Generation of an epigenetic signature by chronic hypoxia in prostate cells

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Increasing levels of tissue hypoxia have been reported as a natural feature of the aging prostate gland and may be a risk factor for the development of prostate cancer. In this study, we have used PwR-1E benign prostate epithelial cells and an equivalently aged hypoxia-adapted PwR-1E sub-line to identify phenotypic and epigenetic consequences of chronic hypoxia in prostate cells. We have identified a significantly altered cellular phenotype in response to chronic hypoxia as characterized by increased receptor-mediated apoptotic resistance, the induction of cellular senescence, increased invasion and the increased secretion of IL-1β, IL6, IL8 and TNFα cytokines. In association with these phenotypic changes and the absence of HIF-1α protein expression, we have demonstrated significant increases in global levels of DNA methylation and H3K9 histone acetylation in these cells, concomitant with the increased expression of DNA methyltransferase DMNT3b and gene-specific changes in DNA methylation at key imprinting loci. In conclusion, we have demonstrated a genome-wide adjustment of DNA methylation and histone acetylation under chronic hypoxic conditions in the prostate. These epigenetic signatures may represent an additional mechanism to promote and maintain a hypoxic-adapted cellular phenotype with a potential role in tumour development.

INTRODUCTION

Environmental factors are known to alter the fundamental epigenetic programming of the human genome. For example, an increasing number of epigenetic disorders have been described with artificial reproduction technology, where in vitro culturing conditions influence the establishment of global epigenetic signatures (1). Similarly, tissue hypoxia that is known to occur as a natural feature of our aging organs (2,3), or indeed hypoxia within the context of the tumour microenvironment, could equally have an effect on such global epigenetic programming. The development of prostate cancer is related to increased age with an ~35% increase in the instance of prostate cancer foci in 50- to 59-year-old men compared with men aged between 20 and 29 years (4). One of the main environmental changes within the aging prostate is exposure to increasing levels of tissue hypoxia. Two lines of evidence supporting the concept that hypoxia is a natural feature of the aging prostate are: (i) declining blood flow to the prostate through an aging cardiovascular system, or accumulated damage to the vascular region of the prostatic transitional zone (5,6), and (ii) age-related prostatic tissue remodelling resulting in a local increase in the consumption of oxygen (7). Such alterations in the tissue microenvironment may impact normal prostate cells by inducing the expression of many pro-survival proteins that may promote cancer development. Indeed, recent studies have shown significant hypoxia-induced disruption to the global transcriptome resulting in the differential expression of many transcription factors and their targets. These genome-wide effects have been highlighted both in vitro and in vivo in urological tumours (8). Such hypoxic changes may be due to alterations in the epigenetic profiles.

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Epigenetic profiles both at a global and gene-specific level are disrupted in human cancers, resulting in genomic instability, the aberrant expression of oncogenes and transcriptional silencing of tumour suppressors (9, 10). Chromatin modifying complexes are likely candidates in the regulation of epigenetic modifications and chromatin structure as they are involved in the covalent modification of chromatin components. Such modifications are controlled by a delicate equilibrium of chromatin modifying enzymes depositing methyl groups directly onto the DNA or alternatively may occur in conjunction with modifications of the N-terminal tail regions of histone proteins, such as histone acetylation, altering the accessibility and targeting of transcription factors to gene promoter sites in order to regulate gene expression (11). Unlike structural genomic changes (mutations, LOH), aberrant epigenetic modifications can be restored to normal by targeting the epigenetic machinery using agents such as DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors, some of which are being used clinically to treat haematological malignancies, with others under clinical trial (12, 13). In prostate cancer specifically, epigenetic modifications play a role in its development both globally and in the regulation of specific genes involved in early stage disease (14).

In this study, we have used the PwR-1E benign prostate epithelial cell line and an equivalently aged hypoxic-conditioned PwR-1E sub-line mimicking in vitro hypoxic changes which may occur in normal prostate epithelia. Using this model, we have identified large-scale phenotypic changes in apoptotic sensitivity, invasion potential and cellular senescence. In addition to these phenotypic changes, we have also found significant alterations in both global and gene-specific epigenetic modifications.

