMT regulator (Twist; Supplementary Fig. S2E). Furthermore, Seahorse analysis and MIT assay demonstrated a more glycolytic state as well as chemotherapy resistance to cisplatin treatment in isolated BTICs (Supplementary Fig. S2F and S2G; refs. 31, 32). Altogether, these results provide a proof-of-concept that typical features of BTICs could be recapitulated upon hypoxia and HIF1α overexpression.

**Direct regulation of TET by hypoxia is critical for regulation of BTICs**

Next, we investigated whether certain epigenetic regulators might be required for the hypoxia effect contributing to acquisition of BTIC properties. We found that hypoxia treatment significantly increased the expression of TET1 and TET3 in primary cultures as well as breast cancer cell lines (Supplementary Fig. S3A–S3D), whereas no significant changes in TET2 expression...
were observed upon hypoxia. In support of these observations, overexpression of HIF1α consistently increased the expression of both TET1 and TET3 in cancer cell lines (Supplementary Fig. S3E and S3F). To investigate the molecular mechanisms by which hypoxia regulates the expression of TET1 and TET3, we analyzed TET1/TET3 promoter sequences and identified putative HIF1α–binding sites (33) (Fig. 2A). Luciferase reporter assays further showed increases in the promoter activity of TET1 and TET3 as a response to hypoxia as well as upon overexpression of HIF1α. Directed mutagenesis of the putative HIF1α–binding site in TET1/TET3 promoters abolished luciferase activity under hypoxic conditions as well as upon ectopic expression of HIF1α (Fig. 2B). qChIP analysis confirmed that HIF1α bound to both TET1 and TET3 gene promoters containing HRE in hypoxic cells, whereas no significant HIF1α bindings on a distal promoter without HRE were observed (Fig. 2C). Increased binding of HIF1α to the promoter regions of TET1 and TET3 genes was readily observed in breast cancer cell lines, MCF7 or MDA-MB-231, upon overexpression of HIF1α (Supplementary Fig. S4A and S4B). Furthermore, knockdown of HIF1α reduced the hypoxia effect on activation of both TET1 and TET3 genes in breast cancer cell lines (data not shown). Taken together, these results indicate that HIF1α directly regulates the transcription of TET1 and TET3 as a response to hypoxia.

The significant increase of TET1 and TET3 expression in isolated BTICs suggested a role for TET proteins in hypoxia-induced...
BTIC properties (Supplementary Fig. S3G and S3H). For further investigation, we performed shRNA-mediated gene knockdown experiments for TET1 and TET3. Downregulation of either TET1 or TET3 reduced the upregulation of stem cell markers as a result of hypoxia (Supplementary Fig. S5A and S5B), whereas silence of TET1 or TET3 did not affect HIF1 activity, as shown by HIF1α binding on the VEGF promoter in hypoxic cells (Supplementary Fig. S5C and S5D). Likewise, knockdown of TET in isolated BTICs reduced BTIC properties, including CD44+/CD24–/CD133+ and ALDH+ populations, as well as the EMT process (Fig. 3A–C). Mammosphere-forming assays further showed compromised self-renewal in isolated BTICs when TET genes were silenced (Fig. 3E). Moreover, cosilencing of TET1 and TET3 sensitized isolated BTICs to paclitaxel, a widely used chemotherapy drug for the treatment of breast cancer (Fig. 3F). To confirm the role of TET proteins in vivo, we performed subcutaneous and orthotopic tumor formation assays. The subcutaneous xenograft study showed that HIF1α-overexpressing cells and isolated BTICs led to the formation of larger tumors as compared with control cells. Inversely, knockdown of TET1 and TET3 expression in isolated BTICs reduced tumor growth (Fig. 3G and Supplementary Fig. S6A and S6B). To further determine the role of TET1/3 in tumor-initiating capability, we performed limited dilution experiments. As expected, the results indicated a more malignant phenotype for HIF1α-overexpressing cells as well as isolated BTICs. Downregulation of TET1 and TET3 resulted in decreased tumorigenicity in isolated BTICs (Fig. 3H and Supplementary Fig. S6C). Serial transplantation, a gold standard for defining cancer stem cell characteristics in vivo (34), demonstrated that isolated BTICs led to secondary tumor formation. Inversely, knockdown of TET1/TET3 impaired secondary tumor formation (Fig. 3I and Supplementary Fig. S6D). Together, these results demonstrate that TET proteins play a critical role in the acquisition of BTIC characteristics upon hypoxia.
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TET1, TET3, and 5hmC levels are correlated with tumor hypoxia, tumor malignancy, and poor prognosis of patients

