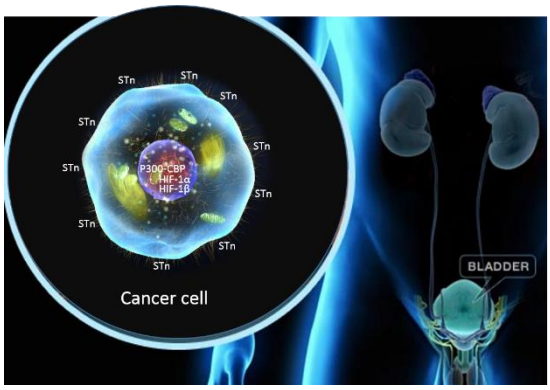

Hypoxic Regulation of Glycosylation in Bladder Cancer

Andreia Filipa Ferreira Peixoto
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Departamento de Química e Bioquímica
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Orientador
José Alexandre Ferreira, Doutor, Universidade de Aveiro
Co-orientador
Maria José Oliveira, Professor Doutor, Faculdade de Ciências

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

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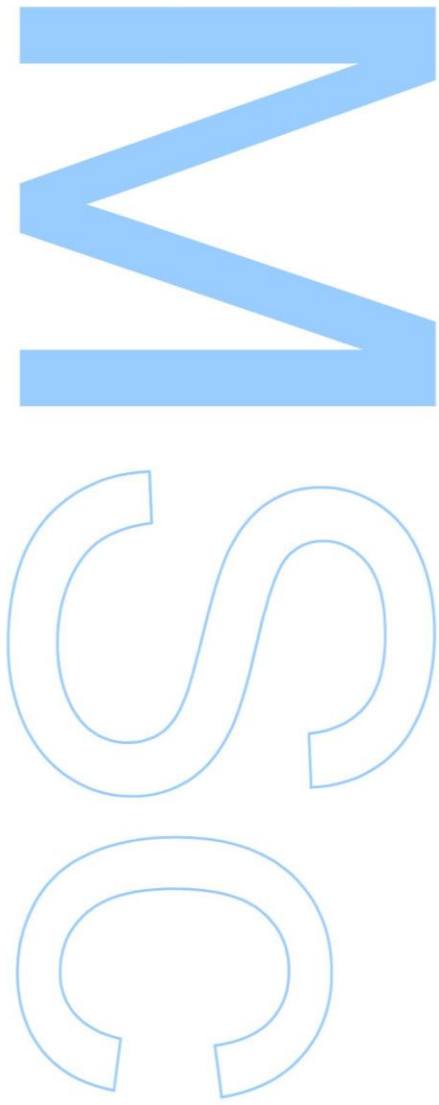

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Sumário

O cancro de bexiga músculo-invasivo figura um dos tipos de cancro genito-urinários mais comuns e com maior taxa de mortalidade. A principal terapêutica utilizada consiste em regimes quimio-terapêuticos baseados em cisplatina, que falham na prevenção da recorrência e disseminação da doença. Assim, são necessários novos bio-marcadores que permitam a estratificação dos doentes e novas estratégias terapêuticas.

Mais de 70% dos tumores de bexiga musculo-invasivos expressam o antigénio Sialil-Tn (STn) em resultado da terminação prematura do processo de elongação das cadeias de O- glicanos das proteínas da superfície celular. O antigénio STn demonstrou-se promotor da invasão celular, da evasão ao sistema imunitário e possivelmente da quimio-resistência, constituindo um bio-marcador importante dos fenótipos celulares mais agressivos de cancro de bexiga. Contudo, o conhecimento acerca dos acontecimentos que promovem esta profunda desregulação dos padrões de glicosilação proteica é escasso. Uma vez que a hipóxia é também uma característica proeminente dos tumores de bexiga avançados, este trabalho visa compreender de que forma os níveis de oxigénio influenciam o glicofenótipo das células tumorais de bexiga, enfatizando a expressão do antigénio STn.

Três linhas celulares tumorais de bexiga com backgrounds genéticos e moleculares distintos (T24, 5637 e HT1376) foram submetidas a hipóxia (0.1% O2). Com o objetivo de identificar processos mediados pelo HIF-1α, as experiências foram conduzidas simultaneamente na presença do estabilizador do HIF-1α Mesilato de Deferoxamina (DFX). Em ambas as condições, todas as linhas celulares sobre- expressam o HIF-1α e a AC IX, uma proteína altamente HIF-1α – regulada, bem como aumentam a biossíntese de lactato, denotando a conversão de metabolismo celular aeróbio para anaeróbio. A hipóxia compromete a proliferação celular e potencia a mobilidade/ invasão celular em matrigel. Todavia, não se verificam diferenças significativas na atividade das MMP avaliadas em hipóxia para todas as linhas celulares, sugerindo estratégias alternativas de mobilidade/ invasão. Fizeram-se observações idênticas aquando da exposição das células ao DFX, sugerindo que se tratam de eventos HIF-1α- mediados. A análise de um painel de 21 genes associados a fenótipos estaminais, epiteliais, de transição epitélio-mesenquimal e mesenquimal por qRT-PCR revelou que a hipóxia promove a ativação de programas de

genes de transição epitélio-mesenquimal e estaminais. Adicionalmente, todas as linhas celulares sobre- expressam o antigénio STn de forma HIF-1α- dependente. Porém, o aumento dos níveis de STn não está associado à sobre- expressão da principal enzima responsáveis pela sua biossíntese, a ST6GalNAC.I, mas sim com a sub-expressão das enzimas envolvidas na elongação das cadeias O-glicosídicas. A exposição de células em hipóxia ao anticorpo monoclonal anti-STn TKH2 promove um decréscimo dramático na mobilidade/ invasão celular, corroborando um papel chave do STn na disseminação da doença. A associação entre a hipóxia, evidenciada pela sobre-expressão de HIF-1α, e a sobre-expressão de STn foi clinicamente confirmada em tecidos tumorais por imuno-histoquímica.

No seu conjunto, estes dados indicam que a hipóxia não só favorece a aquisição de um fenótipo mesenquimal mas também compromete a O-glicosilação proteica como uma forma de conferir ás células maior mobilidade/ capacidade invasiva. Por fim, uma análise glicoproteómica preliminar por western blot, sob o ponto de vista da expressão de STn, revelou que células em hipóxia sobre- expressam proteínas STn positivas de baixo peso molecular (<50 kDa) em relação à normóxia, o que poderá permitir atingir seletivamente células em hipóxia prevenindo a disseminação da doença.

Em resumo, o presente trabalho reafirma a relevância clinica do antigénio STn no cancro de bexiga musculo-invasivo. Apresenta-se ainda um novo mecanismo, baseado na modulação da O-glicosilação proteica e na expressão de STn, através do qual a hipóxia contribui para a agressividade dos tumores de bexiga. Adicionalmente, este trabalho realça a necessidade de se explorar o glicoproteoma de células hipóxicas visando o desenvolvimento de bio- marcadores capazes de melhorar a gestão do cancro de bexiga.

Palavras-chave

Cancro de bexiga; Hipóxia; Sialil-Tn; Glicosilação e metabolismo; Glicosilação proteica, Glicanas associados a cancro, HIF-1α

Abstract

Muscle invasive bladder cancer (MIBC) is amongst the most common and deadliest genitourinary cancers. The mainstay treatment is surgery and cisplatin-based regimens, which fail in avoiding tumour relapse and disease progression, urging for novel biomarkers for accurate patient stratification and new therapeutics.

Over 70% of MIBC express the cell-surface tumour-associated carbohydrate antigen Sialyl-Tn (STn) that stems from a premature stop in the O-glycosylation of cell surface proteins. STn was found to be a promoter of cell invasion, immune escape, and possibly chemotherapy resistance, making it an attractive biomarker for aggressive cellular phenotypes. However, there is scarce information about the events underlying this profound deregulation in protein glycosylation. Based on the fact that hypoxia is also a salient feature of advanced stage bladder tumours, this work devotes to understanding how oxygen levels influences the glycophenotype of bladder cancer cells, with emphasis on the STn antigen.

Three bladder cancer cell lines with distinct genetic and molecular backgrounds (T24, 5637 and HT1376) were submitted to hypoxia (0.1% O_2). In an attempt to determine HIF-1α-mediated events, experiments were also conducted in the presence of the HIF-1α stabilizer Deferoxamine Mesilate (DFX). In both conditions all cell lines overexpressed HIF-1α and its regulated protein CA IX, and increased lactate biosynthesis, denoting a shift to an anaerobic metabolism. Hypoxia also impaired cell proliferation and enhanced motility/invasion in matrigel; however no significant differences were observed in MMP activity in hypoxia for all cell lines, denoting alternative motility/invasion-promoting strategies. Similar observations were made in the presence of DFX, suggesting these are HIF-1α-mediated events. The analysis of a panel of 21 genes associated with stem, epithelial, epithelia-to-mesenchymal (EMT) and mesenchymal phenotypes by quantitative polymerase chain reaction (qPCR) showed that hypoxia led to the activation of EMT/stem cell programs. Concomitantly, all cell lines overexpressed the STn antigen, in an HIF-1α-dependent manner. However, STn elevations did not associate with an overexpression of its main biosynthesis enzyme ST6GalNAC.I, but with a significant down-regulation of the enzymes involved in *O*-glycan elongation. The exposure of hypoxic cells to anti-STn

monoclonal antibody TKH2 promoted a dramatic decrease in cellular motility/invasion, supporting a key role for STn in disease dissemination. The associations between hypoxia, based on the nuclear overexpression of HIF-1α, and STn in MIBC were further confirmed by immunohistochemistry in patient samples.

Collectively this data shows that hypoxia not only favours a mesenchymal phenotype but also antagonizes protein *O*-glycosylation as a strategy to endow cells with higher motility/invasive potential. Finally, a preliminary STn-glycoproteomic analysis by western blot highlighted that hypoxic cells overexpressed low molecular weight STn-proteins (<50 kDa) in relation to normoxia, that may offer potential to selectively target hypoxic cells and consequently avoid disease dissemination.

In resume, the present work reaffirms the clinical relevance of STn antigen in MIBC. It also presents a new mechanism, based on the modulation of protein O-glycosylation and STn expression, by which hypoxia contributes to bladder cancer aggressiveness. Furthermore, it highlights the need to mine the glycoproteome of hypoxic cells for highly specific theranostic biomarkers capable of improving bladder cancer management.

Key-words

Bladder cancer; Hypoxia; Sialyl-Tn, Glycosylation and metabolism, Protein glycosylation, Cancer-associated glycans, HIF-1α.