RESULTS

Chronic hypoxic conditioning impacts apoptotic potential, levels of cellular senescence, migration and cytokine production in PwR-1E cells

Low density microarrays have previously demonstrated significant alterations in the proliferation and apoptotic transcriptional phenotype of the chronic hypoxic cultured PwR-1E sub-lines (manuscript in preparation). Our results have shown that chronic exposure of this benign, androgen-dependent prostatic cell line to low O2 alters its sensitivity to receptor-mediated apoptosis. PwR-1E normoxic and chronic hypoxic sub-lines were treated with 10 ng/ml recombinant soluble Fas ligand (FasL) or a combination of 25 ng/ml tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and 1 μg/ml cycloheximide (CHX). Assessment of apoptosis by flow cytometry showed that in response to both FasL and TRAIL/CHX, there was a significant reduction in the percentage of the chronic hypoxic cell population undergoing apoptosis compared with the equivalently treated normoxic counterparts (Fig. 1A). Hoechst staining of the hypoxic cell populations also identified a decrease in the membrane fragmentation associated with the progression of apoptosis compared with the normoxic cells (Fig. 1B).

To investigate the impact of chronic hypoxic conditioning on cellular senescence, both sub-lines were assessed for β-galactosidase activity. Cells stained positively for β-galactosidase (blue stain) were counted and calculated as a percentage of the total number of cells (Fig. 2A). Increased levels of cellular senescence were identified in the hypoxic population of PwR-1E cells compared with their normoxic counterparts (P < 0.0001). Cellular migration was then assessed on these populations by allowing PwR-1E normoxic and hypoxic cell populations to migrate through an 8 mm pore for 24 h and by measuring the proportion of migrated cells within each population. As we detected a 48% reduction in growth in the chronic hypoxic line compared with the normoxic cells, the migration data were normalized to the level of growth in both sub-lines. Hypoxic PwR-1E cells displayed a significant two-fold increase in cell migration compared with equivalently aged normoxic cells (P = 0.01) (Fig. 2B). In addition to changes in migration, the protein levels of key cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour...
necrosis factor (TNFα) were determined in the media of the sub-lines using a multiplex ELISA system. Interestingly, the levels of all four cytokines were significantly increased in response to chronic hypoxia (P = 0.001) (Fig. 2C).

PwR-1E cells were assessed by western blotting analysis for the presence of HIF-1α (Fig. 2D). Normoxic cells were exposed to 1% O2 for 1, 3, 6, 12 and 24 h and were assessed alongside the chronic hypoxia sub-line. As expected, HIF-1α expression was found to increase in a time-dependent manner under hypoxic conditions, until 6 h of exposure, with lower levels being evident following 12 and 24 h of hypoxia. Interestingly, HIF-1α expression was absent in PwR-1E cells grown and maintained in chronic hypoxia, suggesting the need for another mechanism in order to maintain the hypoxia-adapted phenotype.

Global DNA hypermethylation and H3K9 hyperacetylation induced by chronic hypoxia

In order to assess the impact of prolonged hypoxic exposure on global epigenetic profiles of H3K9 acetylation and DNA methylation, PwR-1E normoxic and hypoxic cells were stained with monoclonal antibodies specific for 5’Methyleytidine (5’MeC) and the lysine 9 acetylated N-terminal tail of histone H3 (AcH3K9) and assessed by
Flow cytometry and immunofluorescence (Fig. 3A and B). Flow cytometric analysis was performed in order to quantify levels of DNA methylation and H3K9 acetylation and mean channel fluorescence (MCF) values were calculated within each cell population following the subtraction of IgG control MCF values. Our results showed significantly increased levels of both DNA methylation ($P < 0.001$) and H3K9 acetylation ($P < 0.0001$) in the chronic hypoxic sub-line compared with the similarly aged normoxic cells. The concurrent analysis of total histone 3 levels in hypoxic PwR-1E cells showed no change, proving that the observed changes in the levels of these two epigenetic markers are not a reflection of increased nuclear size (Fig. 3A).

