Bladder cancer cell lines adapt their aggressiveness profile to oxygen tension

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Abstract. During the process of tumor growth, cancer cells will be subjected to intermittent hypoxia. This results from the delay in the development of the vascular network in relation to the proliferation of cancer cells. The hypoxic nature of a tumor has been demonstrated as a negative factor for patient survival. To evaluate the impact of hypoxia on the survival and migration properties of low and high-grade bladder cancer cell lines, two low-grade (MGHU-3 and SW-780) and two high-grade (SW-1710 and T24) bladder cancer cell lines were cultured in normoxic (20% O_2) or hypoxic atmospheric conditions (2% O_2). The response of bladder cancer cell lines to hypoxic atmospheric cell culture conditions was examined under several parameters, including epithelial-mesenchymal transition, doubling time and metabolic activities, thrombospondin-1 expression, whole Matrix Metallo-Proteinase activity, migration and resistance to oxidative stress. The low-grade cell line response to hypoxia was heterogeneous even if it tended to adopt a more aggressive profile. Hypoxia enhanced migration and pro-survival properties of MGHU-3 cells, whereas these features were reduced for the SW-780 cell line cultured under low oxygen tension. The responses of tested high-grade cell lines were more homogeneous and tended to adopt a less aggressive profile. Hypoxia drastically changed some of the bladder cancer cell line properties, for example matrix metalloproteinases expression for all cancer cells but also switch in glycolytic metabolism of low grade cancer cells. Overall, studying bladder cancer cells in hypoxic environments are relevant for the translation from *in vitro* findings to *in vivo* context.

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Introduction

Cancer statistics for 2021 estimated there were 83,730 new cases of bladder cancer (64,280 men and 19,450 women) in USA, leading to 17,200 deaths (12,260 men and 4,940 women) (1). Despite surgical treatments (transurethral resection or radical cystectomy) that reduce the quality of life of the patients, bladder cancer can not only be recurrent but also spread via metastasis, becoming a highly life-threatening disease (2).

When a non-invasive tumor grows beyond the limit of oxygen and nutrient diffusion (>1-mm), a new blood supply is required to allow subsequent growth of the tumor. The cells are then placed in a hypoxic environment with the risk of necrosis for unsupplied cells (3,4). The metabolism of cells may be altered and growth stopped (5,6). Nevertheless, cells can also react to this change in their environment (7,8) and escape from the tumor to reach a more hospitable location (9-11). In order to produce metastases, cancer cells have to migrate through the extracellular matrix, enter the blood/lymph vessels and disperse. This process involves the following: i) The decrease of cell-cell attachment, ii) the expression of factors promoting or helping migration, both are part of epithelial-mesenchymal transition (EMT); iii) the degradation of the extracellular matrix (ECM) through the secretion and activation of matrix metalloproteinases (MMP); iv) the ability to survive in the blood/lymph environment (for example, in oxidative stress and anoikis); and v) the ability to colonize other organs (12). Cancer is a complex biological phenomenon, and all aspects are linked; therefore, investigating a specific effect without considering other biological responses should be avoided. For example, a change in the metabolism (Warburg or reverse Warburg) is linked to EMT (13), cell proliferation, angiogenesis (14) and migration, in addition to metastases (15) and apoptotic resistance (16). Thus, non-invasive bladder cancer must remain localized to be efficiently cured. EMT, proliferation, migration and resistance to apoptosis remain the key

elements to control in order to prevent cancer cell dispersion from a localized tumor (17,18).

As in numerous other types of cancer, the presence of hypoxia markers have been recognized as predictors of poor prognosis for bladder cancer evolution and treatment (19,20). It was previously demonstrated that hypoxic bladder cancer cells enhance their malignant nature (proliferation, migration, invasion and chemotherapy resistance) by remodeling their tumor microenvironment (9,10) and by enhancing glycolysis (21). Therefore, it is important to study the effect of hypoxia on bladder cancer cells to obtain clinically relevant results. One of the novelties of the present study is the choice of two grade 1 bladder cancer cell lines compared with two grade 3 bladder cancer cell lines; whereas, to the best of our knowledge, the majority of previous studies only used grade 2 and 3 bladder cell lines. Indeed, low-grade bladder cancer can progress to more aggressive ones and potentially recur after initial transurethral resection (22). Also, in order to control the progression of bladder cancer, understanding the difference between low-grade and high-grade tumor responses to their environment could be helpful. Specifically, it has been demonstrated that hypoxia can predispose metastases in several different cancer types (23,24).

The present study aimed to illustrate that standard cell culture parameters are inadequate to mimic cancer biology and that the use of such models could explain the relatively unsuccessful translation to humans of discoveries in cancer research (25). Modifying only one cell culture parameter, such as O_2 concentration, resulted in substantial changes in several aspects of cell biology. A number of parameters differ between standard cell culture and actual tumour microenvironment. Recently, research on the effect of hypoxia in cell biology have been awarded the Nobel prize, paving the way for more research focusing on this important aspect of the cancer microenvironment in the future (https://www.nobelprize.org/prizes/medicine/2019/advanced-information/; accessed 27 January 2022).

Materials and methods

Cell lines. The present study used four bladder cancer cell lines: Dr Y Fradet's lab generously provided MGHU-3 from the transitional cell carcinoma of a 76-year-old Caucasian male (grade 1; CVCL_9827) (26), SW-780 (grade 1; CRL-2169; American Type Culture Collection) from the transitional cell carcinoma of an 80-year-old Caucasian female, SW-1710 (grade 3; ACC 426; German Collection of Microorganisms and Cell Cultures GmbH) from bladder tumor of an 84-year-old Caucasian female, and T24 (grade 3; HTB-4; American Type Culture Collection) from the transitional cell carcinoma of an 81-year-old Caucasian female. Cells were cultivated in Dulbecco-Vogt modification of Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% bovine growth serum (HyClone; Cytiva), 100 U/ml penicillin (Sigma-Aldrich; Merck KGaA), 25 μg/ml gentamicin (Schering AG) in 8% CO_2 at 37˚C. Media were changed three times a week.

Hypoxic culture conditions. A desiccator cabinet (Nalgene; Thermo Fisher Scientific, Inc.) was modified to become a hypoxic chamber (27). The hypoxic chamber was placed in a standard cell culture incubator at 37˚C. A gas canister (Linde Canada, Inc.) containing an O_2-reduced gas mix of 2% O_2, 8% CO_2 and 90% N_2 (2%) was connected to the chamber. After all operations in an open chamber, the atmosphere was renewed with the appropriate O_2-reduced gas mix. During the gas exchange, a pipe evacuated the old gas from the incubator. O_2 pressure was confirmed using a Pac 5500 O_2 (Drägerwerk AG & Co. KGaA). Time of exposure of bladder cancer cells to hypoxia or normoxia was set at 72 h to mimic a chronic exposure.