To evaluate the clinical significance of the aforementioned results, we analyzed the levels of three members of the TET family in 165 cases of patients with breast tumors. As expected, we found that the levels of 5hmC, TET1, and TET3 were significantly associated with tumor hypoxia (Fig. 4A and Supplementary Fig. S7; Supplementary Table S1), as indicated by staining for HIF1α, whereas no significant correlation with hypoxia for TET2 expression was observed (data not shown). The fact that hypoxia is tightly associated with high-grade breast tumor and poor prognosis of patients with breast cancer prompted us to investigate whether TET1, TET3, and 5hmC levels correlate with malignant phenotypes of breast tumor. Our results indicated that TET1 and TET3 were highly expressed in both high-grade (comedo-type) ductal carcinoma in situ (DCIS; P < 0.001 and P < 0.001, respectively, Fig. 4A and B) and grade 3 IDC samples (P < 0.05 and P < 0.05, respectively, Fig. 4A and B). In line with TET1/3 expression patterns, high levels of 5hmC were observed in high-grade DCIS and IDC (P < 0.001 and P < 0.001, respectively, Fig. 4A and B). Histologic analysis demonstrated the presence of TET1 and TET3 as well as 5hmC in the invasive front of breast...
tumors, an infiltrative phenotype correlated with tumor malignancy (Fig. 4C; ref. 35). Furthermore, the levels of TET1, TET3, and 5hmC all showed a significant association with poor patient overall survival (OS) and disease-free survival (DFS; Fig. 4D and E). Collectively, these results indicate that TET1, TET3, and 5hmC levels correlate with tumor hypoxia, tumor aggressiveness, and poor prognosis of patients with breast cancer.

Genome-wide changes in DNA hydroxymethylation resulting from hypoxia are associated with activation of TNFα–p38–MAPK signaling

TET1 and TET3 proteins possess methylcytosine hydroxylase activity to mediate the conversion of 5mC to 5hmC (19). Accordingly, we next asked whether 5hmC levels contribute to the hypoxia-induced aggressiveness of breast cancer. Dot blot analysis indicated a global increase in the levels of 5hmC (Fig. 5A). Immuno precipitation with antibodies against 5hmC (hMeDIP) followed by deep sequencing demonstrated increased 5hmC levels over broad chromatin domains, most notably in gene-rich regions (Fig. 5B). These domains bore striking similarity to chromatin found in the "active" compartment of the genome as determined by Hi-C, a method that uses spatial proximity ligation to measure the conformation of the genome (27). Principal Component Analysis of published Hi-C data (36) compared to our own ChIP data suggested a correlation between 5hmC and the spatial segregation of open and closed chromatin (Fig. 5B and C), with large increases in 5hmC levels found exclusively in the active compartment (positive PC1 values). Together, these results indicate that hypoxia results in genome-wide changes in DNA hydroxymethylation.

We next identified 1,794 gene promoters, in which hypoxia led to an increase in 5hmC levels (≥1.5 fold). In agreement with a
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Figure 5.
Genome-wide changes of 5hmC in response to hypoxia. A, global level of 5hmC in MCF7 cells treated with hypoxia by dot blot assay. Methylene blue staining was used as a total genomic DNA loading control. B, representative data showed the correlation of transcriptionally active (+) and inactive (−) compartment assignment based on PCA (PC1 values) and 5hmC change in a region of chromosome 1. C, genome-wide comparison of Hi-C PC1 values with 5hmC change induced by hypoxia. D, gene ontology results for the comparison of 5hmC change in hypoxia vs. normoxia. E, RNA-seq results show top ranked functional gene clusters and H) as well as in HIF1α-overexpressing cells and isolated BTICs (Supplementary Fig. S8C). However, there were no significant changes in 5hmC levels as well as RNA polII binding at the TSS in genomic regions of the TNFα gene consistently showed an increased 5hmC a role for hypoxia in regulating gene-specific 5hmC changes (Supplementary Fig. S9A). In addition, in line with aforementioned results, hypoxia resulted in the upregulation of TNFα in both primary cultures and cancer cell lines (Fig. 6A–D), whereas TNFα gene expression was not changed in serum-starved cells (Supplementary Fig. S9B). Increased levels of TNFα were observed in HIF1α-overexpressing cells and isolated BTICs (Fig. 6E–H). Together, these results indicate that gene-specific changes of 5hmC were associated with the expression of TNFα gene. As TNFα activation has been described to induce p38–MAPK signaling, an important regulator in cancer progression (38, 39), we next examined the role of hypoxia in this context. Western blot analysis demonstrated increased phosphorylation of...
MCF7 or MDA-MB-231 cells. Data, means ± SD (n = 3). *P < 0.05, compared with controls.

