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Hypoxia

Hypoxia disrupts the Fanconi anemia pathway and sensitizes cells to chemotherapy through regulation of UBE2T

Chantal H.M.A. Ramaekers [a,b,1], Twan van den Beucken [a,b,1], Alice Meng [a], Shaqil Kassam [a], John Thoms [a], Robert G. Bristow [a,c,d], Bradly G. Wouters [a,b,c,d,*]

[a] Ontario Cancer Institute and Campbell Family Institute for Cancer Research, University Health Network, Toronto, Canada; [b] Maastricht Radiation Oncology (MaastRO) Lab, Maastricht University, The Netherlands; [c] Department of Radiation Oncology; [d] Department of Medical Biophysics, University of Toronto, Canada

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ABSTRACT

Background and purpose: Hypoxia is a common feature of the microenvironment of solid tumors which has been shown to promote malignancy and poor patient outcome through multiple mechanisms. The association of hypoxia with more aggressive disease may be due in part to recently identified links between hypoxia and genetic instability. For example, hypoxia has been demonstrated to impede DNA repair by down-regulating the homologous recombination protein RAD51. Here we investigated hypoxic regulation of UBE2T, a ubiquitin ligase required in the Fanconi anemia (FA) DNA repair pathway.

Materials and methods: We analysed UBE2T expression by microarray, quantitative PCR and western blot analysis in a panel of cancer cell lines as a function of oxygen concentration. The importance of this regulation was assessed by measuring cell survival in response to DNA damaging agents under normoxia or hypoxia. Finally, HIF dependency was determined using knockdown cell lines and RCC4 cells which constitutively express HIF1α.

Results: Hypoxia results in rapid and potent reductions in mRNA levels of UBE2T in a panel of cancer cell lines. Reduced UBE2T mRNA expression is HIF independent and was not due to changes in mRNA or protein stability, but rather reflected reduced promoter activity. Exposure of tumor cells to hypoxia greatly increased their sensitivity to treatment with the interstrand crosslinking (ICL) agent mitomycin C.

Conclusions: Exposure to hypoxic conditions down-regulates UBE2T expression which correlates with an increased sensitivity to crosslinking agents consistent with a defective Fanconi anemia pathway. This pathway can potentially be exploited to target hypoxic cells in tumors.

Solid tumors frequently display extreme heterogeneities in oxygenation within their microenvironment which arise from poorly developed vasculature [1]. From clinical studies it is evident that hypoxia correlates with poor prognosis [2–5]. This is in part due to the resistance of hypoxic cells to standard cancer treatments like radio- and chemotherapy and therefore has stimulated efforts to increase radiotherapy efficacy by reducing the hypoxic fraction [6,7]. In addition tumor hypoxia is associated with a more aggressive disease. For example, patients with high levels of tumor hypoxia have a poor prognosis when treated with surgery alone. Hypoxic tumors have also been associated with increased angiogenesis, increased autophagy, pH regulation as well as with higher metastatic and invasive potential [4,8–14]. Several mechanisms have been demonstrated to contribute to the association of hypoxia with more aggressive disease. For example, both transient and stable genetic changes can contribute to cellular adaptation to hypoxia that promotes overall tumor growth [15,16]. The first demonstration of this mechanism was the selective outgrowth of p53 and apoptosis-deficient cells cultured under repeated conditions of hypoxia [17]. In addition to causing increased cell survival, resistance to apoptosis in the hypoxia tolerant cells can contribute to increased genetic instability and the potential for selection of even more aggressive disease [18]. This is amplified by recent observations that hypoxia suppresses the expression of key proteins involved in DNA mismatch repair (MMR) and homologous recombination (HR) repair. The MMR proteins MLH1, MLH2 and MSH6 are down-regulated under hypoxic conditions [19–22]. Likewise, several studies have shown that HR activity is impaired by hypoxia and is associated with reduced expression of the HR repair proteins RAD51, BRCA1 and BRCA2 [23–25]. Reduced RAD51 and BCRA1 mRNA levels have been observed after prolonged anoxic conditions (<0.02% O_2 for 48 h) and could be attributed to E2F4/p130 mediated transcriptional repression at the proximal promoter regions of both genes [25,26]. Decreased RAD51 and BRCA1 protein levels have also been reported under moderate hypoxia (0.2% O_2 for 72 h). Interestingly, this was not due to decreased

* Corresponding author at: 610 University Ave., Toronto, ON, Canada M5G 2M9.
E-mail address: bwouters@uhnresearch.ca (B.G. Wouters).

[1] These authors contributed equally to this article.