Semi-quantification by western blotting. Cells cultivated for 72 h in the normoxic atmosphere (20% O_2) or hypoxic conditions (2% O_2) were rinsed two times with PBS and lysed directly in the cell culture plate in a buffer (31 mM Tris, 10% glycerol, 3% SDS, pH 6.8) and collected with cell scrapers. Cell lysates were sonicated [3 pulse of 3 sec at an amplitude of 20% in an ice water bath with a digital sonifier (Branson Ultrasonics Corporation)]. Protein amount was determined using MicroBCA protein assay kit according to the manufacturer's protocol (Themo Fisher Scientific, Inc.). All experiments were performed with independent triplicates for each of the four cell lines. An equal amount of proteins, 10 μl (1 μg/μl) of each sample, were separated by SDS-PAGE (10% gels) and transferred onto PVDF membranes (Bio-Rad Laboratories, Inc.). Non-specific binding sites were blocked for 1 h at room temperature with 5% (w/v) non-fat powdered milk (BioBasic, Inc.) before an overnight incubation at 4˚C with the specific antibodies (Table I). Secondary antibodies were immunopure antibodies (Thermo Fisher Scientific, Inc.) goat anti-rabbit (cat. no. 31460) or anti-mouse (cat. no. 31430) IgG (H+L) conjugated to horse radish peroxidase. These antibodies were incubated at room temperature for 1 h at the concentration indicated in the Table I. A SuperSignal™ West Dura Extended Duration substrate (Thermo Fisher Scientific, Inc.) was used, and the light signal was detected using Fusion Fx7 (Vilber Lourmat). ImageJ version 1.53e software (National Institutes of Health) was used to estimate the relative amount of protein.

Doubling time determination. The four cancer cell lines were seeded in 12-well plates at a density of 20,000 cells per well (12% confluence). They were cultivated in a normoxic atmosphere (20% O_2, control) or hypoxic conditions (2% O_2). The next day (Day 1), water-soluble tetrazolium (WST)-1 assay (Roche Diagnostics) was used following the manufacturer's instructions. Relaxing time of 1 h in normoxic conditions was allowed for all cultures before being treated with WST-1 to avoid inhibition of the mitochondrial oxidative chain. Cell density was also measured at days 2 and 3. Doubling time was determined by the classic formula, D=Ln(2)/g, where g is the exponential coefficient of the slope. Overall, three independent experiments in octuplicate were performed for each of the four cell lines.

Acidity measurement. Cell culture medium (DMEM supplemented with 10% bovine growth serum, 100 U/ml penicillin, 25 μg/ml gentamicin) conditioned for 72 h by cells in the normoxic atmosphere (20% O_2, control) or hypoxic conditions (2% O_2) were collected from plate into tubes. The pH was measured using MQuant pH indicator strips (MilliporeSigma).

Table I. Antibodies used for western blotting.

Target protein	Host species	Company	1st antibody dilution	2nd antibody dilution	Cat. no.
β-actin	Mouse	Sigma-Aldrich; Merck KGaA	1/20,000	1/20,000	A5441
E-Cadherin	Mouse	Abcam	1/1,000	1/1,000	Ab1416
EpCAM	Rabbit	Abcam	1/500	1/1,000	Ab32392
N-Cadherin	Mouse	MilliporeSigma	1/1,000	1/1,000	05-915
TSP-1	Rabbit	Cell Signaling Technology, Inc.	1/1,000	1/1,000	37879
Vimentin	Rabbit	Abcam	1/2,500	1/2,000	Ab45939

EpCAM, epithelial cell adhesion molecule; TSP-1, thrombospondin-1.

The pH was used to determine the level of acidity associated with metabolism. All experiments were performed in independent triplicates for each of the four cell lines.

Cell viability measurement. For cell viability measurements, WST-1 (Roche Diagnostics) was used following the manufacturer's instructions. All cell cultures were performed in the normoxic atmosphere (20% O_2) or hypoxic conditions (2% O_2). Cells were plated at 50% confluence. Subsequently, 24 h later, DMEM containing ascorbic acid (Sigma-Aldrich; Merck KGaA) at various concentrations (0, 1, 2 or 4 mM) was added. The culture was continued for 24 h, and then WST-1 was added. A relaxing time of 1 h in normoxic conditions was allowed for all cultures before being treated with WST-1 to avoid inhibition of the mitochondrial oxidative chain. Incubation with this reagent took place in normoxia and measurements were collected after 2 h, when the maximal value of optical density was between 0.8 and 1.2. A total of three independent experiments in octuplicate were performed for each of the four cell lines.

MMP activity measurement. Supernatant from 72 h cell cultures in the normoxic atmosphere (20% O_2) or hypoxic conditions (2% O_2) was collected. Total MMP activity was determined in cell culture supernatants using the SensoLyte™ 520 Generic MMP assay kit (cat. no. AS711-58; AnaSpec). This kit can simultaneously detect the activity of MMP-1, 2, 7, 8, 9, 12, 13 and 14. APMA was not used; therefore, inactive forms of MMP were not detected. The results reflect the overall active MMP and metalloproteinase inhibitor balance. A total of three independent experiments in triplicate were performed for each of the four cell lines.

Cell migration assay. The four bladder cancer cell lines were seeded at 80% confluence and cultured for 48 h in a normoxic atmosphere (20% O_2, control) or hypoxic conditions (2% O_2). After 24 h, the cell culture medium (DMEM supplemented with 10% bovine growth serum, 100 U/ml penicillin, 25 μg/ml gentamicin) was exchanged for serum-free cell culture medium (DMEM supplemented with 100 U/ml penicillin, 25 μg/ml gentamicin) and culture continued for another 24 h. At this time, a scratch was performed using a 200 μl-pipette tip. Plates were rinsed three times with PBS at 37˚C to remove detached cells and culture was continued in serum-free medium for 24 h. At the indicated time points, images were captured using a CKX41 light microscope (Olympus Corporation) with an E-620 camera (Olympus Corporation) at a magnification of 40X and analyzed using ImageJ software (National Institutes of Health). All experiments were performed two times in quadruplicate for each of the four cell lines. For MGHU-3 and SW-1710 bladder cancer cell lines, additional experiments were performed using factors to modulate migratory responses to hypoxia: MG-132 (20 μM; Enzo Life Sciences, Inc.), cobalt chloride (200 μM; Sigma-Aldrich; Merck KGaA), hypoxia-inducible factor (HIF)-2 antagonist (10 μM; Sigma-Aldrich; Merck KGaA) and rottlerin (2 μM; Santa Cruz Biotechnology, Inc.). These factors was added at the indicated concentration at the same time as cell culture medium containing serum was exchanged for serum-free cell culture medium and maintained after the cell monolayer was scratched.