Figure 6. Hypoxia induces TNFα–p38–MAPK signaling in breast cancer cells. A, qRT-PCR analysis for TNFα gene expression in hypoxic primary breast cancer cells. B, Western blot analysis for protein level of TNFα and phospho-p38–MAPK in primary cells treated with hypoxia. C and D, hypoxia increased expression of TNFα gene in mRNA level and protein level as well as the level of phospho-p38–MAPK in either MCF7 or MDA-MB-231 cells. E and F, activation of TNFα–p38–MAPK signaling in either MCF7 or MDA-MB-231 under hypoxia–overexpressing cells. qRT-PCR analysis (E) for TNFα gene expression and Western blot analysis (F) for protein level of TNFα and phospho-p38–MAPK. G and H, the qRT-PCR analysis and Western blot analysis showing TNFα–p38 MAPK signaling was activated in isolated BTICs. L, ELISA analysis showed an elevated level of secreted TNFα protein in hypoxic primary cells. J and K, the level of secreted TNFα protein was increased in hypoxic MCF7 or MDA-MB-231 cells as well as in their corresponding isolated BTICs. N, normoxia; H, hypoxia. Sorted cells represented isolated BTICs from MCF7 or MDA-MB-231 cells. Data, means ± SD (n = 3). *P < 0.05, compared with controls.

TET proteins are essential for hypoxia-induced TNFα–p38–MAPK signaling

To further investigate whether TET proteins mechanistically mediate the activation of TNFα–p38–MAPK signaling in response to hypoxia, we knocked down either TET1 or TET3 expression in breast cancer cell lines. As expected, TNFα expression and phosphorylation of p38–MAPK were suppressed upon knockdown of either TET1 or TET3 in hypoxic condition (Supplementary Fig. S10A–S10D). Likewise, silencing TET1 or TET3 repressed the TNFα–p38–MAPK signaling cascade in isolated BTICs (Supplementary Fig. S10E–S10H). hMeDIP analysis further indicated an attenuation in the levels of 5hmC in the promoter region of TNFα upon TET knockdown as a response to hypoxia (Supplementary Fig. S11A and S11B). Consistently, silence of TET reduced 5hmC levels in HIF1α–overexpressing cells and isolated BTICs (Supplementary Fig. S11C–S11F). To more stringently assess the role of TET proteins in hypoxia-induced TNFα–p38–MAPK signaling, the rescue experiment was performed using either wild-type or inactive mutant forms of TET1 and TET3. Re-expressing wild-type of TET1, but not inactive TET1 mutant, was capable of restoring the hypoxia effect on induction of 5hmC in TET1 knockout cells (Supplementary Fig. S12A and S12B). Likewise, the induction of 5hmC by hypoxia was restored by re-expression of the wild-type of TET3, but not the inactive TET3 mutant, in TET3-deficient cells (Supplementary Fig. S12C and S12D). Furthermore, as shown by Western blot analysis, re-expression of either...
wild-type of TET1 or TET3 was capable of restoring the activation of TNFα-p38–MAPK signaling, whereas inactive TET mutants were unable to rescue this phenomenon (Supplementary Fig. S13A–S13D). These results indicate an essential role for TET1 and TET3 in hypoxia-induced TNFα–p38–MAPK signaling.