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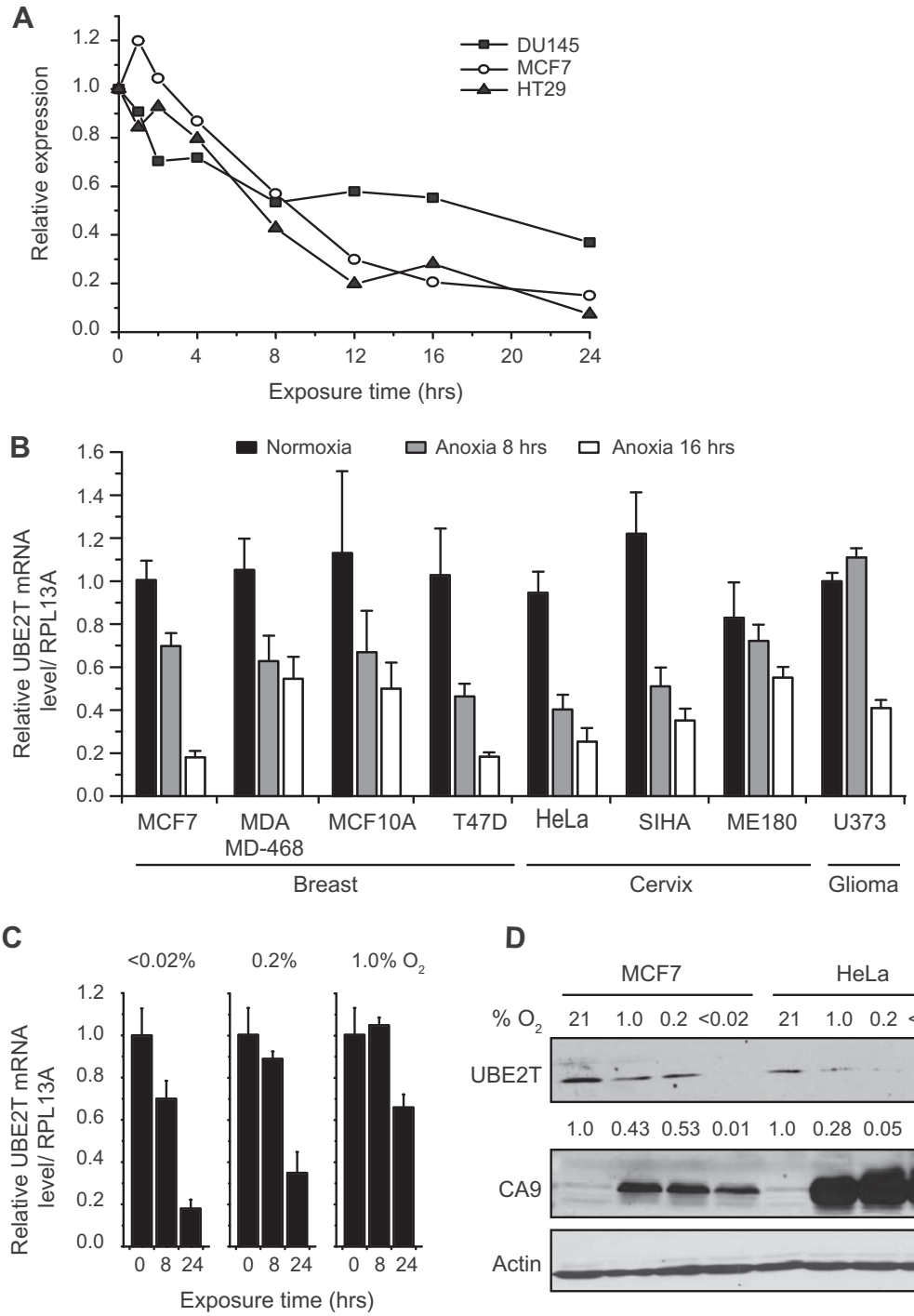

Fig. 1. Hypoxia down-regulates UBE2T expression. (A) Analysis of UBE2T mRNA expression in MCF7, HT29 and DU145 cells during anoxia using Affymetrix gene arrays. (B) Down-regulation of UBE2T mRNA levels after 8 and 16 h of anoxia determined by Q-PCR. (C) Oxygen dependency of UBE2T down-regulation on the mRNA level in MCF7 cells. (D) UBE2T protein levels determined by western blotting at 21%, 1.0%, 0.2% and <0.02% O_2 in MCF7 and HeLa cells. Indicated intensities of UBE2T were normalized to loading control actin and expressed relative to 21% O_2 control for each cell line independently. CA9 served as positive control for hypoxia.

mRNA levels but was rather the result of impaired RAD51 mRNA translation [24].

Fanconi anemia (FA) is a rare, recessive genetic disorder in which patients exhibit congenital defects, bone marrow failure and cancer susceptibility [27,28]. Patients are diagnosed by their cellular phenotype of chromosomal instability and hypersensitivity to DNA interstrand crosslinks (ICLs) induced by agents such as mitomycin C or cisplatin. Thus far, 13 distinct complementation groups have been identified in Fanconi anemia and the gene products cooperate in a common DNA repair pathway to remove ICLs. Inactivation of these genes is not only found in Fanconi anemia but also in a variety of cancers in the general population [29]. ICLs are very toxic lesions in proliferating cells as they obstruct separation of DNA strands necessary for replication and transcription. A crucial aspect in the initiation of ICL repair by the FA pathway is carried out through mono-ubiquitylation of FANCD2 and FANCI [30,31]. The core complex, containing FANCA, B, C, E, F, G, L and M together with the FA associated proteins FAAP24 and FAAP100, functions as a