Statistical analysis. A bilateral unpaired Student's t-test assessed differences between values. The data were analyzed using Excel 2003 (Microsoft Corporation). For datasets containing ≥3 groups, ordinary one-way ANOVA followed by Tukey's multiple comparison test were performed (GraphPad Prism v.9.0.2 Software; GraphPad Software, Inc.). All data were expressed as mean ± standard deviation, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

The effect of hypoxia on EMT potential of bladder cancer cells. When placed in hypoxic cell culture conditions (2% O_2), T24 and SW-780 bladder cancer cell lines did not present a significant change in epithelial cell adhesion molecule (EpCAM) expression compared with the cells cultured in normoxic conditions (20% O_2). MGHU-3 bladder cancer cell lines revealed a significant decrease in EpCAM expression when cultured in hypoxic cell conditions compared with the cells cultured in normoxic conditions, whereas it significantly increased in SW-780 cultures exposed to hypoxia (Fig. 1). When placed in hypoxic cell culture conditions, SW-1710, T24 and MGHU-3 bladder cancer cell lines did not present a significant change in

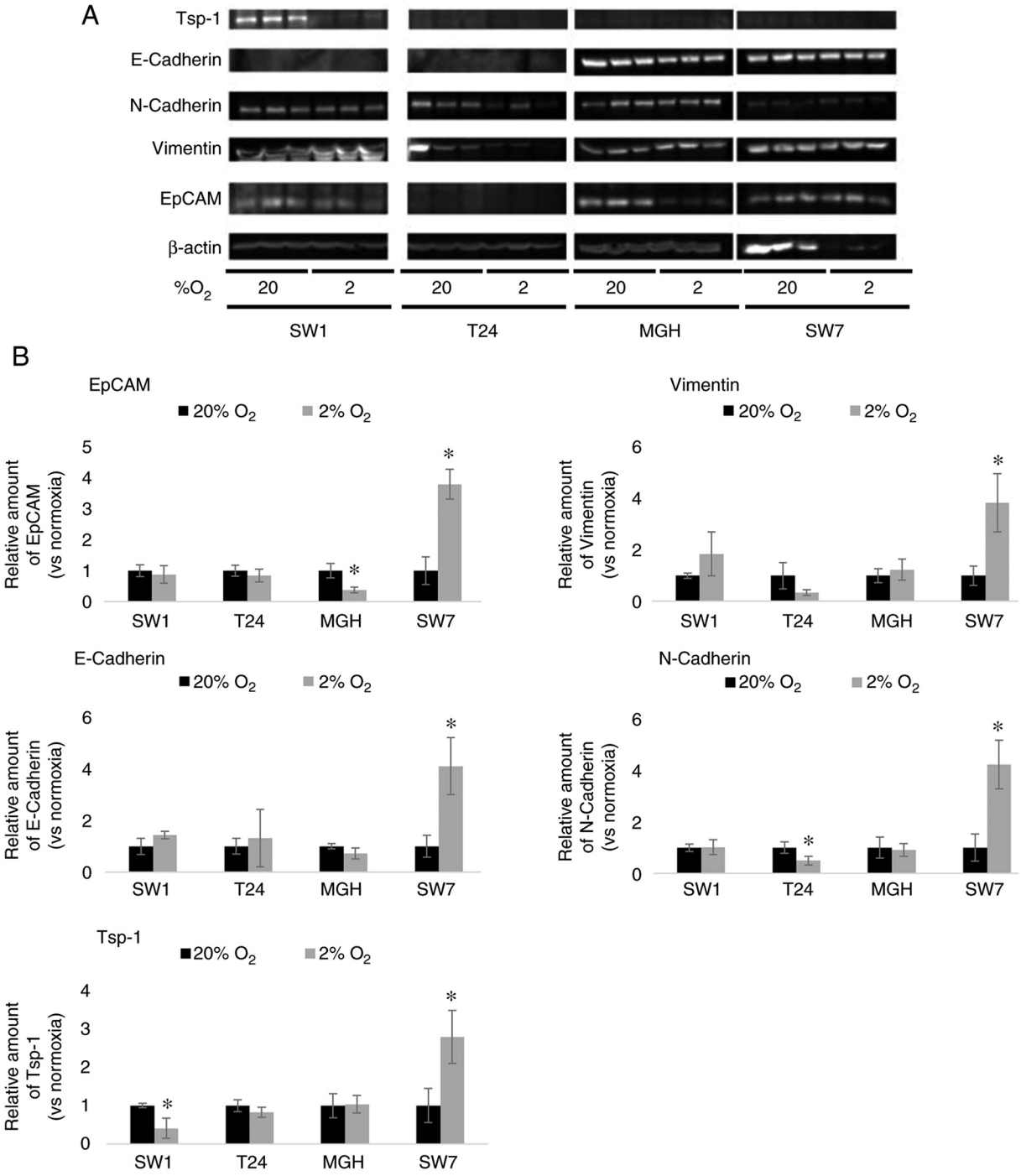

Figure 1. Effect of hypoxia on the expression of epithelial-mesenchymal transition markers and thrombospondin-1 by bladder cancer cell lines in normoxic or hypoxic cell culture conditions. (A) Representative images captured by Fusion FX7 of PVDF membranes. (B) Semi-quantitative determination of protein expression normalized to β-actin content. Values are expressed as % of normoxic condition protein expression. White bars are for normoxic conditions and black bars for the hypoxic atmosphere. N=3, n=1. *P<0.05 vs. normoxic conditions. SW1, SW-1710 cells; MGH, MGHU-3 cells; SW7, SW-780 cells; EpCAM, epithelial cell adhesion molecule; Tsp1, thrombospondin-1.

vimentin expression compared with normoxic conditions. By contrast, SW-780 cells demonstrated a significant increase in vimentin expression when cultured in hypoxic cell conditions compared with the SW-780 cells cultured in normoxic conditions (Fig. 1). Expression of E-Cadherin remained unchanged in SW-1710, T24 and MGH-U3 cancer cell lines cultured in hypoxic conditions compared with normoxic conditions; whereas it demonstrated a significant increase in SW-780 cells in hypoxic conditions compared with normoxic conditions. Nevertheless expression levels of E-Cadherin were very low, near the limit of detection, in both of the high grade cancer cell line cultures (T24 and SW-1710). Expression of N-Cadherin revealed no significant difference in SW-1710 and MGH-U3 cancer cell lines cultured in hypoxic conditions compared with normoxic conditions; however, expression was significantly decreased in T24 and significantly increased in SW-780 cancer cell line cultures in hypoxic conditions compared with the normoxic conditions (Fig. 1).