Index of contents

Index of figures

Index of tables

Abbreviations

BC Bladder cancer

BCG Bacillus Calmette-Guérin

CA IX Carbonic anhydrase IX

CDH1 E-cadherin (epithelial)

CDH2 N-cadherin (neuronal)

CIS Carcinoma in situ

DC Denditic cells

DFX Deferoxamine mesylate salt

DNA DSBs DNA double-strand breaks

DNA SSBs DNA single-strand breaks

DPCs DNA–protein crosslinks

DSP Desmoplakin

EMT Epithelial-mesenchymal transition

EPCAM Epithelial cell adhesion molecule

FGFR3 Fibroblast Growth Factor 3

FIH-1 Factor Inhibiting HIF-1

FN1 Fibronectin 1

GalNAc N-acetiylgalactosamine residue

GAPDH Glyceraldehyde 3-phosphate dehydrogenase

GlcNAc N-acetylglucosamine residue

GlcNAc-T GnT N-acetylglucosaminyltransferases

HIF Hypoxia-inducible factor

HPRT Hypoxanthine-guanine phosphoribosyltransferase

HRAS Harvey rat sarcoma viral oncogene homolog

iPSCs Induced pluripotent stem cells

IR ionizing radiation

KLF Kruppel-like factor gene

LIN28A Lin-28 homolog A gene

MAPK mitogen-activated protein kinase

MET Mesenchymal-epithelial transition

MHC Major histocompatibility complex

MIBC Muscle invasive bladder cancers

MMP Matrix metalloproteinase

MYC V-myc avian myelocytomatosis viral oncogene homolog

NANOG Homeobox transcription factor NANOG

NMIBC Non-muscle invasive bladder cancer

OCT-4 (POU5F1) POU class 5 homeobox 1 gene

PERK Endoplasmic reticulum kinase

PHDs Prolyl hydroxylase domain enzymes

PI3K/ AKT phosphatidylinositol-3-OH kinase/ Protein kinase B

PI3KCA phosphoinositide-3-kinase

ppGalNAc-Ts N-acetylgalactosaminyl- transferases

PTM Post-translational modification

pVHL Von Hippel-Lindau protein

qPCR Quantitative polymerase chain reaction

RB Retinoblastoma

RUNX Runt-related transcription factor

SNAI Snail family zinc finger

SOX-2 SRY (sex determining region Y)-box 2 gene

SPARC Secreted protein, acidic, cysteine-rich (osteonectin)

ST6GalNac.I GalNAc α-2,6-sialyltransferase or ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase

STn Sialyl-Tn antigen

T antigen Thomsen-Friedenreich antigen

TAMs Tumour-associated macrophages

TB-α α-Tubulin

TCC Transitional cell carcinoma

TIS Tumours in situ

TNM Tumour-node-metastasis staging system

TUR Transurethral resection

TWIST Twist family bHLH transcription factor

UBC Urinary bladder cancer

UPR unfolded protein response

VHL Von Hippel-Lindau tumour suppressor E3 ligase complex

VIM Vimentin

VNTR Variable number tandem repeat

WHO World Health Organization

ZEB Zinc finger E-box binding homeobox

β3/4 Gal-Ts β3/4-galactosyltransferases

β3/4 Gn-Ts N-β3/4-acetylglucosaminyltransferases

I. Introduction

1.1. Bladder cancer epidemiology and disease information

1.1.1. Epidemiology

Bladder cancer (BC) is the most common malignancy of the urinary tract and the 7th most common cancer in men and the 17th in women (1). The incidence of BC increases with age, reaching a peak between 50 and 70 years, and is three times more common in men than in women (2).

Environmental factors are thought to play a significant role in BC initiation. Chemical or environmental/occupational exposures and chronic inflammation are well known risk factors for the development of BC and they may lead to genetic and molecular changes, which irreversibly convert a normal urothelial cell to one with a malignant phenotype. Chemical and environmental exposures include aromatic amines, aniline dyes, nitrites and nitrates, acrolein, coal, and arsenic. Other causal factors include indwelling catheters, Shistosomiasis (*Schistosoma haematobium* infection*)*, and pelvic irradiation (3). Notwithstanding all these factors, in Western world cigarette smoking is the most relevant risk factor, accounting for approximately 50% of BC cases (1).Genetic predisposition also has a significant influence on BC, especially via its impact on susceptibility to other risk factors (4).

1.1.2. Pathophysiology and progression of Bladder Cancer

Urothelial cancers arise through two distinct but somewhat overlapping pathways that are driven by different genetic changes: papillary and non-papillary. Approximately 80% to 85% of urothelial cancers are papillary lesions, which arise from hyperplastic epithelium with the expansion of a preneoplastic clone, which shows minimal phenotypic

deviation from normal urothelium. The continuous growth of such clone results in the development of low-grade superficial papillary tumours that harbour frequent mutations in the Fibroblast Growth Factor 3 (FGFR3) (~70%), Harvey rat sarcoma viral oncogene homolog (HRAS) (30-40%) and phosphoinositide-3-kinase (PI3KCA) genes (5). Non-papillary and invasive tumours usually arise from severe dysplasia or carcinoma in situ (CIS). In a non-papillary pathway, a successive subclone of the initial hyperplasia develops genetic instability with frequent loss of major tumour suppressor genes as Retinoblastoma (RB) and p53 (~ 50%). The vast majority of invasive bladder cancers occur in patients without a prior history of papillary tumours (6).

Macroscopic (frank or gross) haematuria is the commonest presenting sign of BC, occurring in about 75% of patients. More than 90% of diagnosed bladder cancers are transitional cell carcinomas (currently classified as high-grade urothelial cell carcinomas according to WHO guidelines), 5% are squamous cell carcinomas, and less than 2% are adenocarcinomas. Of all newly diagnosed cases of urothelial carcinomas, about 70% are superficial tumours (stages Ta, T1, or tumours in situ [Tis]), but as many as 50–70% of those superficial tumours will recur and roughly 10–20% will progress to muscularis propria invasive disease (T2–4) usually with metastasis (as well as localized persistent disease) within a median of 2 years if managed only by transurethral resection and intravesical therapy (7). Consequently, non-muscle invasive bladder cancer (NMIBC) is a chronic disease with varying oncologic outcomes requiring frequent follow-up and repeated treatments, making the cost per patient from diagnosis to death the highest of all cancers (8). Increasing tumour grade, stage, size and multifocality have been associated with an increased risk of progression.

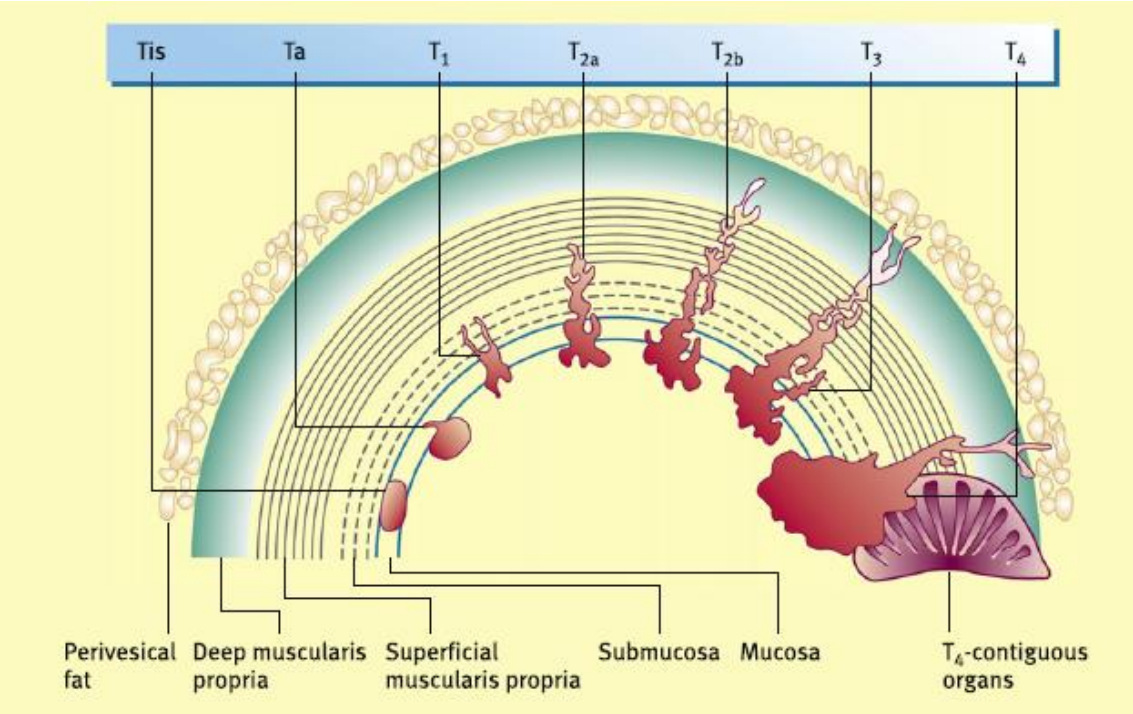


Figure 1. Transitional cell carcinoma staging by the tumour-node-metastasis (TNM) staging system, in which the stage of the primary tumour (T) is based on the extent of penetration or invasion into the bladder wall (9).Tis, Tumour in situ: ''flat tumour''; Ta, Non-invasive papillary carcinoma; T1, Tumour invades subepithelial connective tissue; T2, Tumour invades muscle; T2a, Tumour invades superficial muscle (inner half); T2b, Tumour invades deep muscle (outer half); T3, Tumour invades perivesical tissue; T4, Tumour invades any of the following: prostate, uterus, vagina, pelvic or abdominal wall.

1.1.2.1. Diagnosis, management and therapeutics of BC

Primary treatment of Ta/T1 transitional cell carcinoma is endoscopic resection of the bladder tumour. Further management is based upon risk factors discussed above and stage/grade of the disease. Depending on these, a patient may need only surveillance, single-installation of intravesical chemotherapy, a course of intravesical chemotherapy or immunotherapy or further surgery. The most commonly used and recommended intravesical therapy is the administration of the live attenuated bacillus Calmette-Guérin (BCG) causing an extensive local inflammatory reaction in the bladder wall. While the precise mechanism of BCG action is unclear, studies have implicated natural killer cells and T lymphocytes as critical mediators of the anti-tumour immune response (10). However, only two thirds of patients respond to BCG and one third of the responders will have recurrent disease. Recurrence after BCG treatment is associated with a poor prognosis (11).

In patients with low grade Ta disease, the 15-year progression-free survival is 95% with no cancer-specific mortality. Patients with high grade Ta tumours have a progression-free survival of 61% and a disease-specific survival of 74%, whereas patients with T1 disease have a progression-free survival of 44% and a disease-specific survival of 62%, lending support to the view that invasion of the lamina propria is a prognostic indicator for risk of disease progression and reduced survival (3).

Once transitional cell carcinoma invades the muscle of the bladder wall (T2), perivesical tissue (T3) or adjacent pelvic organs (T4), it cannot be controlled by endoscopic resection and intravesical treatment alone (12). The standard surgical approach to treatment of muscle-invasive bladder cancer is radical cystectomy. For patients who undergo total or partial resection of the bladder, treatment with adjuvant (after surgery) or neoadjuvant (before surgery) chemotherapy is also an option (12).