Confirmatory immunofluorescent staining and fluorescence microscopy was also performed (Fig. 3B). Increased levels of DNA methylation were clearly observed across the hypoxic cell population, whereas AcH3K9 staining showed more varied levels across the cell population. The presence

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**Figure 3.** Chronic hypoxic conditions induce significant alterations in global epigenetic signatures of histone acetylation and DNA methylation. (A) Normoxic and chronic hypoxic PwR-1E cells were stained with anti-acetylated histone 3 lysine 9 (AcH3K9), anti-5'-Methylcytidine (5'MeC) or anti-histone 3, subsequently incubated with an FITC-tagged secondary antibody and assessed quantitatively using flow cytometry. Appropriate isotype control samples were also applied. The data represent the mean channel fluorescence ± standard deviation. PwR-1E cells grown under hypoxic conditions exhibited a significant global H3K9 hyper-acetylation ($**P < 0.0001$) and DNA hypermethylation ($**P < 0.001$) when compared with equivalently treated normoxic counterpart. Total histone 3 levels were not significantly altered. (B) Immunofluorescent ×40 magnification images of PwR-1E cells stained with anti-5'MeC, anti-acetylated H3K9 and IgG1 isotype controls with a FITC-tagged secondary antibody and DAPI counterstain. Visualization confirms global increases in 5’-methylcytidine and H3K9 acetylation in hypoxic cell populations. An increase in nuclear size was also evident in hypoxic PwR-1E cells. (C) Flow cytometric analysis of DNA methylation in response to 72 h treatment with 1 μM 5-aza-2’-deoxycytidine (5-aza-dC). Data represent the mean channel fluorescence ± standard deviation. This treatment caused a reversal of global DNA methylation levels in hypoxic populations to equal normoxic levels.
of large nuclei in the hypoxic PwR-1E cells is also of interest, as this may be indicative of chromatin decondensation in the nucleus which has been previously demonstrated in regions of histone hyperacetylation.

In order to determine if the observed hypoxia-induced global hypermethylation is reversible both normoxic and hypoxic sub-lines were treated with 1 \( \mu \text{M} \) 5-aza-2'-deoxycytidine (5-aza-dC) and incubated in the respective oxygen environments for 72 h. Flow cytometry was subsequently carried out to measure the impact of this treatment on global levels of 5\(^{\text{MeC}}\). Our results showed that treatment with 5-aza-dC caused an overall reduction in DNA methylation in both normoxic and hypoxic cell populations. Furthermore, in the hypoxic sub-line where 5\(^{\text{MeC}}\) was found to be naturally elevated, 5-aza-dC treatment caused methylation levels to return to levels resembling those of the normoxic counterpart prior to treatment, suggesting a full recovery from hypermethylation (Fig. 3C).

**Histone acetyltransferase and DNMT activity**

To investigate the underlying mechanisms that may account for global epigenetic alterations in histone acetylation and DNA methylation, the activity of epigenetic modifying enzymes were assessed using ELISA-like assays. Here we have shown no significant increase in the activity of histone acetyltransferase (HAT) or DNMT enzymes in response to chronic hypoxia (Fig. 4A). To further assess the impact of hypoxia on the components required for DNA methylation, an assay was performed which allowed the measurement of the methyl-donor S-adenosylmethionine (SAM). Results showed a significant decrease in levels of SAM from ~180 \( \mu \text{M} \) in normoxic cells to 80 \( \mu \text{M} \) SAM present in the hypoxic sub-line. This may suggest an increase in SAM usage in the hypoxic cells which corresponds with DNA hypermethylation (Fig. 4B). In order to determine the levels of DNMT1, DNMT3a and DNMT3b, RT–PCR analysis was performed. Interestingly, a 1.6-fold increase in the expression of DNMT3b was observed in the hypoxic PwR-1E sub-line suggesting that de novo DNMT activity by DNMT3b is enhanced in response to chronic hypoxia and may play a role in establishing global hypermethylation. No significant change was observed in the expression of DNMT1 or DNMT3a (Fig. 4C).

**Karyotyping**

Karyotypic analysis was undertaken on both PwR-1E normoxic and hypoxic cells. The karyotyping proved that both were derived from the same original cell line and that there was still a close karyotypic relationship between the normoxic and hypoxic lines. In both lines, two distinct clones could be defined, the major line in both showed high-hyperdiploidy with a chromosome number range of 54–58 chromosomes.
(normoxic) and 56–64 (hypoxic). A minor hyperdiploid line was also observed in both, with a chromosome number of 50–52 (normoxic) and 47–51 (hypoxic), although no clone was grossly abnormal. The only aberrations that were present in all four clones identified were gains of chromosomes 14 and 20 and gain of a der(15)t(8;15)(q13;q14).