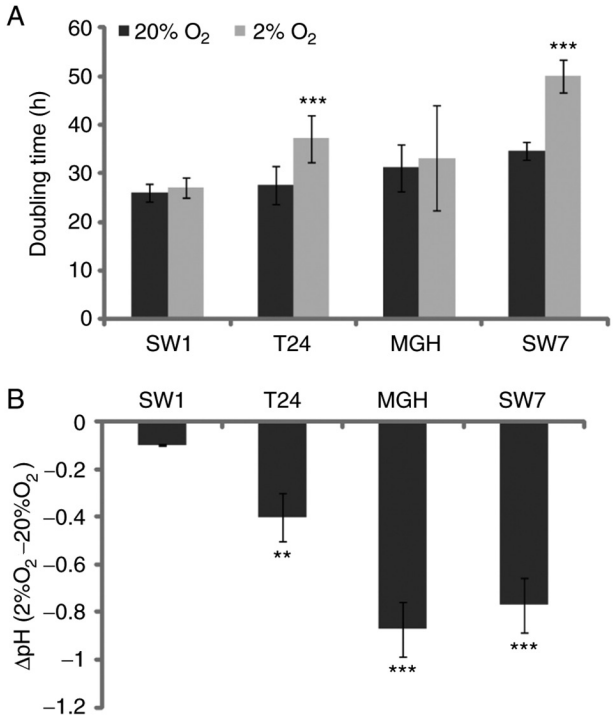

Figure 2. Effect of hypoxia on the proliferation and metabolism of bladder cancer cell lines. (A) Doubling time of cancer cells. White bars are for normoxic conditions and black bars for the hypoxic atmosphere. (B) Differences in pH measurement between 72 h hypoxic and normoxic cell culture supernatants. N=3; n=1. **P<0.01, ***P<0.001 vs. normoxic conditions. SW1, SW-1710 cells; MGH, MGHU-3 cells; SW7, SW-780 cells.

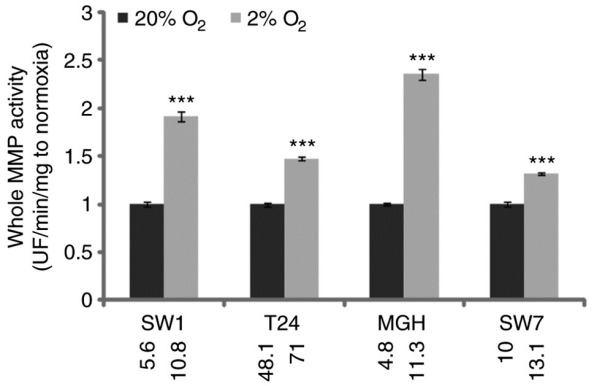

Figure 3. Effect of hypoxia on the activity of MMPs in bladder cancer cell line cultures. Whole MMP activity is measured in arbitrary units/min/mg proteins. White bars are for normoxic conditions and black bars for the hypoxic atmosphere. Numbers under the x-axis are the absolute value of fluorescence in arbitrary fluorescence unit per min per mg proteins. N=3, n=3. ***P<0.001 vs. normoxic conditions. MMPs, matrix metalloproteinases; SW1, SW-1710 cells; MGH, MGHU-3 cells; SW7, SW-780 cells.

The effect of hypoxia on thrombospondin-1 (TSP-1) synthesis by bladder cancer cells. Hypoxia induced a significant reduction of the TSP-1 expression in the SW-1710 grade 3 bladder cancer cell line; by contrast, the T24 and MGHU-3 cells lines revealed no significant difference between the normoxic and hypoxic conditions. Grade 1 bladder cancer cell line SW-780 demonstrated a significant increase of TSP-1 expression in hypoxic conditions compared with the normoxic cells (Fig. 1).

The effect of hypoxia on bladder cancer cell growth and metabolism. In order to determine the tumor growth capacity under hypoxic conditions, bladder cancer cell number was determined on three consecutive days in control atmospheric conditions, 20% O_2, and hypoxic conditions, 2% O_2. Subsequently, doubling times were calculated from the obtained growth curves (Fig. 2A). Doubling times were significantly increased for SW-780 and T24 bladder cancer cells in hypoxic conditions as compared with the control, indicating that these cells proliferated less rapidly. By contrast, doubling times remained unaffected or little affected for the two other bladder cancer cell lines. The pH was measured in 3-day bladder cancer cell culture supernatants (Fig. 2B). Except for SW-1710, where the pH remained relatively unchanged, for all three of the other bladder cancer cell line cultures, the pH was significantly decreased with the reduction in O_2 pressure.

The effect of hypoxia on whole MMP activity secreted by bladder cancer cells. Next, 3-day bladder cancer cell culture supernatants were collected and whole MMP activity was determined by cleavage of a fluorometric substrate (Fig. 3). Whole MMP activity was significantly increased in hypoxic conditions in all four cell lines, increasing the potential for the migration of cancer cells through the ECM to the vascular/lymph network. Nevertheless, SW-1710 and, notably, MGHU-3 demonstrated the most significant increases, whereas T24 and SW-780 slightly increased their potential for ECM degradation.

The effect of hypoxia on bladder cancer cell migration capacities. The migration ability of bladder cancer cells was examined in normoxia (20% O_2) and hypoxia (2% O_2). As presented in Fig. 4A and B, MGHU-3 cells demonstrated a significantly increased ability to migrate in a hypoxic environment compared with that in a normoxia environment. By contrast, SW-1710 and SW-780 had a significantly reduced capacity to migrate in a hypoxic environment, while T24 migration potential revealed no significant change. Mimicking or inhibiting hypoxia could be achieved by the use of chemicals. For SW-1710, ANOVA indicated a P-value <0.0001. The non-covered area at day 1 after scratching the confluent monolayer cell culture in normoxia condition was significantly reduced compared with the hypoxia condition (P=0.0046) and the normoxia + $CoCl_2$ condition (P=0.0001); however, it was not significantly different not from the one in hypoxia + rottlerin group (P=0.9013). The non-covered area at day 1 after scratching the confluent monolayer cell culture in hypoxia condition was significantly increased compared with the one in hypoxia + rottlerin condition (P=0.0251), but not the one in normoxia + $CoCl_2$ condition (P=0.2684).