Approximately 30% of patients with urothelial cancer present muscle-invasive disease, and about half relapse after radical cystectomy, depending on the pathological stage of the primary tumour and the presence of loco-regional or distant metastasis. Local recurrence accounts for approximately 30% of relapses, whereas distant metastasis are more common. Ten to fifteen percent of patients are already metastatic at diagnosis. Before the development of effective chemotherapy, patients with metastatic urothelial cancer rarely had a median survival that exceeded 3-6 months (13).

Advanced bladder cancer may metastasize to lymph nodes (usually pelvic), or via vascular spread to the liver, lung, bone and intestines or other organs. Patients with good renal function may be suitable for systemic chemotherapy with cisplatin-based combinations (methotrexate, vinblastine, cisplatin, doxorubicin, i.e. the MVAC regime or gemcitabine/cisplatin, GC). MVAC and GC prolonged survival up to 14.8 and 13.8 months, respectively, compared to monotherapy and other combinations. Response rates were 46% and 49% for MVAC and GC, respectively. The major difference between the above-mentioned combinations is toxicity. The lower toxicity of GC has resulted in it becoming a new standard regimen. Even though chemotherapy is efficient against highly proliferative malignant cells that form the tumor bulk, the five-year overall survival does not exceed 25% and many patients die prematurely from adverse drug reactions (14) urging for effective and safe targeted therapeutics.

Modest disease control rates, with sporadic marked chemotherapy responses has led to the investigation of biomarkers for assessment of postoperative prognosis and the potential value of perioperative chemotherapy, and as predictors of response to

chemotherapy or its monitoring. Most of the biomarkers are associated with tumour angiogenesis. Small studies, usually retrospective, have investigated microvessel density, altered p53 tumour expression, serum vascular endothelial growth factor, urinary and tissue basic fibroblast growth factor, urinary (wild-type and mutant) and tissue fibroblast growth factor receptor-3, and more recently, thrombospondin-1, circulating tumour cells, and multidrug resistance gene expression. More recently, it was also reported that over 75% of MIBC express the sialyl-Tn antigen that stems from profound alterations in the O-glycosylation (linked to Ser/Thr residues) of cell-surface proteins that appears to have implications in cell behaviour towards invasion and migration (15). Although some biomarkers have shown predictive potential, none has sufficient evidence to support its routine clinical use in diagnostics or to develop novel therapeutics.

Despite the several studies on pathways associated to invasion and metastasis for prognostic and predictive biomarkers of response to chemotherapy, few have addressed the influence of microenvironment factors. In particular, the role of hypoxia, a known promoter of drug resistance, invasion and metastasis in solid tumours (16), remains poorly understood in the context of bladder cancer.

1.2. Hypoxia: an hallmark of cancer progression and dissemination

1.2.1. HIFs and cellular O_2 sensing

Hypoxia is defined as a reduction of oxygen tension available to a cell, tissue or organism. At the atmospheric pressure (150 mm Hg), ambient air consists of 21% O_2 however in most mammalian tissues O_2 percentage is around 2%–9% (on average 40 mm Hg). Hypoxia is usually defined as ≤ 2% O_2 and severe hypoxia (or anoxia) is defined as ≤ 0.02% O_2. Depending on regional and temporal status of blood flow through tortuous vessels, hypoxia can vary from moderate to severe, acute to chronic, and intermittent to persistent. Low O_2 tensions are normally associated to intense inflammation or necrotic regions as well as to less vascularized regions of the bone marrow. In solid tumours, hypoxia results from of an inadequate supply of oxygen, due to exponential cellular proliferation and an inefficient vascular structure. Both acute and chronic hypoxia co-exist

within a tumour, resulting in differential gradients of oxygen consumption and contributing to intra-tumour heterogeneity.

Although hypoxia is toxic to both cancer and normal cells, when tissue metabolism exceeds metabolic supply, the hypoxic response triggers a series of changes in gene transcription or protein posttranslational modifications, conducting to changes of cell metabolism, ultimately protecting against cell death. This response to the decline of O_2 levels is in part mediated by heterodimeric transcription factors known as hypoxia-inducible factors (HIFs) comprised by an α and a β subunit. In mammals, there are three O_2-regulated HIFα subunits. While HIF-1α is ubiquitously expressed, HIF-2α is restricted to vascular endothelium, liver parenchyma, lung type II pneumocytes and kidney epithelial cells and HIF-3α is found at high levels in the thymus, cerebellar Purkinje cells and the corneal epithelium of the eye. Heterodimers that contain HIF-1α or HIF-2α seem to have overlapping but distinct specificities, with regard to physiological inducers and target gene activation. The third related protein, HIF-3α, might function primarily as an inhibitor of HIF-1α. Instead, HIF1β is constitutively expressed and insensitive to changes in O_2 levels (17–21).

1.2.2. HIF-1-alpha and mechanism of hypoxia sensing

Under normal O_2 tensions, prolyl hydroxylase domain enzymes (PHDs) hydroxylate two conserved proline residues (405 and 531 in HIF-1α) within the O_2-dependent degradation (ODD) domain of the HIF-α subunit in an O_2- and 2-oxoglutarate-dependent manner. After hydroxylation, the von Hippel-Lindau (VHL) tumour suppressor E3 ligase complex polyubiquitinates HIF-α and targets it for degradation by the 26S proteasome. Moreover, the O_2-dependent hydroxylation of the asparagine (N) residue 803 in HIF-1α by the enzyme FIH-1 (Factor Inhibiting HIF-1) blocks the binding of p300 and CBP to HIF-1α and therefore inhibits HIF-1α gene transcription. Under low O_2 tension, HIFs are no longer modified by PHDs, but instead dimerize with ARNT/HIF-1β through HLH and PAS domain interactions, translocate to the nucleus, and recruit co-activators such as CBP/p300. HIF heterodimers bind and recognize hypoxia-response elements (HREs), with the consensus sequence G/ACGTG, within the promoter regions of target genes and drive adaptive gene transcription. Some well-known examples include vascular endothelial growth factor

(*VEGF*; involved in angiogenesis), *GLUT1* (also known as *SLC2A*; a glucose transporter involved in glycolysis) and carbonic anhydrase IX (*CA9*; a regulator of cellular pH) (22–24).

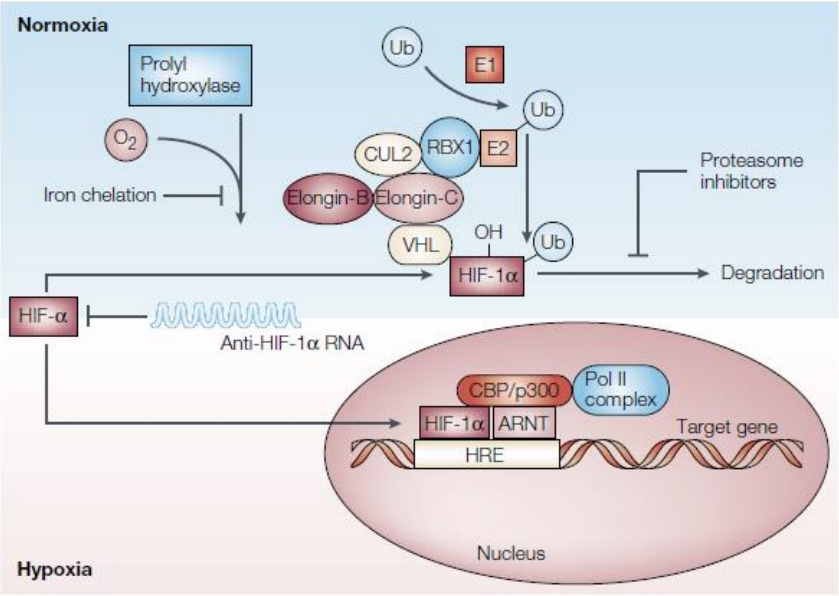


Figure 2. HIF regulation is primarily based on post-translational modification and protein stability (25). CBP, cyclic AMP response-element binding protein; CUL-2, cullin-2; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; HIF-α, hypoxia-inducible factor alpha; ARNT/HIF-1β, hypoxia-inducible factor-1beta; HRE, hypoxia response element; O2, oxygen; OH, hydroxyl ions; p300, a histone acetyltransferase closely related to CBP; Poll II, RNA polymerase II; Rbx1, really interesting new gene (RING)-box protein 1 (or regulator of cullin 1); Ub, ubiquitin; VHL, von Hippel–Lindau protein; Anti-HIF-1α RNA, αHIF, a natural anti- sense transcript (NAT) complementary to the 3' untranslated region of the HIF1α, negatively regulates the expression of HIF1α. Overexpression of αHIF triggers HIF1α mRNA decay and HIF1α and αHIF constitute a negative feedback loop(26).

It is relevant to evidence that the activation of HIF is not only induced by hypoxic conditions. Moreover, whereas hypoxia ubiquitously increases HIF-1α levels by decreasing its molecular degradation, growth factors, cytokines and other signalling molecules, also induce *HIF-1α* expression in a cell-type specific manner. HIF-1α levels can increase by dysfunctional tumour suppressor genes (e.g., *VHL, SDHB, SDHC, SDHD, FH, IDH1, P53, TSC2, PTEN* and *LKB1*) as well as by proteins of oncogenic viruses (e.g., EBV latent membrane protein 1, hepatitis B virus X protein, human papillomavirus E6/E7 protein, human T-cell leukemia virus tat protein, KSHV G protein-coupled receptor, KSHV latency-associated nuclear antigen and KSHV viral interferon regulatory factor 3). Furthermore, HIF can be activated by two additional mechanisms: oncogenic signalling, including the

mitogen-activated protein kinase (MAPK: RAS/MNK) and PI3K/AKT pathways and constitutive oncogene induced signalling, including the loss of tuberous sclerosis complex 1 or 2 (TSC1 or 2) function, inactivation of VHL (in the Von Hippel-Lindau disease), accumulation of fumarate (in hereditary leiomyomatosis renal-cell cancer syndrome [HLRCC]) or accumulation of succinate (in hereditary paraganglioma syndrome).

It is also worth mentioning that although HIFs are crucial mediators of the transcriptional response to hypoxia, a number of HIF-independent pathways also respond to changes in O_2 tension. These responses allow cells to acutely adapt to the energetic demands of decreased O_2 availability by reducing and limiting energy-consuming processes such as protein synthesis. There are two distinct pathways leading to this inhibition of translation. The first is rapid, HIF1α-independent, and mediated by the 'unfolded protein response' (UPR). On its turn UPR mediates the phosphorylation of the eukaryotic initiation factor 2α (EIF2α) by the endoplasmic reticulum kinase PERK (also known as EIF2A kinase 3 (EIF2AK3)), leading to inhibition of mRNA translation initiation. The second pathway consists of a delayed response, only activated after prolonged (chronic) hypoxia. This response is associated with disruption of the mRNA cap-binding complex, EIF4F, which inhibits the transcript recruitment step of mRNA translation (27).