Overall, the hypoxic line showed a marginally higher level of karyotypic instability to the normoxic line, however, no obvious clonal selection was observed in response to chronic hypoxia and no further mutations were present.

**Gene-specific changes in imprinting regions**

To quantitatively analyse methylation at specific loci, we used a pyrosequencing assay for 12 well-known differentially methylated regions (DMRs) associated with imprinted genes (Fig. 5). The advantage of using imprinted genes in this study is that both a gain and loss of methylation could be assessed, since the DMRs chosen are methylated on only one parental allele. In the normoxic sub-line, seven DMRs were already hypomethylated and one DMR was hypomethylated, which is consistent with aberrant imprinting at several loci in this sub-line. This is not surprising in the light of the karyotypic anomalies in the cell line and the tendency for cells to undergo changes in methylation over time. More important was the observation of significant methylation changes after chronic hypoxia in seven of twelve DMRs examined. Three DMRs, which were within the expected range of normal methylation [IGF2 DMR0; KCNQ1 (KVDMR) and GNAS], had reduced methylation after hypoxia, whereas a further two DMRs (NAPIL5 and MKRN3), which were hypomethylated to start with, had even further reduced methylation levels. Two hypomethylated DMRs (SNRPN and USP29) had increased methylation after hypoxia. These data indicate that in tandem with global hypermethylation, gene-specific changes in methylation occur in response to chronic hypoxia (Fig. 5).

**DISCUSSION**

Using a novel system of normoxic and hypoxia-conditioned PwR-1E benign epithelial sub-lines, we have characterized phenotypic changes in apoptotic-resistance, cellular senescence, invasion and cytokine secretion in response to chronic hypoxic exposure. Moreover, we have identified concurrent epigenetic alterations that may trigger these cellular changes in the absence of HIF-1α, thereby playing a key role in the establishment and maintenance of the hypoxia-conditioned PwR-1E phenotype.

The phenotypic changes identified here in response to chronic hypoxia represent characteristics that may precede prostate cancer development. This study has demonstrated for the first time that chronic hypoxic PwR-1E cells acquire apoptotic resistance in response to treatment with TRAIL/CHX and FasL. In support of this, Walsh et al. (16) were the first to consider the impact of hypoxia on apoptotic resistance in benign prostate cells following acute hypoxia. These results suggest that early acquired resistance to receptor-mediated apoptosis may have implications for immune surveillance and therefore promote the early development of prostate cancer (16). In cells with a reduced potential for apoptosis, an alternative cell fate is cellular senescence. In this study, we have reported significantly increased levels of senescence in chronic hypoxic cell populations. Current literature suggests that senescence may be triggered by the activation of oncogenes (17,18) as an alternative tumour suppressor mechanism to apoptosis (19). Such activation of oncogenes may also be attributed to early epigenetic changes that are known to occur in the early stages of prostate cancer (20).

In a study by Collado et al. (21), following oncogene activation, senescent cells could be found in pre-malignant lesions but not in malignant tumours. Furthermore, senescent cells may have the ability to interfere with their own microenvironment by secreting proteases and mitogenic, anti-apoptotic and angiogenic factors, promoting carcinogenesis in neighbouring cells (22).

In this study, we have also shown that exposure to chronic hypoxia results in a significant increase in cell migration in PwR-1E cells. Previous studies have suggested that hypoxia may play a role in increasing the motility of tumour cells through a number of HIF-1α-mediated mechanisms (23–25) and that this increased migratory potential may act as a survival mechanism allowing cells to migrate away from areas of low oxygen levels (26). However, few studies have focused on the impact of hypoxic environments on non-cancerous cells. We have shown that benign prostate cells exposed to long periods of hypoxia may already harbour a greater migratory potential in the absence of HIF-1α. Furthermore, we have determined a significant increase in the production of a range of important cytokines (IL-1β, IL-6, IL-8 and TNFα), which have been previously reported to promote the growth and survival of prostate cancer cells (27,28).