For MGHU-3, ANOVA indicated a P-value=0.0006. The non-covered area at day 1 after scratching the confluent monolayer cell culture in normoxia condition was significantly increased compared with the hypoxia condition (P=0.0013) and the normoxia + MG-132 condition (P=0.0099), but not the one in from hypoxia + rottlerin condition (P=0.1598). The non-covered area at day 1 after scratching the confluent monolayer cell culture in hypoxia condition was significantly decreased compared with the one in hypoxia + rottlerin condition (P=0.0536), but not from

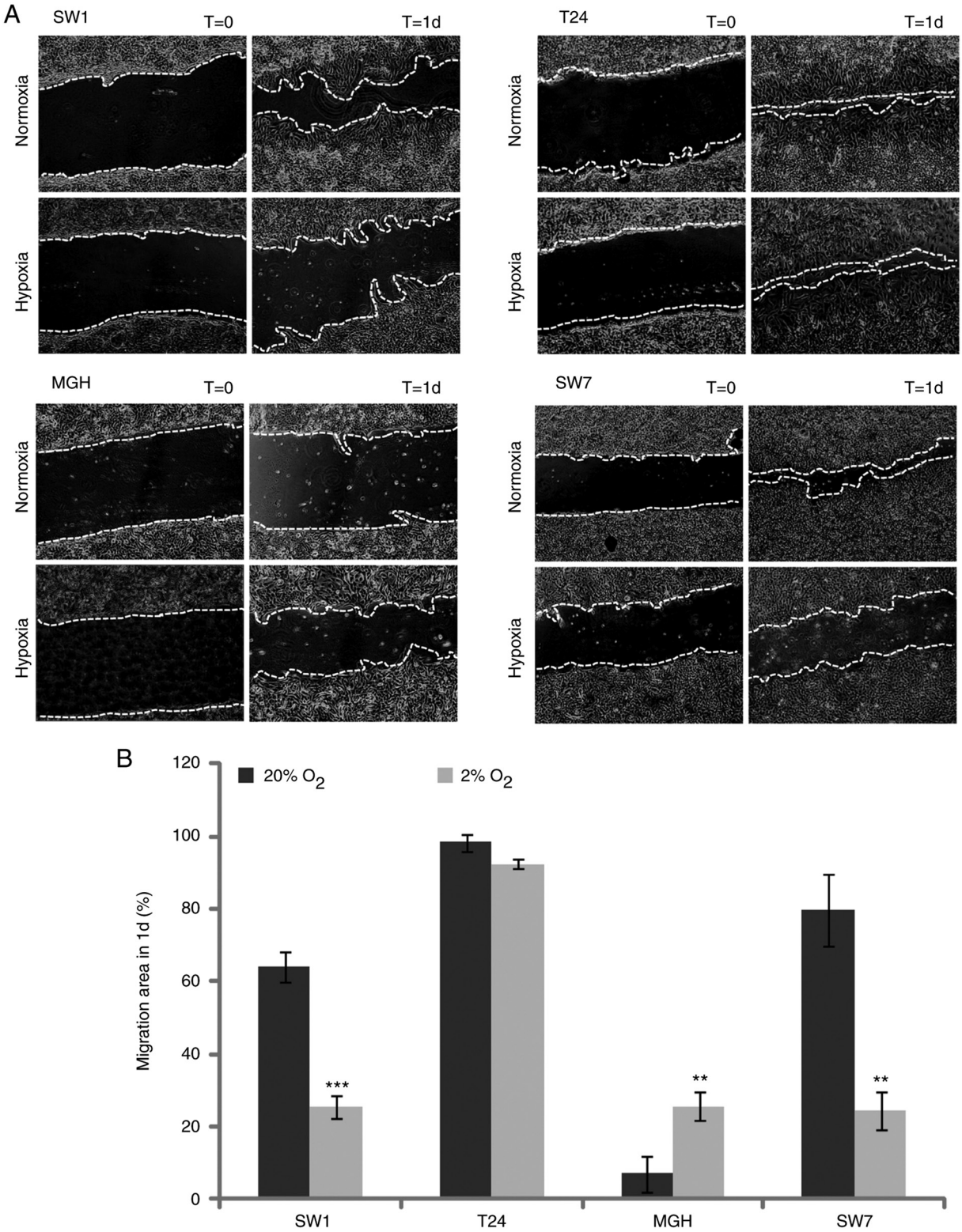

Figure 4. Effect of hypoxia on the migration capacity of bladder cancer cell lines. (A) Microscopic observation of migration of scratched cells, 1 day after the scratch. Normoxic (20% O_2) and hypoxic (2% O_2) conditions were tested (magnification, x40). (B) Migration index determination. The newly populated area (in pixels) gives the migration index. White bars are for normoxic conditions and black bars for the hypoxic atmosphere. N=2, n=4. **P<0.01 and ***P<0.001 vs. normoxic conditions. SW1, SW-1710 cells; MGH, MGHU-3 cells; SW7, SW-780 cells.

the one in normoxia + MG-132 condition (P=0.6242). SW-1710 migrated faster in normoxia compared with in hypoxia, activating hypoxic pathways slowed SW-1710 migration whereas inhibiting hypoxic pathway accelerated the migration. Inverse results were found for MGHU-3 (Fig. 5A and B).

The effect of hypoxia on the resistance of bladder cancer cells to oxidative stress-induced apoptosis. The resistance of cells to oxidative stress-induced apoptosis was tested in bladder cancer cell lines in normoxic (20% O_2) or hypoxic (2% O_2) cell culture conditions (Fig. 6) by determining their viability