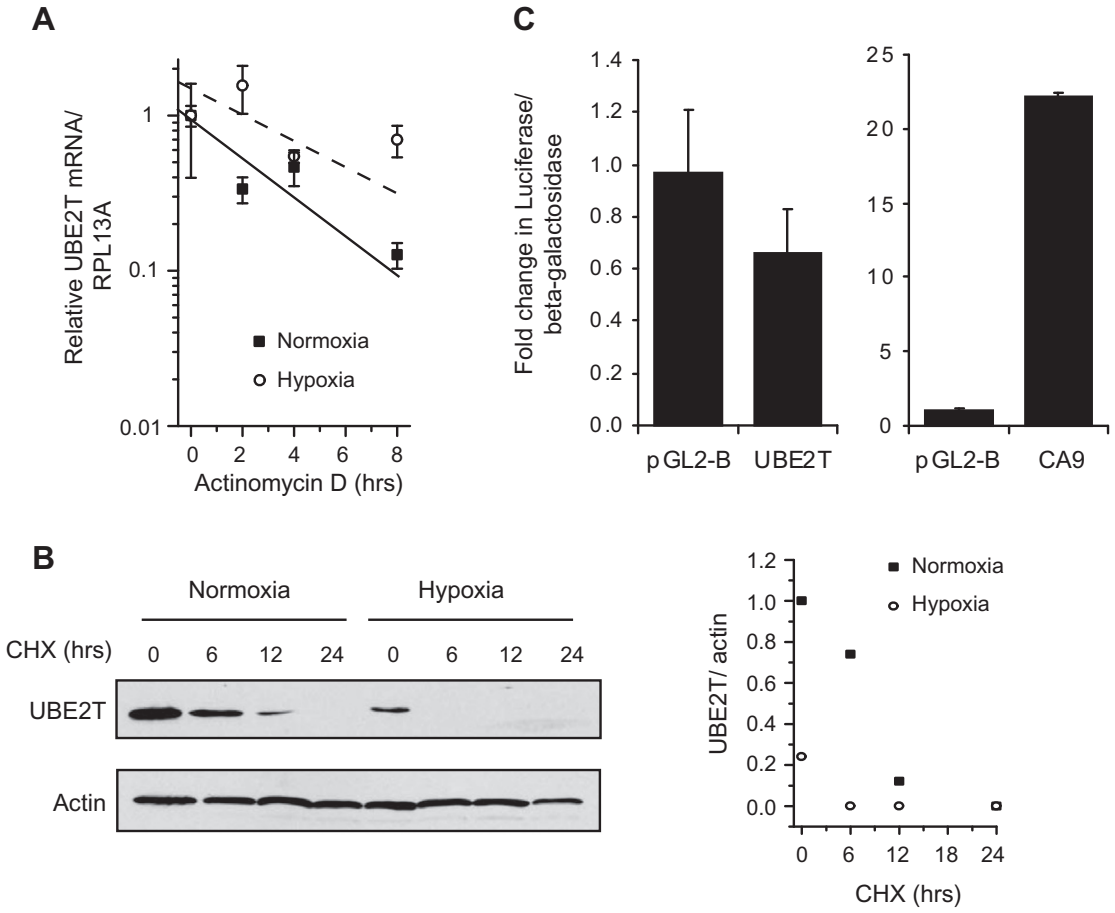

Fig. 2. Impaired UBE2T expression is due to decreased promoter activity. (A) UBE2T mRNA stability was determined using Q-PCR in MCF7 cells that were pre-exposed for 24 h to 21% or 0.2% O_2 and then treated for indicated times with actinomycin D ($n = 3$). (B) Protein synthesis was blocked using cycloheximide to assess UBE2T protein half-life under aerobic conditions or after exposure to 0.2% O_2 for 24 h. (C) UBE2T or CA9 promoter constructs were transiently cotransfected with pcDNA3-LacZ into HeLa cells. Luciferase activity was measured relative to β-galactosidase expression after 24 h of 0.2% O_2 after which fold induction was calculated for each construct.

multi-subunit E3 ubiquitin ligase [32,33]. This complex together with the novel E2 conjugating enzyme UBE2T facilitates mono-ubiquitylation of FANCD2 and FANCI [34]. The exact mechanistic function of FANCD2 and FANCI ubiquitylation is not clear, however it seems to serve as a targeting signal to DNA damage, as it retains the complex on chromatin and colocalizes with DNA repair proteins in distinct foci at sites of DNA lesions [30,35–40].

Here we show that the ubiquitin conjugating enzyme UBE2T is rapidly down-regulated at both the mRNA and protein levels during hypoxia in a HIF independent manner. This down-regulation correlated with an increased sensitivity to ICL inducing agents under hypoxic conditions. Hypoxic inhibition of the FA pathway represents a novel mechanism for increased genetic instability in cancer, but can also provide an opportunity for use of synthetic lethal approaches to selectively target hypoxic cells in aggressive tumors.