Many studies have demonstrated the direct epigenetic regulation of IL-8 (29,30), IL6 (31) and TNFα (32), and further studies have shown that cytokines such as IL-6 may themselves epigenetically modulate gene expression both through the regulation of DNMT activity (33,34) and indirectly through the regulation of miRNAs (35). Therefore, it is possible that the impact of epigenetic alterations in response to chronic hypoxia may be augmented by increased cytokine production.

The phenotypic changes that we have observed in the hypoxia-conditioned PwR-1E cells are characteristics often associated with aggressive cancers. It has previously been shown that the disruption of epigenetic modifications may be early events in the development of prostate cancer, where global alterations in histone acetylation patterns and in chromatin structure have been previously identified in low-grade prosthetic lesions and in regions of cancer associated normal tissue (12,36,37). Although it is well known that HIF-1α initiates a cascade of hypoxic response pathways following acute hypoxia, only a small number of hypoxia-induced gene expression changes can be accounted for by the direct effects of HIF-1α (38). Furthermore, due to the transient nature of HIF-1α expression, other mechanisms must be involved during the chronic hypoxic response in order to complete and maintain an adaptive phenotype. We suggest that the maintenance of this hypoxia-adapted phenotype may be supported by genome-wide epigenetic changes. Evidence for this can be found by studying the associations of HIF-1α
Figure 5. Chronic hypoxic conditions induce alterations in local DNA methylation at imprinted gene loci. Methylation levels at 12 imprinted differentially methylated regions (DMRs). Data represent two biological replicates and two technical replicates. The mean methylation is shown when between 3 and 12 individual CpG’s are measured within a DMR. Box plots show maximum and minimum values and the inter-quartile range. The $P$-values were calculated using the non-parametric Wilcoxon match pairs test ($\pm$DMRs possibly affected by abnormal karyotype).
with epigenetic modifying enzymes, many of which have been found in direct contact with HIF-1α during the initial cellular response to hypoxia. The HAT enzymes CBP and p300, and steroid receptor cofactor (SRC-1) are components of the HIF-1α complex and therefore potentiate the HIF-1α gene expression response to hypoxia (39,40). HDAC-7 has also been found to enhance transcriptional activity within this complex (41) and HDAC-1 and HDAC-3 regulate HIF-1α stability and transcriptional activity (42). The role of epigenetics in HIF-1α regulation may also be demonstrated by the fact that HIF-1α is a direct target of the SWI/SNF chromatin remodelling complex (43).

In this study, we have identified a global H3K9 hyperacetylation and DNA hypermethylation in PwR-1E cells in response to chronic hypoxia which may signify a new epigenetic signature indicative of the altered gene expression profile of a hypoxia-adapted cell. The acetylation of H3K9 is known to promote gene expression and may reflect the enhanced transcriptional activity of a plethora of genes associated with the hypoxic response. In association with this hyperacetylation an increase in nuclear size was also observed in these cells, possibly reflecting a global opening of chromatin structures, resulting in the promotion of gene expression. Several lung carcinoma studies have reported a global hypoacletylation in response to hypoxia (44–46). However, we have found that in normal prostatic cells, in the absence of the confounding phenomenon of cancer-associated epigenetic changes, that this is not the case. In addition to acetylation changes, we have demonstrated a reversible global hypermethylation in PwR-1E cells in response to chronic hypoxia. Shahrzad et al. (47) have also addressed global levels of DNA methylation in response to hypoxia and although they observed a global hypomethylation, there are several important differences between Shahrzad’s study and this study, including their use of a cancer model and the exposure to cells to anoxic conditions for short periods of time, in contrast to our non-cancerous prostatic chronic hypoxia model. Also, in the current study, we have employed a novel method of DNA methylation analysis using flow cytometry which allows the quantification of average levels of DNA methylation per analysed cell. Moreover, in support of our findings of global hypermethylation, we have identified a significant increase in DNMT3b expression and a decrease in the levels of SAM, which may be associated with increased usage during the addition of new methyl groups. DNMT3b is one of the de novo methyltransferase enzymes and is therefore responsible for establishing new patterns of DNA methylation (48). Although the levels of this enzyme are normally low in adult somatic cells, levels of DNMT3b are often deregulated during many disease processes including cancer development (49,50). Therefore, the presence of increased DNMT3b may be responsible for the establishment of a DNA methylation pattern that represents the hypoxia-adapted nuclear phenotype.