1.2.3. Role of hypoxia in the hallmarks of human cancer

Hypoxia has been recognized as one of the fundamentally important features of solid tumours, exerting a critical role in various cellular and physiologic events, including cell proliferation and differentiation, survival, angiogenesis, immunosurveillance, and metabolism. Moreover, it also influences tumour invasion, metastasis and resistance to radiotherapy and certain chemotherapies. Overall, hypoxia may contribute to promote and select for a more aggressive phenotype of tumour cell and it is an adverse prognostic indicator in cancer.

Apoptosis evasion

In contrast to normal cells, cancer cells can break the balance between pro- and anti-apoptotic factors to escape HIF-1α-mediated apoptosis under adverse conditions, such as hypoxic stress. Several pro-apoptotic (e.g. BNIP3, NIX, and NOXA) as well as

anti-apoptotic factors (e.g. BaK, Bax, Bcl-xL, Bcl-2, Bid, Mcl-1, NF-kB, p53 and survivin) are regulated by HIF-1α. However, in the vast majority of transformed cells, HIF-1α functions as a major suppressor of apoptosis (28). For example, hypoxia can up-regulate the expression of the p53-negative regulator MDM2, increasing cell resistance to apoptosis *in vivo* and promoting metastasis or induce the expression of the anti-apoptosis protein IAP-2. Additionally, hypoxic cancer cells also activate the PI-3K/Akt survival pathway, a critical regulator of cell survival and proliferation (29–31). During hypoxia-induced apoptosis, only the phosphorylated HIF-1α binds to HIF-1β. By contrast, the dephosphorylated form of HIF-1α binds to and stabilizes p53, inducing a p53-dependent apoptosis, which is mediated by APAF-1 and caspase-9. Moreover, p53 directly interacts with HIF-1α and blocks HIF-1α's ability to activate transcription of anti-apoptotic genes. Hypoxia acts then as a selective pressure during tumour growth, eliminating cells with wild-type p53 and selectively and clonally expanding cells with mutant or otherwise inactive p53, therefore evading p53-dependent apoptosis and transcription blockage (32).These and many other experimental results give further evidence for the major role played by hypoxia in facilitating tumour cell survival by evading the apoptotic pathway.

Limitless replicative potential

Human somatic cells normally have a finite replicative potential. However, cancer cells can overcome this obstacle, fail to senesce and acquire limitless replicative potential by increasing telomerase activity in a HIF-1α-dependent manner (27,33). Despite numerous descriptions of telomerase expression within human tumours and cancer cell lines, the mechanisms of regulation are largely unknown. In a hypoxic microenvironment, cancer cells can also overexpress mitogenic growth factors such as EGF, insulin, IGF-1, IGF-2, and PDGF, or constitutively activate the downstream pathways of these growth factors in order to maintain their proliferative potential. Moreover, HIF-1α is involved in the autocrine growth-factor stimulation of cancer cells, through which binding of growth-factors ligands, such as IGF2 and TGF-α, to their cognate receptors stimulates the expression of HIF-1α. This leads to increased HIF-1α transcriptional activity of target genes, which include those that encode IGF2 and TGF-α (34).

Angiogenesis induction

Normally, tumour vasculature is structurally and functionally inefficient, leading to abnormal perfusion and tumour hypoxia. In response to this challenging microenvironment, a number of angiogenic factors are induced such as VEGF, IL-8, angiogenin, FGF, and PDGF (16). Besides promoting up-regulation of VEGF and one of its receptors, VEGF receptor 1 (VEGFR1/FLT-1), HIF-1α activity can also decrease the activity of the angiogenic inhibitor thrombospondin to create a pro-angiogenic microenvironment. Moreover, there are two different models that explain the initiation or acceleration of angiogenesis in a hypoxic microenvironment, the hypoxic crisis model and the acceleration model (35). In the vascular crisis model, hypoxia can induce VEGF to promote the initiation of angiogenesis. On the other hand, the acceleration model proposes that hypoxia is not responsible for initiation of angiogenesis and that this initiation is driven by non-hypoxia-mediated mechanisms, such as the induction of VEGF by oncogene activation. Once angiogenesis is initiated, cancer cells grow quickly, resulting in additional hypoxia, while HIF-1α levels increase to accelerate angiogenesis (17,19,23,36–38). Hypoxia-induced angiogenesis is blocked by inhibitors of oncogenic signalling pathways, such as RAS, epidermal growth factor receptor, and the tyrosine kinase receptor ERBB2 (HER2/neu) inhibitors. This indicates that there is crosstalk between oncogenic and hypoxia-response pathways.

Immunosurveillance evasion

The tumour microenvironment involves several cell types, including cancer cells, endothelial cells, immune cells, and stromal elements. The co-existence of tumours and anti-tumour immune cells (“Hell- strom Paradox”) has been a challenging problem for a long time (39). This paradox prompted speculations that tumour microenvironment *in vivo* may prevent anti-tumour $CD8^+$ T cells cytotoxic activity (40). Monocytes are also continually recruited into tumours where they differentiate into tumour-associated macrophages (TAMs), and accumulate in these hypoxic areas. It is now known that macrophages respond to the levels of hypoxia found in tumours by acquiring a pro-tumour phenotype in which they up-regulate HIF-1α and HIF-2α which in turn activate a broad

array of mitogenic, pro-invasive, pro-angiogenic, and pro-metastatic genes (41). Hypoxia may also inhibit the ability of macrophages to phagocyte and present antigens to T cells, reducing their pro-inflammatory capacity (42). The complex mechanisms by which cancer cells evade the immune response under hypoxic microenvironment are still poorly understood. However, one possibility may be that cancer cells secrete various immunosuppressive factors by HIF-1α-dependent pathways, such as hypoxia-induced extracellular adenosine. Other possibility is the activation of HIF-independent pathways, that protect cancer cells from immune damage by the same mechanism which protects normal tissues from the excessive collateral damage by overactive immune cells during acute inflammation (40). These and other findings suggest that not only malignant cells but also immune cells adapt to hypoxic microenvironments and promote the expression of factors that are necessary to escape immunosurveillance (43). Additionally, inflammatory cytokines are significantly enhanced in hypoxic or inflammatory states, and their constitutive expression is a characteristic of many malignant tumours and often associates with tumour progression, metastasis and poor prognosis (44). Inflammatory cytokines including TNFα, TGFβ, IL-1, IL-6 and IL-8, secreted by inflammatory cells in tumours sites such as neutrophils, lymphocytes, macrophages and myeloid-derived suppressor cells (MDSC) might play an essential role in a hypoxia-induced phenomenon called epithelial-mesenchymal transition (EMT) that will be latter on explored (45).

Radioresistance and Chemoresistance

Under normoxic conditions, ionizing radiation (IR) produces DNA double-strand breaks (DNA DSBs), DNA single-strand breaks (DNA SSBs), DNA base damage, and DNA–DNA and DNA–protein crosslinks (DPCs). The ionizing radiation generates ROS that lead to DNA DSB and consequently cell death. Under hypoxic conditions, O_2 shortage translates into decreased ROS production, which negatively affects treatment efficacy (46). It is thought that fractionated radiotherapy partially overcomes intratumoural radioresistance because radioresistant hypoxic cells become reoxygenated, and therefore resensitized between individual dose fractions. Hypoxic genes expression can also modify cellular radioresponse, in a HIF-1α-dependent manner, by promoting ATP metabolism during fractionated radiotherapy, proliferation, p53 activation, and by stimulating endothelial cell survival (27). A number of factors associated either directly or indirectly with tumour hypoxia also contribute to an overall decrease of the efficacy of

chemotherapeutic agents *in vivo*. Some factors include; i) decreased drug action in the absence of O_2, ii) decreased effect of agents in hypoxic cells that are poorly proliferating or have altered pH gradients, iii) induction of gene amplification, and iv) an overall decreased drug diffusion and delivery to cells distant from functional vasculature (47). Moreover, HIF-1α is able to activate the multidrug resistance 1 (*MDR1*) gene that encodes a membrane-resident P-glycoprotein (P-gp) that belongs to the family of ATP-binding cassette (ABC) transporters. On its turns, P-gp decreases the intracellular concentration of several chemotherapeutic drugs by acting as drug efflux pump. Multidrug-resistance-associated protein 1 (MDRP1) is another ABC transporter related with HIF-1α-mediated drug resistance (28). Earlier studies also reported that transient hypoxia induces DNA over-replication and amplification of a drug resistant gene encoding dihydrofolate reductase. HIF-1-mediated alteration in cell proliferation and survival represent other pivotal causes for drug resistance. The relative importance of each factor might depend on the duration of hypoxic exposure (such as acute versus chronic) (27,32).

Genetic instability

The cause of human cancers is imputed to the genetic alterations at nucleotide and chromosomal levels of ill-fated cells. It has long been recognized that genetic instability is responsible for the cellular changes that confer progressive transformation on cancerous cells (48). Hypoxia is one of the most important microenvironmental factors that can drive genetic instability and its role in gene amplification, DNA breaks at chromosomal fragile sites and disruption of DNA damage repair is well established (49).

Mutation in human DNA mismatch repair (MMR) genes such as *MLH1*, *MSH2*, and *MSH6* are associated with the development of both hereditary and sporadic cancers and it's well known that hypoxia/ ischemia impairs the MMR system by inhibiting gene expression (50,51). Moreover, it has been reported that *MutSα*, *MSH2* and *MSH6* genes responsible for MMR initiation, are specifically down-regulated by hypoxia via the HIF-1α-Myc pathway (52). Hypoxic stress can also suppress the nucleotide excision repair (NER) pathway. It is hypothesized that the unique metabolic conditions induced by hypoxia (e.g., decreased adenosine triphosphate [ATP] production) may impair the enzymatic activity of specific NER proteins, leading to a functional decrease in NER repair under these conditions (53). In addition to genetic changes at the nucleotide levels, chromosomal instability is more commonly observed in cancers. Moreover, cancer-associated hypoxia

induces additional chromosomal breakage and rearrangement during replication stress at regions called chromosomal fragile sites (54). Therefore, hypoxia-induced DSBs may contribute to a later stage of tumour progression, by inducing chromosomal instability mainly due to impairment of DSBs repair (49). DSBs are mostly repaired by homologous recombination (HR) or non-homologous end-joining (NHEJ) pathways in a cell cycle-dependent manner. The proteins RAD51, BRCA1/2 and the MRN complex (MRE11, RAD50, NBS1) together regulate HR during S and G2 phases of the cell cycle and proteins such as KU70/80, DNA-PKcs and DNA-ligase IV function in NHEJ across all phases of the cell cycle (55,56). The majority of HR proteins are repressed by chronic hypoxia through decreased transcription, translation, miRNA modulation and epigenetic silencing (57). Altogether HR pathway repression is associated with substantial suppression of recombinational repair activity in hypoxic cells. Other chromatin responses to hypoxia include global deacetylation and methylation of histones. Moreover, histone deacetylases (HDACs), implicated in alteration of chromatin assembly and tumorigenesis, are activated by hypoxia (58,59). Premature condensation of chromosomes and abnormal chromosome mis-segregation are also common features of hypoxic cells (27).