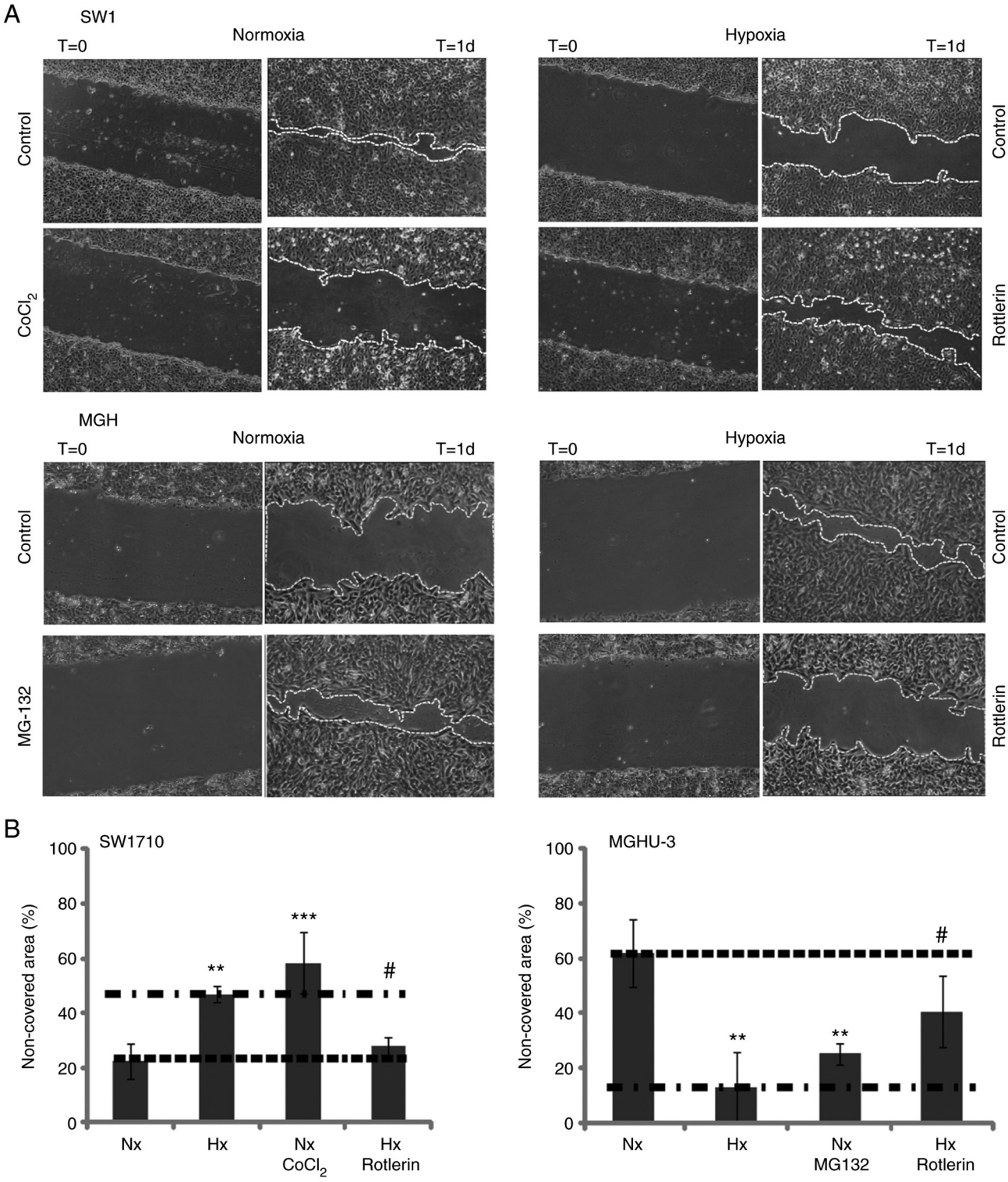

Figure 5. Effect of factors mimicking hypoxia in normoxia and normoxia in hypoxia on the migration capacity of bladder cancer cell lines. (A) Migration assay (scratch test): SW-1710 and MGHU-3 cells were used. Representative photographs of migration after 0 h (T=0) and 24 h (T=1d) at magnification, x40. (B) Uncovered areas were determined from photographs such as presented in panel B. Percentage of uncovered areas are the area uncovered by cells at day 1 divided by the surface of the scratch at day 0. The dotted lines, which represent the normoxic condition (dash) or the hypoxic condition (dash/dot). **P<0.01 and ***P<0.001 vs. normoxic conditions; $^{\#}$P<0.05 vs. hypoxic conditions. Nx, normoxia (20% O_2); Hx, hypoxia (2% O_2); $CoCl_2$, cobalt chloride.

using a WST-1 viability assay. After ascorbate treatment, the viability of bladder cancer cells in hypoxia was significantly reduced compared to the one in normoxia (SW-1710 and MGHU-3 cells for a concentration of 2 and 4 mM ascorbate, T24 cells for a concentration of 1 and 2 mM ascorbate). By contrast, after ascorbate treatment, the viability of the SW-780 cell line in hypoxia was slightly but significantly increased compared to the one in normoxic conditions. The discrepancy in the results for SW-780 should be noted because of its potential impact on therapies.

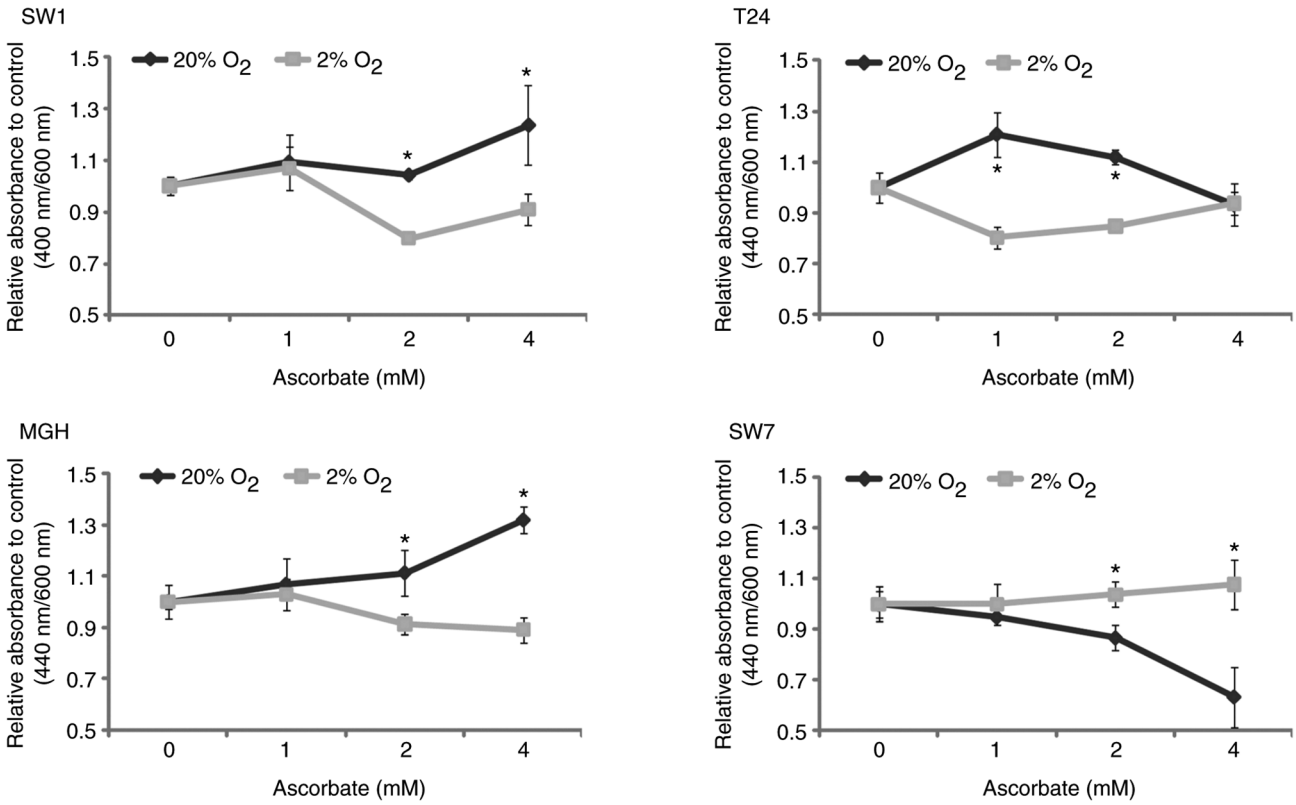

Figure 6. Resistance to oxidative stress-induced apoptosis of bladder cancer cells in normoxic or hypoxic cell culture conditions. After 48 h in normoxic (20% O_2) or hypoxic (2% O_2) conditions, ascorbate was added at a concentration of 0, 1, 2 and 4 mM, and 24 h later WST-1 was added, and optical density was measured to determine the number of living cells. N=3, n=8. In the upper panels, the results are normalized to controls (0 mM ascorbate). Filled lines and filled squares (□) are for hypoxic cell culture conditions; filled lines and close diamonds (♦) are for normoxic cell culture conditions. *P<0.05 vs. normoxic conditions. SW1, SW-1710 cells; MGH, MGHU-3 cells; SW7, SW-780 cells.