Materials and methods

Cell culture

The following cell lines were obtained from ATCC: HT29, DU145, MCF7, MDA-MD-468, U373, HCT116, Hela, ME180 and SiHa. All cell lines were grown according to ATCC instructions. For hypoxic exposure cells were transferred into a MACS VA500 micro-aerophilic workstation. The atmosphere in the chamber consisted of 5% H_2, 5% CO_2, the desired % O_2, and residual N_2. For exposure to stress inducing agents cells were grown for 24 h in deferoxamine (DFO).

Microarray experiments

Microarray experiments were performed as previously described [41]. In short, MCF7, DU145 and HT-29 and cells were grown on glass dishes and exposed to 0, 1, 2, 4, 8, 16 or 24 h of hypoxia (<0.02% O_2). Three independent experiments were performed and equal amounts of total RNA from each biological repeat were pooled and hybridized to Human Genome U133 Plus 2.0 Arrays. Genes with signal intensities lower than 100 on one or more arrays were removed from the analysis.

Plasmids and lentiviral work

Knock-down of UBE2T and HIF-1α was achieved using lentiviral shRNA constructs TRCN0000004386, TRCN000000439 and TRCN0000003808 (TRC consortium). Lentiviral particles were generated by co-transfection of 293T cells with packaging plasmids pCMVdR8.74psPAX2 and pMD2.G together with shRNA vector pLKO.1. Virus supernatant was harvested 48 and 72 h post transfection. MCF7 cells were transduced with lentiviral supernatant in the presence of 8 μg/ml polybrene. Infected cells were selected for 2 days in 2 μg/ml puromycin containing media.

RNA extraction and quantitative RT-PCR

RNA was isolated using Tri-reagent (Sigma) according to manufacturers' instructions. RNA samples were reverse transcribed using q-Script kit as described by manufacturer (Quantas).

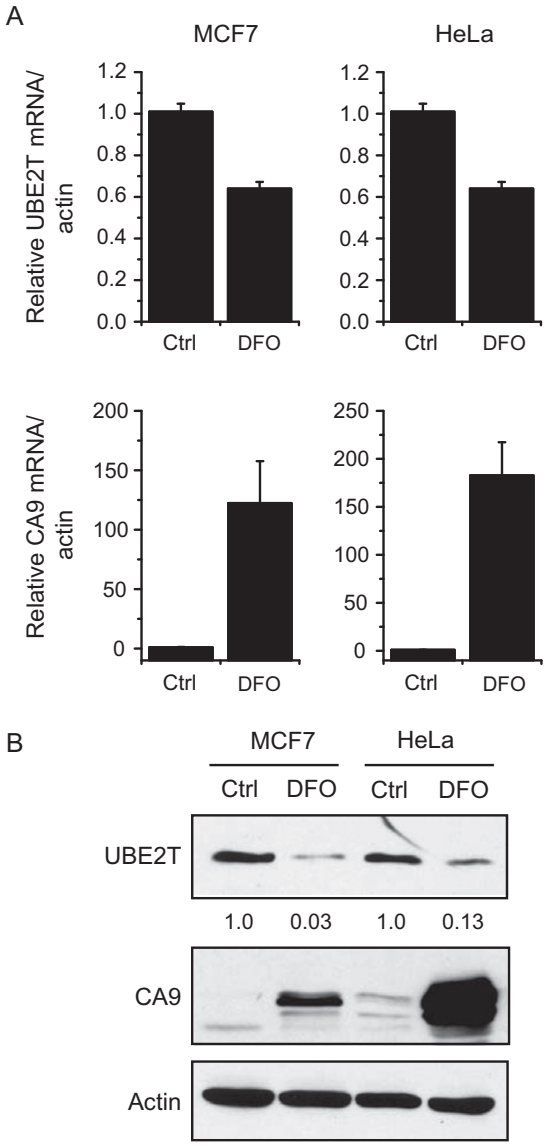

Fig. 3. UBE2T down-regulation in response to DFO treatment. MCF7 and HeLa cells were treated with DFO for 24 h after which UBE2T and CA9 mRNA (A) or protein levels (B) were determined.

Real-time PCR was performed on an Eppendorf Realplex^2 mastercycler using SYBR green (Quantas). Gene expression was normalized to RPL13A under hypoxic or anoxic conditions and to β-actin following treatment with DFO, as the expression of these genes is constant and well within the dynamic range of the Q-PCR under the particular conditions. For determination of UBE2T mRNA half life cells were treated with 5 μg/ml actinomycin D (Sigma) for the indicated times.