Glycolysis and pH regulation

As already stated, cells undergo a variety of biological responses towards hypoxic conditions, however the earliest recognized pathway was that hypoxic cells undergo a shift from aerobic to anaerobic metabolism. Metabolic adaptation represents a canonical response to hypoxia, which includes a more or less dramatic shift toward the glycolytic metabolism that enables the sustained, although less efficient production of energy (60). Hypoxic cancer cells use glycolysis as a primary mechanism of ATP production without the need for O_2-dependent oxidative phosphorylation, and HIFs are master regulators of glucose metabolism during hypoxia. HIF-1α activates transcription of genes encoding glucose transporters (*GLUT1* and *GLUT3*), which mediate cellular glucose uptake, and other glycolytic enzymes such as lactate dehydrogenase A (*LDHA*), phosphoglycerate kinase 1 (*PGK-1*), and hexokinase 1 (*HK1*) and thus plays an important role in the glycolytic switch (61,62). Hypoxia and HIF-1α increase glucose/carbon flux through the glycolytic pathway while minimizing input into the tricarboxylic acid cycle (TCA) and oxidative phosphorylation. The up-regulation of pyruvate dehydrogenase kinase (PDK1) which in turn deactivates pyruvate dehydrogenase (PDH), the enzyme responsible for

conversion of pyruvate to acetyl-CoA, redirects glucose away from the oxidative phosphorylation pathway (61,63). HIF-1α has also been shown to collaborate with *c-Myc* oncogene to active hexokinase 2 (HK2) as well as PDK1, resulting in increased conversion of glucose to lactic acid (64). Moreover, glycolysis also provides substrates for biosynthetic reactions (e.g. nucleotides, amino acids, and lipids) and thereby facilitates cell proliferation in the process of tumour expansion (65). In addition, hypoxia leads to selection of inherently glycolytic cells developed through oncogene-mediated, hypoxia-independent HIF-1α stabilization (66). These are the reasons why tumour cells maintain glycolytic metabolism even in the presence of oxygen and why they do not switch back to oxidative phosphorylation under aerobiosis. Nevertheless, some tumour cells strongly rely on uptake of glutamine and glutaminolysis, which supports the mitochondrial TCA cycle and pentose phosphate pathway and thereby facilitates the synthesis of fatty acids, nonessential amino acids and nucleosides (67). Although HIF-1α strongly links aerobic glycolysis to carcinogenesis, it would be premature to conclude that the glycolytic phenotype in cancer cells is invariably due to deregulation of the HIF system.

In addition, glycolysis is thought to be the main mechanism by which tumours lower their pH, through generation of lactic acid. Carbonic Anhydrases (CA), which are metalloenzymes that catalyse the reversible hydration of carbon dioxide to bicarbonate ions and protons, might also be involved. CA IX is one of the 15 human isoforms of the carbonic anhydrase family and it is not expressed in the majority of normal tissues. Actually, it is abundant only in the stomach and gallbladder epithelia. On the other hand, it is very often and strongly expressed in tumours, generally in the more aggressive forms (68). HIF-1α is the essential transcription factor of the *CA9* gene driving its transcription in response to intra-tumour hypoxia or inactivating mutation of the *VHL* tumour suppressor gene. Recently, the hypoxia-induced *CA9* transcription was found to be dependent on the cooperation between HIF-1α and an intracellular domain of Notch3 (NICD3), a transcription factor involved in cell-fate determination, morphogenesis, and oncogenesis (69). Importantly, hypoxia also regulates *CA9* expression by post-transcriptional mechanisms through splicing and mRNA stabilization. Accumulating experimental evidence supports the direct participation of CA IX in many hypoxia- and acidosis-induced features of tumour phenotype, including increased adaptation of tumour cells to microenvironmental stresses, resistance to therapy, increased tumour cell migration and invasiveness. Moreover, increased focal adhesion during cell spreading, destabilization of intercellular contacts, maintenance of stem cell phenotype, tumour–stroma crosstalk,

signal transduction and possibly other cancer-related phenomena (22). For example, extracellular acidosis mediated by CA can activate the proteolytic enzymes (some of them being transcriptionally induced by hypoxia) that degrade extracellular matrix and facilitate invasion of tumour cells across the basal membrane and through the surrounding normal tissue (70,71). CA IX also contributes to therapy resistance in a way that tumours expressing high CA IX levels are less responsive to experimental therapy, and inhibition of CA IX catalytic activity significantly improves their chemo- or radiosensitivity (68). Hypoxia and extracellular acidosis also contribute to cancer cell dedifferentiation toward the stem cell-like phenotype and CA IX was shown to be involved in this phenomenon in breast (72), bladder (73) and esophageal carcinoma models (73). Expression of CA IX correlates with the expression of a stem cell marker CD44 and CA IX targeting by inhibition of its catalytic activity results in the inhibition of breast cancer stem cell expansion in hypoxia, indicating that pH-regulating function of CA IX is required for the maintenance of stemness (22,74).

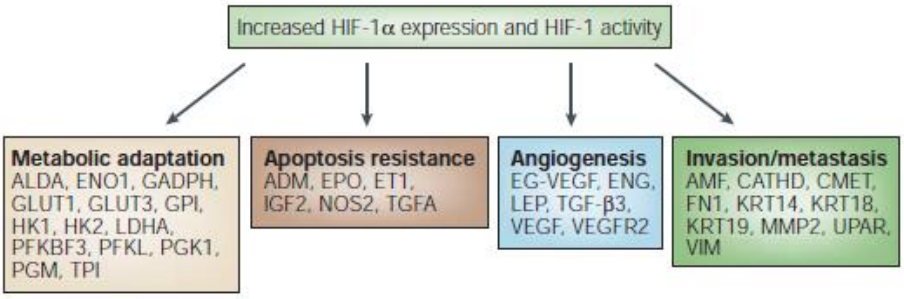


Figure 3. Genes that are involved in many essential processes are direct HIF-1α targets (75). Four groups of direct HIF-1 target genes particularly relevant to cancer encode: angiogenic factors, glucose transporters and glycolytic enzymes, survival factors and invasion factors. ADM, adrenomedullin; ALDA, aldolase A; AMF, autocrine motility factor; CATHD, cathepsin D; EG-VEGF, endocrine gland-derived VEGF; ENG, endoglin; ET1, endothelin-1; ENO1, enolase 1; EPO, erythropoietin; FN1, fibronectin 1; GLUT1, glucose transporter 1; GLUT3, glucose transporter 3; GAPDH, glyceraldehyde-3-P-dehydrogenase; HK1, hexokinase 1; HK2, hexokinase 2; IGF2, insulin-like growth-factor 2; KRT14, keratin 14; KRT18, keratin 18; KRT19, keratin 19; LDHA, lactate dehydrogenase A; LEP, leptin; MMP2, matrix metalloproteinase 2; PFKBF3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3; PFKL, phosphofructokinase L; PGK 1, phosphoglycerate kinase 1; TGF-α, transforming growth factor-α; TGF-β3, transforming growth factor-β3; TPI, triosephosphate isomerase; VEGF, vascular endothelial growth factor; UPAR, urokinase plasminogen activator receptor; VEGFR2, VEGF receptor-2; VIM, vimentin.

1.2.4. Epithelial-mesenchymal transition, hypoxia and acquisition of stem cell traits

The epithelial-mesenchymal transition (EMT) is a multistep process characterized by loss of homotypic adhesion and cell polarity in which epithelial cells lose their epithelial characteristics and gain mesenchymal characteristics, such as motility and invasive properties (76). Type 3 or oncogenic EMT occurs in neoplastic cells that have previously undergone genetic and epigenetic changes, specifically in genes that favour clonal outgrowth and the development of localized tumours (77).

As already described, tissue invasion and metastasis are prognostic indicators for risk of disease progression and reduced survival in bladder cancer, thus understanding EMT may provide insight for the development of novel tumour markers or new therapeutic strategies. In BC and other malignancies, the hallmarks of EMT are the loss of epithelial markers such as E-cadherin (calcium dependent transmembrane glycoproteins found at adherent junctions and responsible for cell-cell adhesion in epithelial tissues), the increase of matrix metalloproteinases (MMPs) involved in basement membrane degradation, the activation of the Rac/ Rho/Cdc42 small GTPase family implicated in cytoskeleton reorganization, and the nuclear translocation of transcription factors. The down-regulation of E-cadherin is associated with the release of β-catenin, which then migrates to the nucleus and activates WNT signalling, thereby resulting in the EMT and metastasis (78).There are multiple mechanisms resulting in E-cadherin mediated cell adhesion inactivation in cancer, such as “cadherin switching” in which the normal expression of E-cadherin is replaced by the abnormal expression of P-cadherin and N-cadherin or N-cadherin expression is increased and E-cadherin levels do not change significantly (79). However, one of the main mechanisms remains the transcriptional repression mediated by transcription factors such as ZEB-1, ZEB-2 (SIP1), Snail-1, Snail-2 (Slug), and TWIST1 as well as microRNAs (80,81). These transcriptional repressors result in epigenetic silencing of the E- cadherin promoter of E-cadherin-encoding gene (*CDH1*) by DNA hypermethylation. miR-200 family members participate in the EMT in a way that the loss of miR-200 expression leads to the accumulation of Zeb-1 and Zeb-2, which is sufficient to silence *CDH1* and promote EMT and tumour invasion. In BC, expression of P-cadherin and N- cadherin, correlates with late stage, high grade disease and increased expression of MMP-9 was associated with a poor clinical outcome in patients with urothelial tumours.

Additionally, vimentin, an intermediate filament protein that induces epithelial cell changes, including their adoption of a mesenchymal shape and their increased motility, is characteristically up-regulated in cells undergoing EMT and was mainly detected in invasive BC (31% in MIBC vs. 7% in NMIBC) and was positively associated with tumour grade and stage (78). Moreover, EMT also seems to have a role in drug sensitivity of primary patient bladder tumours (44,45).

Although both hypoxia and the EMT are separately considered as crucial events favouring the invasion and metastasis of many cancer cells, the two processes are long known to involve few common molecular mechanisms (83). Recently, an increasing amount of evidence demonstrates that alterations in microenvironmental oxygen levels and activation of hypoxic signalling through HIFs are important triggers and modulators of the EMT, which is now thought to take a key role as the convergence point between hypoxia and cancer (84). Under hypoxic conditions, the tumour microenvironment generates and sustains major EMT-triggering pathways for facilitating tumour growth and metastasis such as transforming growth factor (TGF) β and Notch signalling pathway as well as the nuclear factor kappa B (NFκB) pathway (85,86). At the early stages of tumour formation, TGFβ arrests cell proliferation and induces apoptosis. At later stages of tumorigenesis, TGFβ acts as a tumour promoter by increasing tumour cell proliferation, survival, motility and invasion probably by up-regulation of *Snail* and *Slug* that are potent initiators of the EMT in cancer cells (78). HIF-1α and TGFβ co-regulate gene products whose expression is increased as a result of the EMT process. Additionally, HIF-1α interacts with the Notch intracellular domain and increases its transcriptional activity. Moreover, the interplay between HIF-1α and Notch appears to be important for stem cell maintenance under hypoxia. Furthermore, NFκB is a key transcriptional activator of HIF-1α, and basal NFκB activity is required for HIF-1α protein accumulation under hypoxia in cultured cells and in the livers and brains of hypoxic animals. Finally, there is growing experimental evidence that HIFs modulate the EMT by regulating the expression and activity of major transcription factors including those already referred TWIST, SNAIL, SLUG, SIP1 and ZEB1 (83).