Finally, using an imprinting array for DNA methylated regions, we have shown that hypoxia-induced alterations in DNA methylation directly impact on specific gene loci, having identified alterations in DNA methylation within the promoter sequences of a number of imprinted genes. This analysis was performed in association with a full cytogenetic analysis of the PwR-1E normoxic and hypoxic sub-lines and therefore methylation alterations shown here exist in the absence of significant karyotypic changes at these sites.

In conclusion, our results demonstrate a genome-wide adjustment of DNA methylation and histone acetylation under chronic hypoxic conditions in benign prostate cells. We suggest that these phenomena may promote and maintain the expression of regulatory genes and the activation of adaptive pathways that may promote prostate tumour development. Understanding how chronic hypoxia influences global and gene-specific epigenetic programming will provide important insights into the mechanisms of hypoxia-induced cellular changes. The identification of these factors contributing to the initial development of prostate cancer represent important targets for early detection and manipulation in early stage disease.

**MATERIALS AND METHODS**

**Generation of the chronic hypoxic (1% O₂) PwR-1E cell sub-line**

The human prostate androgen-dependent PwR-1E cell line was purchased from the American Type Culture Collection. Both normoxic and hypoxic (10, 3 and 1% O₂) PwR-1E cultures were maintained in keratinocyte serum-free medium supplemented with 50 mg/ml of bovine pituitary extract, 5 ng/ml EGF, 50 U/ml penicillin and 50 mg/ml streptomycin with 500 mg/ml amphotericin B (fungizone) (Gibco), and passed routinely in 75 cm² vented tissue culture flasks. To initiate the hypoxic sub-line cultures, PwR-1E parental cells at passage 11 were maintained at 37°C in a humidified hypoxic (10% O₂) atmosphere using an hypoxic chamber (Coy Laboratories). Medium was replenished every 48 h to avoid accumulation of by-products of anaerobic glycolysis that result in acidification of the medium under hypoxic conditions. Media was also replenished in the normoxic control cells at the same time. Control PwR-1E cells were also maintained under atmospheric normoxia (21% O₂) with a balance of 95% N2/5% CO₂ and passaged in parallel with their hypoxic counterparts such that both cultures were equivalently aged. After a period of 7 weeks, the 10% O₂-viable PwR-1E cell culture was then transferred to an atmosphere of 3% O₂ and further maintained under these conditions for 4 weeks. At this point, the 3% O₂-viable hypoxic PwR-1E cell culture was transferred to an atmosphere of 1% O₂ and further maintained for an additional 3 weeks prior to the start of experimentation at passage 23.

**Treatments**

Normoxic (21% O₂) and chronic hypoxic (1% O₂) PwR-1E cells were treated with 10 ng/ml recombinant soluble FasL or a combination of 25 ng/ml TRAIL and 1 μg/ml CHX for 24 h. Treatment with 5-aza-2′-deoxycytidine (5-aza-2dC) (Sigma) was administered at 1 μM concentrations and replenished every 24 h for 72 h.

**Analysis of apoptosis**

Apoptosis was quantified as the percent of cells with hypodiploid DNA as assessed by propidium iodide (PI) incorporation.
Cells were trypsinized, then centrifuged (200g, 5 min), and resuspended in hypotonic fluorochrome PI solution (50 mg/ml PI, 3.4 mM sodium citrate, 1 mM Tris, 0.1 mM EDTA, 0.1% Triton X-100). Analysis was performed on a CYAN flow cytometer and results assessed using SUMMIT software (Dako). A minimum of 5000 events were collected and analysed. Apoptotic nuclei were distinguished from normal nuclei by their hypodiploid DNA. Cellular debris was excluded from analysis by raising the forward and side scatter thresholds. For validation, cells were stained with 1 mg/ml Hoechst. Cellular morphology was assessed using comparative light or UV microscopy (original magnification, ×10).