Western blot analysis

Cells were washed twice with cold PBS and scraped in 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS supplemented with protease inhibitors (Roche). After centrifugation at 10,000 g supernatants were boiled in Laemmli buffer for 10 min and proteins were resolved by SDS–PAGE. Proteins were subsequently transferred onto PVDF membranes and blocked for 1 h in TBS containing 0.05% Tween-20 (TBS-T) supplemented with 5% skim milk powder. Membranes were probed overnight at 4 °C with antibodies directed against UBE2T (Abnova), FANCD2 (Santa-Cruz), CA9 (M75, or β-actin (Sigma). Bound antibodies were visualized using HRP-linked secondary antibodies (GE healthcare) and ECL luminescence (Pierce).

Luciferase reporter constructs

A 2.2 kb fragment from the 5′ flanking region of the *UBE2T* gene was amplified using the following primers; UBE2T_F 5′CGATG GTACCCCCACCAGTTAACATACCCC 3′, UBE2T_R 5′CGATAAGCTTC AGTGGCTTTTCTAAGGCTCTCC 3′ and subsequently cloned into pGL2-basic vector (Promega, Madison, WI). MCF7 cells were transiently co transfected with the UBE2T or CA9 [42] promotor constructs and pcDNALacZ using Lipofectamine (Invitrogen) as described by the manufacturer. Transfected cells were sub cultured 16 h post transfection, exposed to hypoxia and finally harvested 48 h after transfection. Luciferase and β-galactosidase activity was measured using a commercial kit (Amplied biosystems) and measured on the Fluorstar Optima platereader (BMG Labtech).

Clonogenic assays

Cells were exposed to 21% or <0.02% O_2 for 24 or 48 h and subsequently treated with 1 μg/ml MMC for 1 h at 21% O_2. After the treatment cells were trypsinized and plated for clonogenic survival at a range of 200–10,000 cells per 6-cm dish in triplicate. Subsequently cells were grown for 14 days and resulting colonies were stained with methylene blue (Sigma). Colonies of at least 50 cells were counted.

Statistical analysis

Unpaired Student *t*-test was used to test significance between populations. $p < 0.05$ was considered significant. Points and error bars plotted in graphs of all figures represent the mean ± standard deviation.

Results

Hypoxia down-regulates expression of UBE2T

As part of other ongoing studies, we have assessed changes in the transcriptomes of several cancer cell-lines as a function of time following exposure to anoxic conditions (<0.02% O_2). DU145, MCF7 and HT29 cells were exposed to these conditions for 1, 2, 4, 8, 12, 16 and 24 h to identify both increases and decreases in gene expression associated with acute and chronic anoxia. One of the most rapid and strongly repressed transcripts identified in this study encodes the E2 ubiquitin conjugase, UBE2T. UBE2T mRNA levels were significantly down-regulated in all three cell lines with similar kinetics, reaching approximately 50% within 8 h of anoxic exposure. At 24 h of anoxia UBE2T mRNA levels were reduced to ~10% of starting values in normoxic controls (Fig. 1A). The observed down-regulation of UBE2T mRNA levels is selective as the expression of the majority of genes on the array remains unchanged under these conditions.

To confirm these results, quantitative reverse transcription-PCR analysis was performed on a panel of additional cell-lines consisting of 4 breast cancer lines, 3 cervix cancer lines and 1 glioma line. Significant down-regulation of UBE2T was observed after 8 h of anoxic conditions in all cell-lines except for ME-180 and U373. After 16 h of anoxic exposure, UBE2T was down-regulated in all cell lines and most strongly in MCF7, T47D and HeLa cells (Fig. 1B). UBE2T mRNA levels were normalized to RPL13A as described in the materials and methods. The normoxic levels of UBE2T were similar across the panel of eight lines.