Up until now, EMT has been implicated in two of the most important processes responsible for cancer-related mortality: development of distant metastasis and acquisition of therapeutic resistance. Both of these processes may be linked, in turn, to a third: the generation by EMT of cancer cells with stem cell-like characteristics. As described before, hypoxia has the potential to regulate cell differentiation, which has led to the emergence of

a new paradigm that tumour hypoxia may facilitate the maintenance of cancer stem cell characteristics in a HIF-dependent manner and thus allow a tumour cell with self-renewal potential to accumulate a multitude of genetic and epigenetic changes over a long period of time in order to become increasingly aggressive and resistant (87,88). Within a tumour, stem-like cells reside in defined microenvironments termed “stem cell niches” and hypoxia may be an important feature of those microenvironments. HIF-2α is an upstream regulator of Pou5f1 (Oct4), which is one of the main transcription factors used to generate the first induced pluripotent stem cells (iPSCs). It has been shown that knock-down of HIF-2α but not HIF-1α, leads to a decrease in the expression of *Oct4, Nanog and Sox2*, which are important stem cells markers (89).

In several models of cancer, the induction of EMT potentiates self-renewal and the acquisition of cancer stem-cell properties. Consequently, a common notion is that EMT may be a general feature of cancer stem or progenitor populations. This notion associates local invasiveness with the ability to colonize distant organs as expressions of two tightly interdependent gene programs borne by the same tumour cells. However, other models of neoplasia have found an inverse correlation between local invasiveness and the ability of tumour cells to colonize distant organs. This suggests a dichotomy between these two critical features of the metastatic process, possibly expressed by separate tumour cell subpopulations in which tumour cells that display a strong epithelial phenotype are endowed with the strongest capacity to survive in circulation and to establish distant metastases (76,90).

It is important to note that EMT is usually not an irreversible transition, cells can return to their epithelial phenotype once they have reached the metastatic sites for re-establishing barrier function and for enabling cell proliferation in a process called mesenchymal-epithelial transition (MET). Thus, in the absence of the EMT-inducing signals received from the ‘activated’ stroma, metastatic cancer cells might simply fall back to an epithelial state through an MET. For example, E-cadherin expression is re-induced in metastases, a change that is accompanied by the demethylation of the *CDH1* promoter (77). However, sustained activation of EMT leads to progressive epigenetic alterations in cells, inducing heritable effects that maintain the mesenchymal state even after EMT-initiating signals are no longer present. Hence, under certain conditions, EMTs can yield stable changes in the phenotype and thus lineage identity of cells (77).

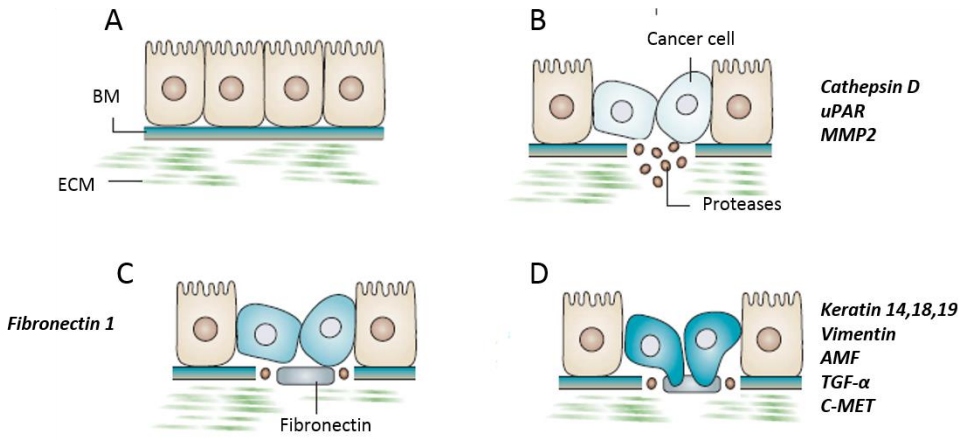


Figure 4. Invasion of the basement membrane is the defining characteristic of epithelial cancers. A. Epithelial cells are normally constrained by cell–cell contacts and by the basement membrane (BM). B. Cancer cells produce proteases, including the urokinase-type plasminogen-activator receptor (uPAR) and matrix metalloproteinase-2 (MMP2), which digest the basement membrane/extracellular matrix (ECM). C. Degraded ECM is replaced by fibronectin and other ECM proteins that are recognized by integrins that are expressed on cancer cells. D. An epithelial-to-mesenchymal transformation occurs in which intermediate-filament production is switched from keratin subtypes, which are characteristic of fixed epithelial cells, to keratins and vimentin, which promote the fluid structure that is required for motility and which is also stimulated by expression of secreted factors such as autocrine motility factor (AMF) and transforming growth factor-α (TGF-α), and surface receptors such as the c-MET tyrosine kinase. HIF-1 target genes that regulate invasion are listed (75).

1.3. Glycosylation and cancer

1.3.1. General features of glycosylation

Glycosylation is the most frequent, complex and plastic posttranslational modification (PTM) of membrane-bound and secreted proteins and results from a coordinated action of nucleotide sugar transporters, glycosyltransferases and glycosidases in the endoplasmatic reticulum and the Golgi apparatus. Glycans play a key role in protein folding, trafficking and stability and act as mediators of cell-cell adhesion, cell differentiation, migration, cell signaling pathways, immune recognition and host-pathogen interactions (91–94). Glycosylation increases the diversity of the proteome to a level unmatched by any other PTM, due to the diversity in sugar compositions, glycosidic linkages, chain length and substitution patterns. Glycosylation is also thought to be the most complex PTM due to the large number of enzymatic steps involved, along with the fact that unlike other cell

processes, such as transcription or translation, glycosylation is non-templated, and thus, all of these steps do not necessarily occur during every glycosylation event. On the other hand, despite all the heterogeneity and enzymes involved, glycosylation mechanisms are highly-ordered and normally individual enzyme activity is dependent upon the completion of the previous enzymatic reactions. As said before, this PTM is characterized by various glycosidic linkages, including N- , O- and C-linked glycosylation, glypiation (GPI anchor attachment) and phosphoglycosylation (95–99). Concerning the different types of glycosylation, they depend on multiple factors such as enzyme availability, amino acid sequence and protein conformation (100).

Focusing on the most common types of glycosylation, two main classes of glycans can be found at the cell-surface, namely N-glycans and O-glycans. Although glycosylation has been so far characterized as a post-translational modification, N-glycosylation often occurs co-translationally during the translation and transport of proteins into the ER. N-glycans are covalently attached to protein asparagine residues by N-glycosidic bonds, of which GlcNAcβ1-Asn is the most common. Precursor N-glycan synthesis begins on the cytosolic face of the ER and is further elongated after the structure is flipped into the ER lumen. In proteins, the candidates for receiving an N-glycan are called Asn-X-Ser/Thr “sequons”, with “X” being any amino acid residue except proline (92). Oligosaccharide transferase (OSTase) scans the nascent protein polypeptides for this consensus sequence and then transfers the precursor glycan (Glc3Man9GlcNac2-) from dolichol pyrophosphate to the Asn residue. To this point, all N-linked glycoproteins have the same precursor glycan structure. Glycan processing to diversify the glycans on individual glycoproteins occurs in the Golgi and combines both trimming and adding sugars to the structures in a step-wise fashion (101–103). Mature N-glycans chains can be modified by the action of fucosyl and sialyltransferases, yielding sialic acids, Lewis (Le) blood group related antigens (Le^a, SLe^a, Le^x, SLe^x, Le^b and Le^y) or ABO(H) blood group determinants as terminal structures. Other sugar modification may include phosphorylation, O-acetylation of sialic acids and O-sulfation of galactose and N-acetylglucosamine residues, thereby increasing the structural complexity of the glycome.

N-glycosylation does not preclude the second most common type of glycosylation, O-glycosylation, from happening, as O-glycosylation commonly takes place on glycoproteins previously N-glycosylated in the ER. O-glycosylation occurs post-translationally by covalently α-linking a GalNAc moiety from a sugar donor UDP-GalNAc to protein serine or threonine residues and is controlled by UDPGalNAc-polypeptide N-

acetylgalactosaminyl- transferases (ppGalNAc-Ts). As opposed to N-glycosylation there is no consensus sequence for the activity of N-acetylgalactosamine transferase and following the first sugar addition a highly variable number of sugars are consecutively added to the growing glycan chain such as galactose, N-acetylglucosamine, fucose or sialic acid but not mannose, glucose or xylose residues. A second level of complexity in O-glycosylation is the processing of carbohydrate chains by other glycosyltransferases. After the first glycan (GalNAc) is added forming the Tn antigen, the core 1 structure is synthesised by Gal-transferase (β(1-3)-galactosyltransferase, C1Gal-T1 or T-synthase) , which adds Gal to GalNAc. The core 1 structure may be also termed T antigen or Thomsen-Friedenreich antigen (Galβ1-3GalNAcα-O-Ser/Thr). Alternatively, Tn and T antigens can be sialylated by sialyltransferases forming the sialyl-Tn, sialyl-T and disialyl-T antigens and the formation of the sialyl-Tn antigen stops any further processing of the oligosaccharide chain. Core 1 may function as a precursor of other core structures (from core 2 to 8), by the addition of different monosaccharides, such as galactose, N-acetylgalactosamine, N-acetylglucosamine and sialic acids. Furthermore, cores 1-4 are the most common in humans. The extension of core units provides a vast array of glycan structures, and is catalysed by N-β3/4-acetylglucosaminyltransferases (β3/4 Gn-Ts) and/or β3/4-galactosyltransferases (β3/4 Gal-Ts), leading to the formation of side chains designated type-1 (Galβ1-3GlcNAc-R) and type-2 (Galβ1-4GlcNAc-R) chains. These chains present a ubiquitous expression, and therefore are widely expressed among epithelial tissues. Mature O-glycans may present terminal structures similar to the ones found in N-glycans (100).