**Senescence-associated-β-galactosidase activity**

A total of 200,000 cells were fixed with 2% formaldehyde (Sigma) and 0.2% glutaraldehyde (Sigma) in water and stained with 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) staining solution (1 mg/ml X-gal, 40 mmol/l citric acid/sodium phosphate pH 6.0, 5 mmol/l potassium ferricyanide, 5 mmol/l potassium ferrocyanide, 150 mmol/l NaCl, 2 mmol/l MgCl2) for 24 h in a CO2 incubator at 37°C. The cells were subsequently counted using a phase contrast microscope (Olympus CK2). Senescent cells were expressed as a percentage of the total number of cells counted. Approximately 200 cells were assessed per field.

**Invasion assay**

A total of 50,000 cells were incubated in the upper chamber of an Innocyte™ migration assay well (Becton Dickinson) and allowed to migrate through an 8 mm pore for 24 h. Migrated cells were detached using a detachment buffer and the number of invaded cells was quantified using a cell-permeable fluorescent dye. Fluorescence was read on a plate reader at excitation and emission wavelengths of 485 and 520 nm, respectively. The number of invaded cells was normalized to cell growth levels.

**Western blotting**

Proteins were extracted using RIPA buffer, quantified and boiled at 95°C in SDS sample buffer for 5 min, run on 8% SDS–PAGE gels and transferred onto nitrocellulose membranes. Membranes were subsequently blocked in 5% skimmed milk and incubated overnight at 4°C with mouse monoclonal anti-HIF-1α. After washing with phosphate buffered saline containing 0.1% Tween 20, membranes were exposed to horseradish peroxidase-conjugated anti-mouse IgG (Pierce) and visualized by chemiluminescence using exposed to horseradish peroxidise-conjugated anti-mouse IgG (Pierce) and visualized by chemiluminescence using exposed to horseradish peroxidise-conjugated anti-mouse IgG (Pierce). The tissue culture media using a four-spot multi array plate (Meso Scale Discovery, USA). The assay was carried out following the protocol provided and read using the Sector Imager.

**Quantification of pro-inflammatory cytokines using the MSD multi-spot human cytokine assays**

Levels of IL-8, IL-1β, IL-6 and TNF-α were measured in tissue culture media using a four-spot multi array plate (Meso Scale Discovery, USA). The assay was carried out following the protocol provided and read using the Sector Imager.

**Flow cytometry—protein quantification**

Cells were harvested by trypsinization and fixed in 70% methanol. Staining was then conducted using monoclonal anti-Actin (Abcam), anti-5′Methylycytidine (5′McC) (Eurogentec) and anti-pan histone H3 antibodies. Prior to staining with 5′MeC cells were pre-incubated with 1 M HCl at 37°C for 1 h. IgG negative controls were used at the same concentration as the primary antibody. Secondary staining was conducted using an FITC conjugated rabbit anti-mouse secondary antibody (Dako). Analysis was performed on a CYAN flow cytometer and results assessed using SUMMIT software (Dako).

**Immunofluorescence**

Cells were grown on coverslips and fixed in methanol. Cells were blocked using 1% Bovine Serum Albumin and stained with monoclonal anti-ACh3K9 (Abcam), anti-5′McC (Eurogentec) and corresponding IgG negative controls. Prior to staining with anti-5′MeC, the cells were pre-incubated with 1 M HCl at 37°C for 1 h. Cells were subsequently stained with FITC-conjugated rabbit anti-mouse secondary antibody (Dako), counterstained with 4′-6-diamidino-2-phenylindole and mounted using p-phenylenediamine anti-fade mounting media. Visualization was performed using an Axioplan 2 Zeiss microscope and images were captured with a Carl Zeiss AxioCam system and Axiovision 3.0.6 software (Carl Zeiss).

**Enzyme activity assays**

Cells were harvested and nuclear proteins isolated using the EpiQuik™ Nuclear Extraction Kit (Epigenetek) following the manufacturer’s instructions. Protein extracts were quantified using a Bradford assay and 10 μg of nuclear protein from each sample was used. Enzyme activity assays were performed for HAT and DNMT enzymes using the EpiQuik™ HAT Activity/Inhibition and DNMT Activity/Inhibition Assay kits (Epigenetek) according to manufacturer’s instructions. Plates were read on a microplate reader at an absorption of 450 nm.