TET1 and TET3 coordinate to mediate the activation of TNFα–p38–MAPK signaling in response to hypoxia

Given that single knockdown of either TET1 or TET3 resulted in similar effects, we next asked whether they could function coordinately in response to hypoxia. TET1 co-immunoprecipitation (co-IP) experiments demonstrated the physical interaction of TET1 and TET3 proteins in hypoxic samples (Supplementary Fig. S14A and S14B). Inverse co-IP experiments, using an anti-TET3 antibody, further confirmed an interaction between TET3 and TET1 (Supplementary Fig. S14C and S14D). Likewise, physical interactions between TET1 and TET3 were also observed in isolated BTICs (Supplementary Fig. S14E and S14F). To examine whether TET1–TET3 complexes occupy the TNFα gene promoter, we performed serial ChiP assays. Indeed, co-occupancy of TET1 and TET3 was observed in breast cancer cell lines as a response to hypoxia (Supplementary Fig. S15A–S15D). Decreased binding of the protein complex to the TNFα promoter was observed upon individual knockdown of either TET1 or TET3 in HIF1α-overexpressing cells and isolated BTICs (Supplementary Fig. S15E–S15L). Collectively, these results indicate that TET1 and TET3 function cooperatively to regulate TNFα–p38–MAPK signaling upon hypoxia.

TNFα–p38–MAPK signaling is essential in maintaining BTIC properties

The TNFα signaling pathway contributes to the acquisition of BTIC properties (33, 40). Likewise, activation of p38–MAPK has been shown to correlate with increased tumor malignancy and poor prognosis of cancer patients (41–43). Therefore, we next attempted to block hypoxia-elicited responses by disrupting this signaling pathway. We found that BTIC phenotypes were suppressed upon treatment with TNFα-neutralizing antibodies (Fig. 7A–C). Similarly, chemical inhibition of p38–MAPK led to compromised BTIC phenotypes in isolated BTICs (Supplementary Fig. S16A–S16C) and resulted in increased cell death upon paclitaxel treatment (Supplementary Fig. S16D). To further confirm these results, we performed TNFα knockdown experiments in isolated BTICs (Fig. 7D). Knockdown of TNFα resulted in reduced phosphorylation of p38–MAPK (Fig. 7G). BTIC properties, including CD44+/CD24− populations, ALDH+ population, EMT process, as well as mammosphere-forming ability, were decreased when TNFα was silenced (Fig. 7E–H). Moreover, downregulation of TNFα sensitized isolated BTICs to paclitaxel treatment (Fig. 7I). Noticeably, xenograft studies showed that TNFα knockdown hampered tumor-initiating capabilities in vivo, suggesting a critical role for TNFα in hypoxia-enhanced tumorigenicity (Fig. 7J). Next, we asked whether inhibition of p38 MAPK signaling might be sufficient for attenuating tumor forming potential in vivo. To this end, cells were injected into mice and then animals were treated with p38–MAPK inhibitor for 3 weeks. Our results demonstrated decreased tumor formation ability, but not decreased tumor growth, upon inhibition of p38–MAPK in both subcutaneous and orthotopic tumor formation assays (Supplementary Fig. S16E and S16F and data not shown). To ensure the hierarchy with respect to the TNFα–p38–MAPK cascade in isolated BTICs, we examined the expression of their downstream targets in this context (44). We found that the expression of selected targets, including IL1β, IL11, MMP3, MMP9, IGFBP2, PTGS2, and TGM2, was upregulated in isolated BTICs. Treatment of TNFα-neutralizing antibodies and p38–MAPK inhibitor reduced the expression level for all of these genes (Supplementary Fig. S17A and S17B). In addition, these genes were regulated by hypoxia, whereas knocking down either TET1 or TET3 abolished this effect (Supplementary Fig. S17C). These data collectively suggest that the TNFα–p38–MAPK signaling cascade, activated by TET proteins in response to hypoxia, plays a vital role in maintaining BTIC capabilities.