Discussion

Hypoxia defines a condition where the oxygen concentration is decreased compared with the physiological concentration called normoxia. However, in the majority of studies, hypoxia refers to a lower level of oxygen compared with that of ambient air (20%). This concentration does not correspond in any way to a physiological condition because, in the human body, the oxygen concentrations are much lower (28,29). What is called hypoxia in the majority of studies is actually physiological normoxia, and what is called normoxia is hyperoxia (28,29). A 2% oxygen concentration was selected in the present study because it is below the concentration found in the bladder or adjacent tissues (30). In the present study, time of exposure of bladder cancer cells to hypoxia or normoxia was set at 72 h to mimic a chronic exposure. Some studies use more prolonged exposure, but for a technical reason, experiments were terminated after 72 h. A home-made hypoxic chamber was utilized, and it required that medium exchange be performed outside the chamber, therefore in normoxia, and it was challenging to maintain the cells in culture for >72 h.

It is also clear that in a tumor mass the concentration of oxygen varies from the center of the tumor, which could be anoxic or severely hypoxic, and the periphery of the tumor, which could be at the same O_2 concentration of healthy tissues or even at a higher concentration in the case of an adequate vascular perfusion (31,32). A metabolic coupling has been demonstrated in bladder cancer between these compartments and tumor aggressiveness (33).

EMT is a hallmark of these changes (34). Hypoxia can modify several parameters such as tumor progression and resistance to therapy (7,35), and these changes can be irreversible after prolonged exposure, especially EMT (36). Mechanisms linking hypoxia and EMT have been extensively described (37-39). Although it is considered to be a cell adhesion molecule only, the epithelial cell adhesion molecule EpCAM is overexpressed in a number of carcinomas (40). Benign epithelia express EpCAM at a variable but generally lower level compared with carcinoma cells (41). EpCAM can modulate cell migration, proliferation and differentiation, and also prevent cell-cell adhesion (42,43). Vimentin is a well-known marker of EMT (44,45). In the present study, SW-1710, T24 and MGHU-3 cells tended to reduce their EpCAM expression, whereas SW-780 cells tended to increase theirs. SW-1710, MGHU-3 and SW-780 cells tended to increase their vimentin expression, whereas T24 cells did not.

Another marker of cancer progression is the metabolic switch. Cancer cell metabolism affects the immediate environment, notably by acidifying the culture medium (46-48). Hypoxia is known to change cell metabolism by up-regulating the glycolytic enzymes (49). Measuring the acido-basic equilibrium can, therefore, reflect changes in metabolism (Warburg effect) (50,51). It should be noted that the cell number in culture also influences this parameter. Recently, a distinct metabolomic profile has been defined for

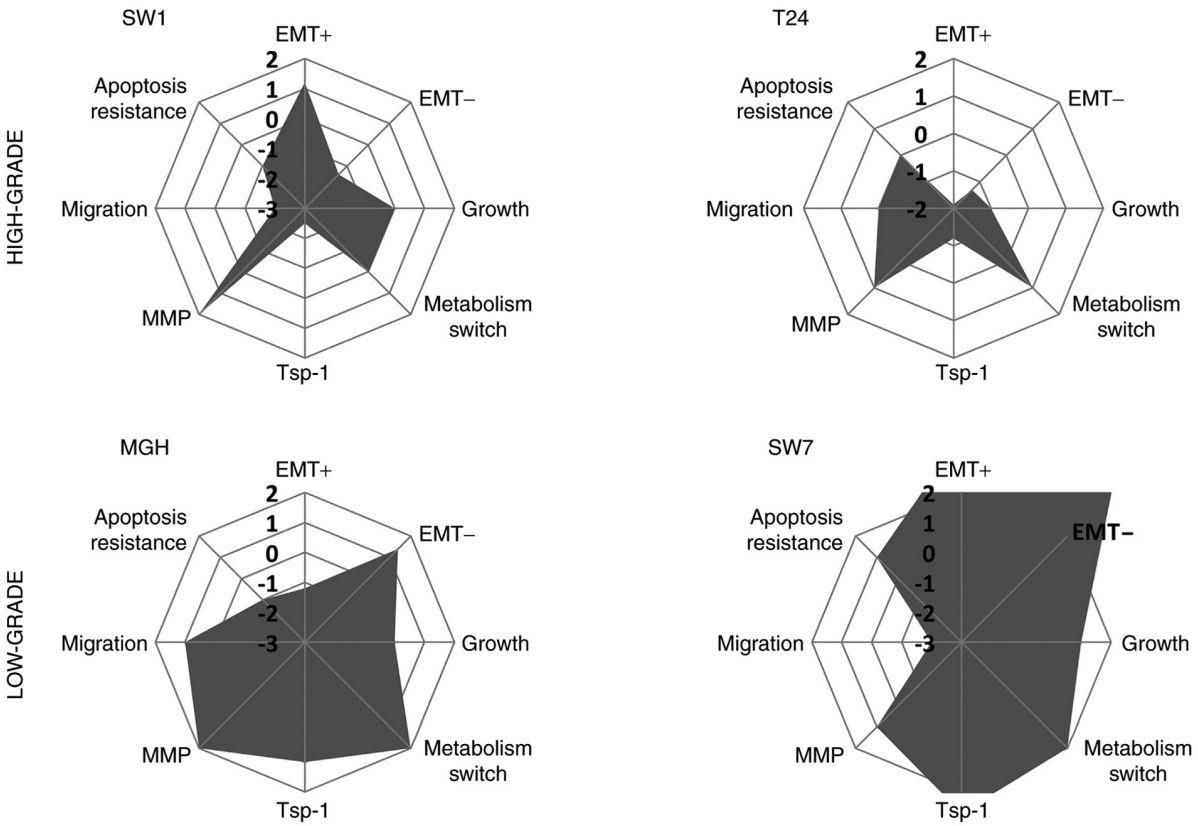

Figure 7. Low-grade bladder cancer cell lines became more aggressive after exposure to hypoxia. For each cell line, the eight investigated parameters are represented in a diagram where the value is positive for increasing aggressiveness (2 or 1 indicating the magnitude of the increase), negative for decreasing aggressiveness (-2 and -1 indicating the magnitude of the decrease) and null (0) if no modification was observed. SW1, SW-1710 cells; MGH, MGHU-3 cells; SW7, SW-780 cells; EMT, epithelial-mesenchymal transition; Tsp-1, thrombospondin-1; MMP, matrix metalloproteinases.

low and high-grade bladder cancer cultured cells, but the impact of hypoxia has not yet been studied to the best of our knowledge (52).