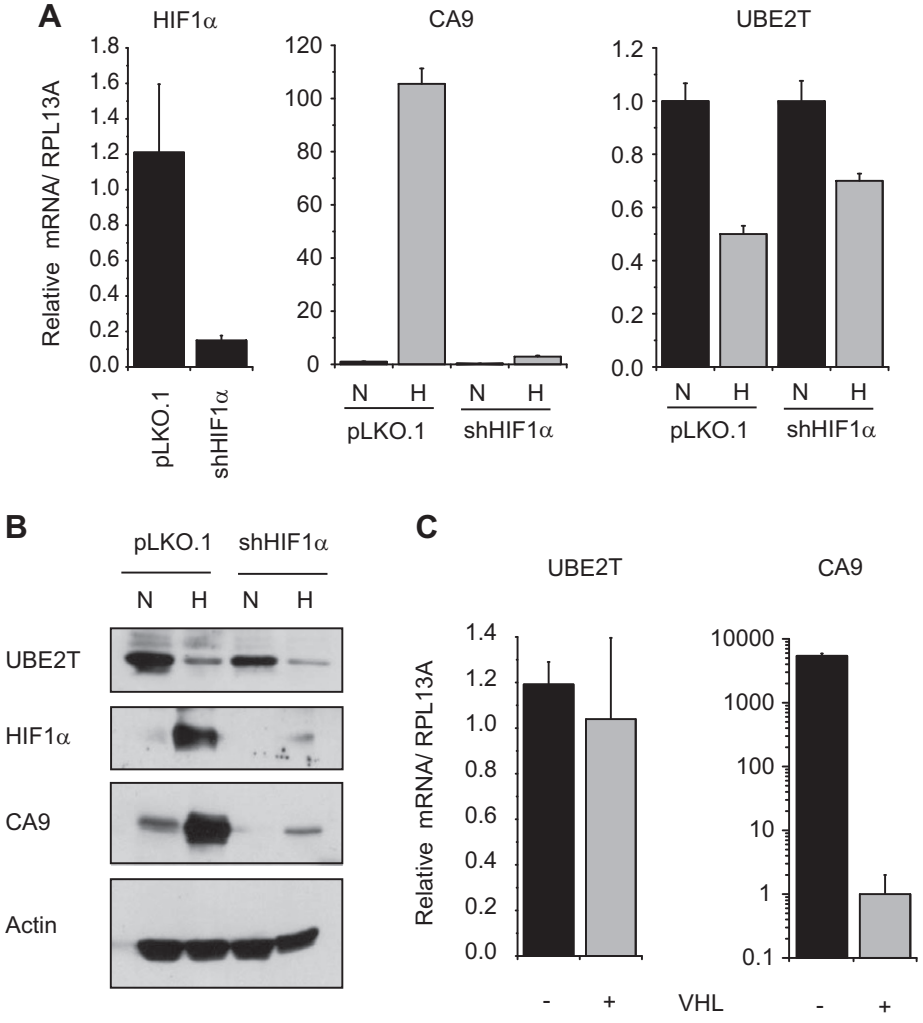

Fig. 4. UBE2T repression under hypoxia is HIF independent. (A) Knockdown of HIF1α was confirmed of the gene directly or via the downstream target CA9 at the mRNA (A) and protein level (B). UBE2T down-regulation in knockdown cells at the mRNA (A) and protein level (B). (C) UBE2T and CA9 mRNA levels in VHL deficient and proficient cells.

Exposure to very low oxygen conditions can influence cell death by inducing apoptosis, or by decreasing cell proliferation and altering cell-cycle distribution, both of which could potentially be influencing UBE2T expression. To investigate if UBE2T mRNA expression is also decreased under more moderate hypoxic conditions that do not induce these effects, we compared UBE2T mRNA levels of MCF7 cells exposed to <0.02%, 0.2% or 1% O_2 for 8 or 24 h. After 8 h of exposure, UBE2T levels were decreased substantially only in cells exposed to <0.02% O_2, whereas all three conditions significantly reduced UBE2T mRNA levels following 24 h exposure. At 0.2% hypoxia, which causes no significant change in cell-cycle or cell survival in these cells (data not shown), UBE2T mRNA was down-regulated to a similar extent to that observed under anoxia after 24 h (Fig. 1C). In agreement with the down-regulation at the mRNA level, we observed strong decreases in UBE2T protein levels in MCF7 and HeLa after 24 h exposure to 1%, 0.2% and <0.02% O_2 (Fig. 1D).

UBE2T is down-regulated by transcriptional repression

Given the relatively rapid drop in mRNA levels, we investigated whether the decreased expression could be explained by a change in mRNA stability. To this end we treated MCF7 cells with the transcriptional inhibitor actinomycin D for different durations under normoxic or hypoxic conditions and subsequently measured mRNA abundance to determine transcript half-life. Under normoxic conditions UBE2T mRNA half-life was estimated to be ~3 h, and under hypoxia this value showed a small increase to ~5 h (Fig. 2A). This suggests that UBE2T is not preferentially degraded under hypoxic conditions and if anything is somewhat more stable than under normoxic conditions (Fig. 2A).

The biological impact of a rapid change in mRNA abundance is influenced by the relative stability of the protein encoded by that particular mRNA, particularly in the case for suppressed genes. The data in Fig. 1 suggest that, at least within 24 h, the drop in mRNA level is sufficient to affect protein levels significantly, suggesting that UBE2T protein may have a relatively short half-life. We were unaware of any published studies on the half-life of UBE2T protein and so we assessed this in cells under normoxic or following 24 h of hypoxic (0.2%) conditions using cyclohexamide (CHX) treatment for various periods of time (Fig. 2B). The data indicate that UBE2T does indeed have a relatively short half-life under normoxic conditions of ~7.5 h, consistent with the measured protein loss observed within 24 h. The half-life could not be determined following pre-exposure to hypoxia, because starting levels were too low. However, the fact that protein levels were reduced in the hypoxia treated samples to undetectable levels following 6 h of cyclohexamide indicates that the half-life is similar or

shorter than that measured in normoxic samples. Consequently, suppression of UBE2T mRNA during hypoxia has a rapid impact on protein availability.