This type of glycosylation is particularly found and modulates the biological role of mucins, a family of high molecular weight glycoproteins rich in repetitive sequences of serine and threonine residues termed tandem repeat domains (VNTR), which are ubiquitously present in mucous secretions, on cell surfaces as transmembrane glycoproteins and in body fluids. Mucins that span the plasma membrane are known to be involved in signal transduction, to mediate cell-cell adhesion or to have an anti-adhesive function (104). Mucins have also been shown to have roles in fertilization and immune responses. Their presence shield the epithelial surfaces against physical and chemical damage and protects against infection by pathogens. The expression of mucin genes is regulated by a large number of cytokines and growth factors, differentiation factors and bacterial products. Recently, a precision mapping of human O-GalNAc glycoproteome has revealed over 6000 glycosites in more than 600 O-glycoproteins, the majority of which

were found at the cell surface (105), thereby greatly expanding the view of the O-glycoproteome and its functional role. Of note, several intracellular proteins, including cytoplasmatic, mitochondrial and nuclear proteins, have also been found expressing this type of posttranslational modification. Despite the need for further analytical validation, these observations may suggest an up until now neglected role for O-GalNAc glycosylation in intracellular events (105). There are also several types of non-mucin O-glycans, such as α-linked O-fucose, β-linked O-xylose, α-linked O-mannose, β-linked O-GlcNAc, among others. O-glycosylation can also occur in the cytosol and nucleus to regulate gene expression or signal transduction through other glycosyltransferases. This nuclear O-glycosylation is performed by a single N-acetylglucosamine (GlcNAc) residue, and appears to perform a signalling role similar to protein phosphorylation.

1.3.2. Alterations in glycosylation as biomarkers of cancer

Aberrant glycosylation in cell surface glycolipids, membrane-associated and secreted glycoproteins occurs in essentially all types of human cancers, and many glycoepitopes constitute tumour-associated antigens. Those found in the surface of cancer cells are easily accessible to antibodies and lectins while those released to the peripheral circulation, either in secreted glycoproteins or by shedding from cell surfaces can be explored in various serological assays. Changes in some oligosaccharide structures associated with human cancer correlate with a precise stage of disease and their detection with lectins or monoclonal antibodies provide useful diagnostic or prognostic information, or both, and in many cases they contribute directly to cancer biology. A long-standing debate is whether aberrant glycosylation is a result or a cause of cancer. Many recent studies indicate that some, if not all, aberrant glycosylation is a result of initial oncogenic transformation, as well as a key event in induction of invasion and metastasis. Moreover, glycosylation promoting or inhibiting tumour cell invasion and metastasis is of crucial importance in current cancer research (106). Generally, the most frequently described cancer related changes in the pattern of glycosylation include the synthesis of highly branched and heavily sialylated glycans, the premature termination of biosynthesis, resulting in the expression of uncompleted forms, and the re-expression of glycosidic antigens of foetal type. In many cases the formation of these aberrant structures depends on altered regulation of one or more key glycosyltransferases (107).

As already described, cell-cell adhesion in epithelial tissues and tumour cells is mediated by E-cadherin in combination with α- and β-catenin and other cytoplasmic components, which link E-cadherin to the cytoskeleton. A decreased intra-tumour cell adhesion results in increased motility of tumour cells and increased metastatic potential. The type of N-glycosylation of E-cadherin modulates cadherin-dependent cell adhesion trough one of the most common forms of glycosylation aberration, namely the increased β1-6 branching of N-glycans in result of enhanced expression of GlcNAcT-V. Enhanced expression of GlcNAcT-III, another glycosyltransferase affecting N-glycan structure by catalysing the addiction of the bisecting GlcNAc branch, has also been reported in certain tumours and has the inverse effect. Enhanced GlcNAcT-V gene activity results in increased β1-6GlcNac antenna, to form multi-antennary structures without bisecting GlcNAc, and decreases cadherin activity. This reduces adhesion between tumour cells promoting metastasis. Additionally, the secreted form of GnT-V *per se* is angiogenic, which also promotes metastasis. On the other hand, overexpression of GlcNAcT-III reduces β1-6GlcNac antenna, resulting in overall structural change, enhanced E-cadherin activity, and reduced malignancy (108).

Among the most common structural features associated with cancer are the altered expression of terminal structures, which includes loss of ABO determinants by secretor individuals, changes in Lewis antigenic patterns and oversialylation. Moreover, the degree of A/B deletion is associated with the degree of metastasis or malignancy (109).

The overall increase in cell-surface sialic acid content is also a tumour cell feature which reduces the attachment of metastatic tumour cells to the matrix and may help protect them from recognition by the alternative pathway of complement activation. Sialoglycoprotein patterns are not determined exclusively by the transcription of biosynthetic enzymes or the availability of target sequons, instead bulk metabolic flux through the sialic acid pathway has the ability to increase the abundance of certain sialoglycoproteins while having a minimal impact on others (110). Apart from the amount and linkage of sialic acids, there can also be significant changes in their modifications as will be exemplified latter. The changes in Lewis antigenic patterns mentioned above are related to over-sialylation of terminal structures, resulting in an over-expression of selectin-ligands sialyl lewis^{a} (SLe^{a}) and sialyl lewis^{x} (SLe^{x}) antigens, which can be found in both N-glycans and O-glycans as well as glycosphingolipids. These sLe^{a} and sLe^{x} antigens result from the O-*3* sialylation of the Gal residue on Le^{a} (Galβ(1-3)GlcNAc[Fucα(1-4)]) and Le^{x} ((Galβ(1-3)GlcNAc[Fucα(1-4)]) residues, respectively. The SLe^{a} and SLe^{x}, are expressed

in cancer cells, mimicking their normal expression on blood cells (monocytes and neutrophils) and also mimicking their potential for migration through binding to endothelial cell selectins, thus conferring a selective advantage for tumours cells interactions with endogenous selectins. The initial adhesion mediated by these molecules triggers activation of integrin molecules through the action of several cytokines and leads to integrin-mediated cell adhesion and extravasation of cancer cells. Moreover, cancer cells also produce humoral factors that facilitate E-selectin expression on endothelial cells, and specific alterations in the transcription of several glycosyltransferases such as ST3O, UGT1 and fucosyltransferase VII and sugar transporters in cancer has also been suggested to be involved in the induction of the selectin ligands on cancer cells (93,111–114). Sialylated Lewis antigens can also be released to the bloodstream by malignant cells, thus, soluble forms of these antigens are also expressed in high amounts in the blood of many cancer patients. Based on these features, the serological evaluation of SLe^a is currently explored in the CA19-9 test for non-invasive assessment of tumour progression and metastatic spread (94).

Also, minimal structural alterations in terminal motifs may significantly alter the biological behaviour of the glycans and be explored has biomarkers of disease. Namely, a glycan epitope that is very similar to SLe^x, the sialyl-6-sulfo $Lewis^x$, was noticed to be preferentially expressed in normal epithelial cells compared to cancer cells. Essentially all genes involved in the synthesis of SLe^x are predicted to be the same as those involved in the synthesis of sialyl-6-sulfo $Lewis^x$, except for the genes engaged in its sulfation. Thus, this finding supports the idea that the reduced expression of sialyl-6-sulfo $Lewis^x$ causes induction of SLe^x expression in cancers. Moreover, a structure very similar to SLe^a termed disialyl-$Lewis^a$ is preferentially expressed in non-malignant cells, and is useful as a marker for tissue injuries occurring in benign diseases. The structural difference between SLe^a and disialyl-$Lewis^a$ is the presence of one extra sialic acid residue attached to the C-6 position of βGlcNAc in the carbohydrate structure of the latter glycan epitope. This implies that α-2-6 sialylation at the GlcNAc moiety is impaired in cancer cells compared to non-malignant cells. This event has been associated with a significant decrease in the transcription of the sialyltransferase gene responsible for α2-6 sialylation of the βGlcNAc moiety in cancers compared to normal cells due to epigenetic silencing (24).

Increased expression of galectins (especially galectin-3) has also been associated with tumour progression. Galectins are N-acetyllactosamine (Galβ1-4GlcNAc)-binding proteins that have either one or two carbohydrate reactive domains and form complexes

that cross-link glycoproteins dependent on their glycan structures and concentrations. They interact with glycoproteins in both extracellular and intracellular environments and regulate various biological phenomena, including cell growth, cell differentiation, cell adhesion, and apoptosis. A molecular significance for the correlation between increased galectin expression and tumour progression is proposed to be the interaction of galectins with poly-N-acetyllactosamines on matrix proteins such as laminin, which aids cellular invasion.

Alterations in O-glycosylation pathways are also a common hallmark of malignant transformations, frequently amplified at the cell-surface as a result of the high number of O-glycosylation sites presented by mucins. Such events are particularly pronounced in adenocarcinomas, due to the overexpression of these molecules. Perhaps the most studied cancer-associated *O*-glycans result from a premature stop in the O-glycosylation of proteins, namely the Tn antigen (the simplest O-glycan), their sialylated counterpart sialyl-Tn (sTn; Neu5Acα2-6GalNAcα-O-Ser/Thr) and the T or core 1 antigen that results from the addition of a Gal residue to the Tn antigen (Galβ1-3GalNAc-Ser/Thr). These antigens have been classically termed simple mucin-type O-glycans reflecting their overexpression in cancer-associated mucins, and stem from the incapability of the cell glycosylation machinery to produce more elongated glycans (104). Namely, several reports attribute the expression of simple mucin-type O-glycans to a disorganisation of secretory pathway organelles (ER and Golgi) in cancer cells, and absence or altered expression and/or activity of glycosyltransferases responsible for the synthesis of glycan core structures (115). In particular, the overexpression of ST6GalNAc-I has been found to promote the premature sialylation of the Tn antigen and consequent formation of the STn antigen, thereby avoiding chain elongation in gastrointestinal tissues (116), breast cancer (117) and bladder cancer (15) models. Mutations on *Cosmc*, a molecular chaperone essential for T-synthase function has been found to promote the accumulation of Tn and STn antigens in neoplastic lesions, including colon cancer and melanoma-derived cells lines (118) as well as in breast carcinomas (119).

While hindered by extended glycosylation in healthy and benign tissues, simple mucin-type O-GalNAc glycans are uncovered in the majority of human carcinomas, particularly in advanced stages of the disease. The Tn, STn and T antigens have been associated with malignant cell phenotypes and disease progression, metastasis and poor prognosis in clinical settings for a variety of different solid tumours, in particular of gastrointestinal nature (116,120,121) but also breast (122,123), lung (124) and ovarian cancers (125) and its role as promoters of aggressive cell phenotypes confirmed *in vitro* (126,127). The fact that these simple glycans are absent, significantly under-expressed or restricted to some cell types in healthy tissues, makes them ideal diagnostic and therapeutic targets (128). Proteins carrying these glycans are often released from the cell-surface and contribute to raised STn concentrations in serum (CA72-4 test) in gastric, colorectal and pancreatic carcinoma patients (129,130) as well as in gastric pre-cancerous lesions (131). The elevation of CA72-4 is considered to be useful as an independent prognostic factor in gastric cancer (132) and of tumour recurrence in gastric (133) and pancreatic cancers (134). Also, several monoclonal antibodies have been developed for STn as well as the therapeutic vaccine Theratope that, despite promising upfront results in pre-clinical and early stage clinical trials for advanced breast tumours (135–137), remains to be comprehensively accessed in clinical settings in other tumours (138).