Metabolism changes can modify the progression potential of tumor cells. The relationship between activation of EMT and modification of the metabolism has been demonstrated in muscle-invasive bladder cancer via lactate dehydrogenase A (LDH-A); the enzyme that catalyzes the transformation of pyruvate into lactate, an acidic component with multiple recognized signaling function (53). More specifically, in the case of bladder cancer, cancer progression is inhibited by the expression of microRNA-200c, which targets LDH-A (54). In the present study, except for SW-1710 where no modifications were demonstrated between normoxic and hypoxic conditions, all other cell lines acidified the cell culture medium faster in hypoxic conditions compared with in normoxic conditions. Even if the SW-780 and T24 cell lines had reduced growth, they still increased the acidity of the cell culture medium, and therefore changes in cell metabolism should be investigated (55,56).

No clear relationship can be established between the measure of pH and the activation of the EMT in these bladder cancer cell lines. It should be noted that the model in the present study could be improved by the addition of co-culture with fibroblasts, or even improved more with cancer-associated fibroblasts in order to investigate the metabolic interplay between bladder cancer cells and their direct microenvironment (57).

Recently, exposure to hypoxia and cell proliferation has been associated with bladder cancer through the expression of long non-coding RNA (58). The growth rates of the bladder cancer cell lines in the present study were also impacted by exposure to hypoxic conditions with a decrease in the proliferation of T24 and SW-780 cell lines and few changes for SW-1710 and MGHU-3 cell line cultures.

Among ECM components, an important player in urothelial cancer is TSP-1. Even though an increased expression of TSP-1 in tumor cells has been associated with an increase or a decrease in microvessel density, depending on the intracellular calcium concentration, TSP-1 expression is known to be positively correlated with cell migration; thus, TSP-1 may play an important role in tumor aggressiveness (59,60). In the present study, the expression of TSP-1 was significantly decreased under hypoxic conditions in SW-1710 high-grade bladder cancer cell line cultures, whereas this expression was significantly increased in SW-780 cells or remained unchanged in low-grade bladder cancer cell line cultures.

To spread in the organism, cancer cells have to migrate in the epithelium, then across the basal lamina and finally through the extracellular matrix until they find blood or lymphatic vessel (61-63). In order to grow and potentially to escape its original location, a tumor has to degrade the surrounding ECM (64). This action is mainly achieved by secreting and activating MMPs. It is also known that ECM remodeling can play a role in the reorganization of the stroma in order to facilitate the cancer cell escape (65). In the present

study, after exposure to hypoxic conditions, all bladder cancer cell lines significantly increased their MMP activities.

After an evaluation of some aspects of the EMT, proliferation, metabolism and ECM synthesis/degradation balance in bladder cancer cell line cultures exposed to hypoxia, the present study aimed to measure their ability to migrate. In contrast to what was noted for the expression of the EMT markers, SW-1710 and SW-780 had reduced migration ability under hypoxic conditions, whereas MGHU-3 increased it and T24 remained unaffected. In order to confirm the involvement of HIF-1α, several factors were added to cell cultures, and migration was subsequently evaluated. To mimic the effects of hypoxia in normoxia, MG-132 and cobalt chloride were used. On the other hand, to mimic the effects of normoxia in hypoxia, rottlerin was used in normoxia. HIF-1α is rapidly degraded in the proteasome after binding with the von Hippel-Lindau factor (66,67). MG-132 inhibits the degradation of proteins through the proteasome (68), whereas cobalt chloride stabilizes HIF-1α by preventing its binding to the von Hippel-Lindau factor (69). In hypoxia, HIF-α (1, 2 or 3) forms a transcriptionally active complex with HIF-1β and p300 (70), which activates PKCδ (71). Rottlerin inhibits PKCδ (72). These experiments highlighted the role of HIF-1α in response to hypoxic stress. A previous study highlighted the link between hypoxia and tumor cell motility through RhoA and ROCK1 (73).

A number of strategies for limiting tumor growth and dissemination use oxidative agents such as ascorbic acid (vitamin C) or menadione (74,75). Even if some studies highlighted that chronic hypoxia promotes chemotherapeutic agent resistance, such as etoposide and vincristine (76), it was hypothesized that cancer cells, which grow in a hypoxic microenvironment, should be more sensitive to cell death induced by oxidative agents. Cancer cell lines are adapted to a hypoxic environment and are thus more sensitive to oxidative agents. This result is consistent with the literature (74). Future studies will test the resistance to oxidative stress in the context of low nutrients, as high glucose levels can affect the response of the cells (77).

Nevertheless, even if the response of each bladder cancer cell line seems different to an exposure to hypoxic conditions in culture, the low-grade bladder cancer cell lines (MGHU-3 and SW-780) seemed to become more aggressive after low O_2 pressure exposure whereas the high-grade bladder cancer cell lines (SW-1710 and T24) became less aggressive (Fig. 7). It should be noted that every cell line had some increase in pro-aggressive parameters and some in anti-aggressive elements; what the exact contribution would be *in vivo* remains to be determined. A total of eight parameters were tested, and variations were revealed in the majority of these when cells were incubated in hypoxic conditions, as compared with results obtained in normoxia (6, 6, 7 and 8 parameters were modified for SW-1710, T24, MGHU-3 and SW-780 cancer cell lines respectively). Studies on bladder cancer cell lines have to consider the microenvironment of the cells, especially hypoxia, in order to produce data that could be used for clinical translation. Other elements of the cellular environment also play a critical role, especially the ECM composition and stiffness, and the development of new models integrating three-dimensional cell-cell and cell-matrix contacts (78) could be very helpful.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SC designed the experiments, acquired, analyzed and interpreted the data and drafted and revised the manuscript. EP, CRG and CC acquired and analyzed the data, and revised the manuscript. FP and SB designed the experiments and revised the manuscript. All authors have read and approved the final manuscript. SC and SB confirm the authenticity of raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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