These data suggest that differences in UBE2T expression are likely due to changes in transcription. To test changes in *UBE2T* promoter activity more directly, we cloned a 2 kb fragment upstream of the transcriptional start site for *UBE2T* into a luciferase reporter construct. Luciferase activity was measured after transient transfection of this construct and subsequent exposure to either 24 h of 21% or 0.2% O_2. Luciferase activity driven from the *UBE2T* promoter was decreased to 68% under hypoxic conditions compared to normoxia (Fig. 2C). In contrast, a *CA9* promoter containing construct showed a 23-fold increase in luciferase activity upon hypoxia. Together, these data imply that the reduction of UBE2T is due primarily to a decrease in transcription, and that this effect rapidly influences protein levels due to a relatively high turnover of the protein.

The effect of DFO on UBE2T expression

To investigate whether UBE2T repression is dependent on the transcription factor HIF1α (hypoxia inducible factor 1α) we treated MCF7 and HeLa cells with DFO (deferoxamine) for 24 h. DFO is a metal chelating agent, which is known to stabilize HIF1α and HIF2α by inhibiting the HIF prolyl hydroxylases (PHD1–3) whose activity is required for HIF1α and HIF2α degradation [43]. As expected, DFO treatment led to strong induction of the HIF-target gene carbonic anhydrase 9 (CA9) at the mRNA and protein levels in both cell lines (Fig. 3A and B). Consistent with a role for HIF in its regulation, UBE2T mRNA and protein levels were significantly decreased in both cell-lines following DFO treatment to a similar extent as to that following hypoxic exposure (Fig. 3A and B).

UBE2T down-regulation is HIF independent

To assess the requirement for the HIF transcription factor more directly, we established stable MCF7 cells expressing shRNA against HIF1α. Knockdown of HIF1α was confirmed at both the mRNA and protein levels, as well as by assessing induction of its transcriptional target CA9 (Fig. 4A and B). The induction of CA9 was severely inhibited in cells expressing the shRNA against HIF1α. In contrast, HIF1α knockdown did not affect the down-regulation of UBE2T observed under hypoxic conditions (Fig. 4A and B). Also, knockdown of HIF2α or HIF1β did not affect UBE2T levels under hypoxic conditions (Supplementary Fig. 1). To assess HIF dependency in a different model, we utilized RCC4 renal cell carcinoma cells which lack functional VHL leading to constitutive expression of HIF1α and HIF2α. RCC4 cells displayed high levels of CA9 compared to RCC4 cells reconstituted with VHL (Fig. 4C). However, UBE2T expression was similar in both VHL deficient and proficient cells (Fig. 4C). Together these data robustly demonstrate that UBE2T down-regulation does not correlate with HIF activity.

Hypoxia sensitizes cells to the interstrand crosslinking agent MMC

UBE2T is the E2 ubiquitin conjugase required for ubiquitylation of FANCD2 and activation of the Fanconi anemia pathway. Since this pathway is essential for the repair of interstrand crosslinks, we first assessed clonogenic survival of cells with UBE2T knockdown after treatment with MMC (Fig. 5A). UBE2T knockdown markedly sensitized cells to MMC, so next we tested if hypoxic exposures sufficient to down-regulate UBE2T would similarly cause sensitization to MMC. Indeed, we found that cells exposed to 24 or 48 h of < 0.02% O_2 were significantly more sensitive to treatment with 1 μg/ml MMC as assessed by clonogenic survival (Fig. 5B). These data are consistent with a hypoxia induced defect

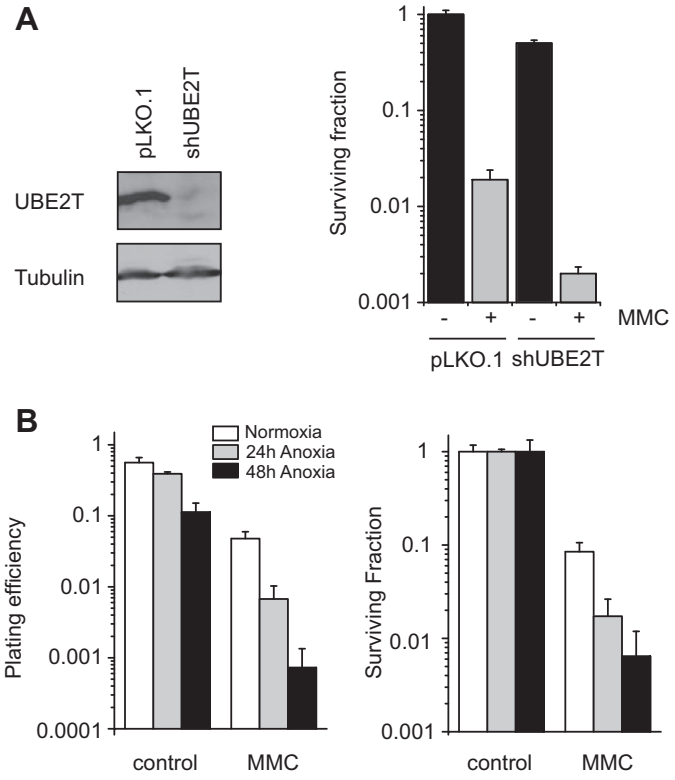

Fig. 5. Reduced UBE2T expression and hypoxia sensitizes cells to the interstrand crosslinking agent mitomycin C. (A) Knockdown efficiency of UBE2T using shRNA was determined by western blot analysis and its effect on clonogenic survival was assessed following treatment with 1 μg/ml MMC for 1 h. (B) Cells were exposed to <0.02% O_2 for 24 or 48 h and subsequently treated with 1 μg/ml MMC for 1 h at 21% O_2. Afterward cells were seeded for clonogenic survival. Plating efficiency for all treatments was determined after 14 days and surviving fraction was calculated.

in the FA pathway under hypoxic conditions and an increased sensitization of cells to MMC.