**SAM quantification**

Cells were harvested by scraping and resuspended in 0.2% perchloric acid solution (Sigma) at a ratio of 100 μl per 1 million cells. Following cell fragmentation, SAM was isolated by centrifuging at 10 000 g for 10 min at 4°C, and collection of the clarified supernatant for analysis. SAM levels were quantified using the Bridge-It® SAM fluorescence assay (Mediomics). The assay was performed according to the manufacturer’s guidelines. The protein concentration of the cell debris pellet was used to adjust results from the SAM quantification assay.

**RT–PCR**

RNA was extracted using TRIzol™ reagent (Sigma), according to the manufacturer’s instructions. Random hexamer
cDNA was synthesized and QPCR was performed using Platinum SYBR Green QPCR SuperMix-UDG (Invitrogen, USA) along with 2 μl of template cDNA and 0.3 μl of each primer (stock of 10 μM) in a total reaction volume of 20 μl. QPCR primers were designed using Primer3 software. One primer in each pair is exon spanning to ensure amplification of only mature mRNA. Primer sequences: Dnm1, 5'-TATCCGAGGGCTACCTG-3' (forward), 5'-CACCT CCCGGTTGAAGC-3' (reverse); Dnm3a, 5'-AGGCCA AGGTCAAGGAGATT-3' (forward), 5'-GTCTTTGCAGT TGGCACA-3' (reverse); Dnm3b, 5'-TCAGATGGGAAG GAGTTTGG-3' (forward), 5'-CTGCAAGAGCTCGGAGAA C-3' (reverse). QPCR reactions were normalized by amplifying the same cDNA with beta-2-microglobulin (B2M) primers, 5'-AGGCTATCCAGCGTACTCCA-3' (forward), 5'-GTCTTTGCAGTT GTCCTTGGCTGAAGACA-3' (reverse). B2M expression levels were not influenced by hypoxia. Amplification and detection were performed using an MX3000P System according to the manufacturer’s instructions (Stratagene). The PCR cycling program consisted of an initial pre-incubation for 10 min at 95°C, followed by 40 three-step cycles of 15 s/95°C, 30 s/58°C and 30 s/72°C. This was followed by 10 min extension at 72°C. In order to avoid non-specific signals, a melting program was carried out after the PCR cycles were completed. The samples were quantified by comparison with a standard calibration curve (generated using serial dilutions of the pooled cDNA samples analysed) and the data were normalized by the B2M internal control.

Cytogenetic analysis
Cytogenetic analysis was performed following standard procedures. Briefly, cells growing exponentially were treated with 40 ng/mL colcemid (KaryoMax; Gibco) for 2 h at 37°C in 5% CO₂. Cells were trypsinized, incubated in hypotonic KCl solution (0.075 M) for 10 min at 37°C, and fixed in 3:1 methanol:acetic acid fixative. The cell suspension was dropped onto glass slides and allowed to dry. Following aging, G-banded chromosomes were prepared by a conventional trypsin-Leishman technique and subsequently analysed using an Olympus BX51 microscope.

Analysis of DMR methylation
We used a standard pyrosequencing assay for 12 different DMRs (15). Each assay included three 11 CpGs. DNA was extracted from normoxic and hypoxic cell lines from two biological replicates. One microgram of genomic DNA was bisulphite treated using EZ DNA methylation kit (Zymo Research) and used as a template in a two round PCR reaction; 10 μl of the biotinylated PCR product was used for each sequencing assay. Pyrosequencing was carried out on a PSQ HS 96 System and PyroMark MD System using Pyro Gold Reagent kits (Biotage, Uppsala, Sweden). Each sample was bisulphite converted and pyrosequenced in duplicate. Methylation was quantified using Pyro Q-CpG Software (Biotage, Uppsala, Sweden) that calculates the ratio of converted C’s (T’s) to unconverted C’s at each CpG and expresses this as a percentage methylation. Median and IQR methylation were analysed using Prism Graphpad. (Specific primers and PCR conditions are available on request).

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Conflict of Interest statement. None declared.

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