Discussion

In the current study, we demonstrated a novel pathway by which hypoxia promotes tumor malignancy through TET-mediated activation of TNFα–p38–MAPK signaling. Hypoxia was found to regulate TET1 and TET3 expression through HIF1α, which led to global changes in DNA hydroxymethylation, associated with tumor malignancy. Upon hypoxia, TET proteins functioned coordinately to activate the TNFα–p38–MAPK signaling pathway, resulting in BTIC properties. Recent findings suggest that hypoxia contributes to the formation of a TICs niche within the tumor where hypoxia influences the behavior of TICs as well as their tumorigenic capacity (45). Consistent with this, as a proof-of-principle, our results demonstrated that hypoxia recapitulated such phenotypes in breast cancer, suggesting hypoxia as a critical factor driving tumor malignancy. Epigenetic alterations occurring in breast cancer cells have been suggested to render heterogeneity to breast tumors, resulting in BTIC phenotypes and tumor malignancy (46). Together with our findings, it indicates that the function of TET proteins is required for hypoxia-induced BTICs, therefore providing a direct link with tumor malignancy for hypoxia-induced deregulation of DNA methylation. Despite this, the role of TET proteins in cancer progression has been debated. Our findings presented here are linked with an oncogenic role for TET proteins; this is consistent with previous reports demonstrating that TET proteins are associated with prognostic epigenetic signature and the regulation for a set of important oncogenic targets (47, 48). Reduced 5hmC level was found in some types of cancer as compared with corresponding normal tissue (49); however, the correlation of 5hmC level as well as the related enzyme–TET proteins with tumor grading in breast cancer has not been thoroughly dissected. Our findings extensively demonstrated that 5hmC as well as the related enzyme–TET proteins with tumor grading in breast cancer has not been thoroughly dissected. Our findings extensively demonstrated that 5hmC as well as the related enzyme–TET proteins with tumor grading in breast cancer has not been thoroughly dissected. Our findings extensively demonstrated that 5hmC as well as the related enzyme–TET proteins with tumor grading in breast cancer has not been thoroughly dissected. Our findings extensively demonstrated that 5hmC as well as the related enzyme–TET proteins with tumor grading in breast cancer has not been thoroughly dissected. Our findings extensively demonstrated that 5hmC as well as the related enzyme–TET proteins with tumor grading in breast cancer has not been thoroughly dissected.
Remodeling DNA methylation profile by hypoxia has been demonstrated in certain cell types, including normal and cancer cells (51, 52). Our results showed that hypoxia led to global changes in 5hmC level through direct regulation of TET1 and TET3, suggesting a novel mechanism by which hypoxia contributes to dynamic change of DNA methylation profile during cancer progression. Increased DNA hydroxymethylation was evident at various pluripotent loci such as Oct4 and Nanog during pluripotent reprogramming (22). We readily observed the occupancy for TET proteins at pluripotent loci upon hypoxia (Supplementary Figs. S18A, S18B, S19A, and S19B), whereas the changes of DNA hydroxymethylation were not significantly correlated with this phenomenon (Supplementary Figs. S18C, S18D, S19C, and S19D), suggesting an intricate mechanism for hypoxia in regulating pluripotency genes. Moreover, our results showed a coordinate function of TET proteins, suggesting the importance of interacting partners in the regulation of the biologic function of TET proteins. As hypoxia targets different histone modifiers that may orchestrate with DNA methylation, contributing to tumor progression (16), it will be interesting to investigate the synergistic function of TET proteins with different epigenetic regulators in regulating the cancer epigenome and tumor malignancy.

Previous studies showed that TNFα stimulates many signaling pathways, resulting in the activation of multiple signaling cascades that contribute to cancer progression as well as BTICs characteristics (33, 40). Likewise, p38–MAPK signaling, a downstream target of TNFα, has been suggested as an important regulator in cancer progression and tumor initiation (41, 53). Our findings unveil, for the first time, a direct mechanistic link between hypoxia–TET proteins and the regulation of the cancer epigenome for the activation of the TNFα–p38–MAPK pathway in regulating BTICs. The results demonstrated that certain downstream targets of TNFα–p38–MAPK signaling known to contribute to cancer stemness, including IL1β, MMP3, MMP9, IGFBP2, and PTGS2, were enriched in BTICs and regulated by hypoxia.
TET–TNFα–p38–MAPK signaling axis have suggested a potential mechanism for this signaling in regulating stemness of breast cancer ( Supplementary Fig. S17 ). However, the precise mechanism needs to be delineated in future studies.

Finally, recent reports describing the influence of hypoxia on regulation of BTICs have suggested tumor hypoxia as an attractive target for therapeutic intervention (12). Acquisition of epigenetic alterations gives tumor cells the ability to expand and adapt to the changing microenvironment and clinical treatment, suggesting a therapeutic strategy targeting epigenetic mechanisms and related downstream signaling pathways. Herein, our results suggest a novel mechanism of epigenetic regulation in cancer cells as a response to hypoxia and additionally highlight TET1 and TET3 as novel prognostic biomarkers for breast cancer. Most importantly, targeting the hypoxia–TET–TNFα–p38–MAPK signaling axis indicates a promising inhibitory effect on tumor malignancy. Thus, our results shed new light on the role of hypoxia in regulating the cancer epigenome leading to breast cancer malignancy, providing novel strategies for the future development of therapeutic strategies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M.-Z. Wu, S.-F. Chen, I. Sancho-Martinez, J.C. Izpisua Belmonte
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