Discussion

Solid tumors are characterized by poor and heterogeneous oxygenation [1], features associated with an aggressive phenotype, genetic instability and poor response to therapy [2–5]. Several mechanisms can contribute to this adverse tumor biology including a reduction in DNA repair activity [18,44]. In particular, previous studies have shown that proteins involved in homologous recombination DSB repair are down-regulated during hypoxic conditions [24,45]. In this study, we demonstrate that the E2 ubiquitin conjugating enzyme UBE2T, a key protein in the Fanconi anemia pathway, is transcriptionally repressed under hypoxia. Rapid reductions in UBE2T expression were due to a reduction in promoter activity mediated by an element within 2 kb of the transcriptional start site. Repression occurred rapidly and was observed in a large panel of cancer cell lines and at a wide range of oxygen concentrations. Importantly, the repression of UBE2T transcription translated into substantial reductions in protein levels, due to the relatively short half-life of the UBE2T protein which was determined to be ~7.5 h. The hypoxic exposures required to observe changes in UBE2T protein levels, and thus influence DNA repair, are relatively short compared with that previously demonstrated to cause RAD51 down-regulation. UBE2T protein levels were reduced to almost undetectable levels following exposure to 24 h of 0.2% or < 0.02% O_2, whereas down-regulation of RAD51 was reported only after ~72 h of 0.2% or 48 h of <0.02% oxygen [24,45].

The ubiquitin conjugase UBE2T mediates ubiquitylation of the Fanconi anemia (FA) proteins FANCD2 and FANCI in concert with the multi-subunit FA "core-complex" E3 ubiquitin ligase [30,31,35,46]. These ubiquitylation events are essential for activation of the FA-pathway required for the repair of interstrand DNA cross-links (ICLs). Consistent with a defective FA-pathway we found that hypoxic cells with reduced UBE2T levels were markedly sensitized to MMC treatment (Fig. 5B). These data provide strong correlative evidence linking down-regulation of UBE2T during hypoxia with sensitivity to cross linking agents. However, the direct contribution of UBE2T to the sensitivity is not yet known, and it remains possible that unrelated effects of hypoxia could contribute to this effect. For example, the increased sensitivity of cells after 24 h of anoxia in Fig. 5B are consistent with a previous study by Chan et al. that showed enhanced cell death in response to treatment with cross-linking agents after exposure to more chronic hypoxia (72 h 0.2% oxygen), an effect which was correlated with loss of RAD51 at this time point [24]. It will be interesting to determine the relative contribution of UBE2T repression to the increased sensitivity of hypoxic cells to MMC.

HIF-1α stabilization occurs through inhibition of prolyl-hydroxylases, which under oxic conditions stimulate VHL mediated ubiquitylation of HIF-1α, thereby targeting it for degradation by the proteasome [47]. Despite the fact that UBE2T was down-regulated in response to the iron chelator DFO, we established that decreased UBE2T expression was not dependent on HIF activity, as knockdown of HIF-1α, HIF-2α or HIF-1β had no effect on UBE2T repression upon hypoxia (Figs. 3 and 4 and Supplementary Fig. 1). Using renal cell carcinoma (RCC) cells, which often experience high levels of constitutive HIF expression due to inactivation of the tumor suppressor VHL, we confirmed in another way that UBE2T repression is not associated with HIF activity (Fig. 4C). Besides deregulating the prolyl-hydroxylases that regulate HIF-1α stability, both DFO and hypoxia also affect the recently identified Jumonji family of hydroxylases [48]. These iron and oxygen dependent enzymes modulate gene expression by histone tail modifications. We speculate that inhibition of this family of enzymes could affect *UBE2T* promoter activity through increased methylation of histone marks that lead to epigenetic silencing.

Our data contributes to the growing evidence that DNA repair pathways are compromised under hypoxic conditions. These DNA repair defects present new opportunities to target otherwise resistant hypoxic cells. One example that has received recent attention is the synthetically lethal effect that PARP-inhibitors have on HR deficient cells due to BRCA1 or BRCA2 mutations. This concept of synthetic lethality can also be applied in a contextual setting to hypoxic tumors which have HR defects due to down-regulation of important HR proteins [44]. The data presented here, suggest an additional contextual synthetic lethal combination of hypoxia with agents that engage the FA pathway, and it will be interesting to determine if ICL agents have preferential activity against aggressive hypoxic tumors in patients. Ultimately this information might increase treatment efficacy and patient outcome.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.radonc.2011.05.059.

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