1.3.3. Glycosylation in BC

Alterations in glycosylation of bladder tumours relate to the loss of ABO blood group determinants in advanced stage carcinomas of secretor individuals (the Se – secretor – locus dictates the capability of an individual to secrete glycoproteins carrying blood group antigens in saliva and other tissues) (139,140). Concomitantly, changes in Lewis antigens patterns, and the over-expression of simple mucin type O-GalNAc glycans have also been reported (141).

The possibility that loss of the genetically predicted blood group antigens precedes the development of recurrent, invasive or metastatic cancer has been extensively explored. The ABO blood group system consists of terminal oligosaccharide antigens carried by glycoproteins or glycolipids in hematopoietic or epithelial cells. Their biosynthesis is presumed to be controlled by the ABO, Se, H, Le, and X gene loci, which code for specific glycosyltransferases. These enzymes add monosaccharides to

precursors in a sequential fashion in which the product of one glycosyltransferase becomes the substrate of the next acting glycosyltransferase. This complex relationship leads to genetic and structural polymorphisms. Studies have shown that loss of tissue ABH (O) antigens in the initial biopsy of bladder carcinomas is predictive of a much greater chance of subsequent invasion than in those tumours in which the ABH antigens are detectable (142). However, a significant number of patients whose initial tumours were reported as blood group antigen negative failed to develop an invasive tumour. It is possible that these conflicting results may, at least in part, be explained by differences in methodology or interpretation, or both. These antigens are present on normal bladder epithelium but not on some low-grade and early- stage papillary transitional cell carcinomas of the bladder. In bladder urothelium the most well examined change has been the deletion of blood group A antigens from A individuals and H antigen from O individuals. Moreover, it has been reported the disappearance of the activity of the A and B gene-encoded transferases in bladder tumours from blood group A and B individuals which explains the deletion of these antigens in bladder tumours (143) .

The A, B, H antigens have biosynthetic and structural similarities with the Lewis antigens, including the type 1 Lewis^a and type 2 Lewis^x antigens. Several authors have also associated the Lewis^a expression patterns with malignant transformations in the bladder, others reported significantly lower expression of this antigen in healthy urothelium (6%), when compared to invasive tumours of the bladder (35%) (144,145). In agreement with this study, others have reported a high expression of Lewis^a antigen in invasive bladder carcinomas (93%). Altogether, one can associate the expression of Lewis^a antigen with a malignant phenotype of bladder tumour. Immunostaining of Lewis^x antigen in the exfoliated cells of a single urine sample is a potentially useful diagnostic test, since up to 90% of bladder tumours express Lewis^x antigen (144). Moreover, apart from their diagnostic utility, the sialylated forms of Lewis^x antigens have been closely linked to the invasive and metastatic potential of bladder cancer (146).The sialylated form of Lewis^a, the SLe^a antigen, has been observed in bladder dysplasia, CIS, non-invasive and invasive carcinomas of the bladder (147). Nevertheless, no correlation was found with invasive or metastatic potential.

The expression of β1-6 branched N-linked oligosaccharides and the GnT-V enzyme responsible by this structural alteration was closely related to low malignant potential in bladder cancer, a finding that could be applied to risk stratification (148).

As already stated, hyaluronic acid (HA) and its degrading enzyme hyaluronidase (HAase) are intricately involved in tumour growth and metastasis. HA is synthesized by the HA synthases HAS1, HAS2, and HAS3. HAS1 expression in tumour tissues is a predictor of bladder cancer recurrence and treatment failure. HA promotes tumour metastasis and is an accurate diagnostic marker for bladder cancer. Moreover, the measurement of HA and HAase (the HA-HA test) has been applied to the screening of bladder cancer. It has been shown recently that HAS1 regulates bladder cancer growth and progression by modulating HA synthesis and HA receptor levels (149).

The galectin protein family was also implicated in bladder cancer. Galectin-1 mRNA levels were markedly increased in most high-grade bladder cancers compared with normal bladder or low-grade cancers. Galectin-3 mRNA levels were also increased in most tumours compared with normal urothelium, but levels were comparable among tumours of different histological grade. Moreover, galectin-7 expression may be related to the chemosensitivity of urothelial cancer (150).

Glycosphingolipids (GSLs), including gangliosides (acidic glycosphingolipids which contain sialic acids), interact with specific membrane proteins, such as growth factor receptors, integrins, tetraspanins (TSPs), and non-receptor cytoplasmic kinases (e.g., Src family kinases and small G proteins), to form glycosynaptic microdomains controlling GSL-dependent or -modulated cell adhesion, growth, and motility. The glycosphingolipid composition of human bladder cancer tissue has been assessed and the results have demonstrated that large amounts of ganglioside GM3 accumulate in superficial bladder tumours, compared with invasive bladder tumours and that exogenous GM3 inhibits the invasive potential of bladder tumour cells. Furthermore, the GM3 overexpression system was applied to bladder tumour therapy and exogenous GM3 inhibited bladder cancer cell invasion as well as locally injected GM3 (151). Additionally, GM3 synthase gene transfection had an antitumor effect on the murine bladder cancer MBT-2 cell line. It has also been reported that the expression of GM2, GM3, or GM2/GM3 complexes affect cell motility and growth in bladder cancer (152).

Finally, increased levels of simple-mucin type *O*-GalNAc glycans have also been observed in bladder cancer. Several reports associate the presence of Tn and T antigens with recurrence and metastasis suggesting these antigens may be surrogate markers of profound cellular alterations (153,154). There is also growing evidences linking the overexpression of the sialyl-T antigen and ST3Gal.I, the enzyme responsible by T antigen sialylation, with bladder cancer aggressiveness and recurrence (155). Adding to these

findings, Ferreira et al. has recently demonstrated that over 70% of high-grade NMIBC and MIBC expressed the STn antigen, whereas 80% of low-grade NMIBC and the healthy urothelium do not. The STn antigen was mostly expressed by cells in non-proliferative tumour areas, known for their high resistance to cytostatic agents currently used to improve the overall survival of advanced stage bladder cancer patients. Studies *in vitro* have further demonstrated that this antigen plays an important role in bladder cancer cell migration and invasion trough mechanisms so far unexplored (15). Also, previous works of our group have demonstrated the presence of STn in metastized ganglia and distant metastasis, suggesting that the expression of STn may reflect on the mobility of cancer cells and the capability to metastasize.

Other studies in STn-expressing bladder cancer cells shown that STn has the ability to down-regulate the anti-cancer immune-response through different mechanisms. First, it hinders the expression of MHC-II and co-stimulatory molecules by dendritic cells (DCs), resulting in impaired ability to present cancer-associated antigens to T cells and making DCs unresponsive to successive activation stimuli. Second, it hinders the expression of inflammatory, Th1-inducing cytokines in DCs, which may result in an attenuation of the Th1 microenvironment and reduced ability to activate and polarize T cells towards the Th1 phenotype. Altogether, these results highlight the expression of STn by cancer cells as a crucial event in the establishment of the tolerogenic microenvironment which allows cancers to escape from the attack of innate and adaptive immunity (156).

Despite the key role of the STn in bladder cancer progression and dissemination, to this date, the promoters leading its biosynthesis remain yet unknown. Nevertheless, preliminary data from our group is showing a correlation between the levels of STn and hypoxic marker HIF-1α in bladder tumours sections, suggesting that hypoxia might be the missing link responsible by STn expression in bladder cancer. More in depth studies are needed to validate these preliminary observations.

1.3.4. Hypoxic regulation of glycosylation in cancer

Glycosylation is kinetically regulated by dynamically changing the portfolio of glycosyltransferases, nucleotide sugars, and nucleotide sugar transporters, which together form a part of what is currently referred to as the “Glycan cycle”. Hypoxic conditions dramatically change gene expression profiles, by activating HIF-1α, which mediates

adaptive cellular responses, which have been extensively addressed in the previous section. As already mentioned, HIF-1α induces the expression of glucose transporters and various types of glycolytic enzymes, leading to shifts in glucose metabolic patterns. This fact strongly suggests that hypoxic conditions are an important factor modulating various nucleotide sugar biosynthetic pathways. Hypoxia-induced glycosyltransferases and nucleotide sugar transporters have also been shown to modulate glycosylation patterns that are part of the mechanism associated with cancer metastasis. For example, hypoxia has an impact in the UDP-GalNAc cycle, as well as in the N-Acetylglucosaminyltransferases V (GnT-V) activity by reducing the activity of a portion of the GlcNAc cycle, including intracellular UDP-GlcNAc levels and GnT-V activity in a HIF-1α-dependent manner (157). Hypoxia also influences galactose metabolism as exemplified by enhanced expression of genes involved in transport, phosphorylation, and transfer of galactose, and this seems to trigger drastic alteration of other monosaccharides including sialic acid (24). The influence of hypoxia on the metabolism of monosaccharides other than glucose, however, is not fully elucidated.

Hypoxia increases the levels of cell surface SLe^x and Sle^a determinants in colon cancer cell lines, as well as the transcription of fucosyltransferase VII (FUT7), sialyltransferase ST3Gal-I and UDP-Gal transporter 1 in colon cancer tissues, which are involved in the synthesis of the carbohydrate ligands of endothelial E-selectin (157–159). Some qualitative changes of glycans are also induced by tumour hypoxia. For example, a part of the sialic acid residues in glycans is replaced by N-glycolyl sialic acid in cancers, while normal glycans usually carry N-acetyl sialic acid residues. This turned out to be due to induction of a gene for sialic acid transporter, Sialin, in cancers by tumour hypoxia. For instance, such cancer cells will frequently express N-glycolyl-SLe^a and N-glycolyl-SLe^x (24).

Among various kinds of glycosaminoglycans (GAGs), hyaluronic acid (HA) and its degrading enzyme hyaluronidase (HAase) are intricately involved in tumour growth and metastasis. Metabolism of hyaluronan is also markedly influenced by hypoxia. Hyaluronan serves as a specific ligand for CD44, and the cell adhesion mediated by the CD44/hyaluronan interaction is heavily involved in cancer cell motility (24). Tumour hypoxia also leads to expression of useful glycolipid tumour markers, such as gangliosides having N-glycolyl sialic acid. Moreover, hypoxia affects not only the glycan moiety of glycolipids, but also their ceramide moiety (160). Nevertheless, despite these findings, no study has

addressed the influence of hypoxia in the expression of simple mucin-type O-GalNAc glycans in cancer.

II. Background, Aims and Experimental outline

Muscle invasive bladder cancer (MIBC) is considered a neglected neoplasia, nevertheless, over 54,000 deaths have been reported in Europe and around 165,100 deaths worldwide in 2012, placing it amongst the deadliest genitourinary cancers (161). Cisplatin-based regimens are the only available therapeutics for invasive and metastatic cases, nevertheless, due to treatment failure, the five-year overall survival does not exceed 25% and many patients die prematurely from adverse drug reactions (2), urging for effective and safe targeted therapeutics.

Even though conventional chemotherapy is efficient against highly proliferative malignant cells that form the tumour bulk, non-proliferative tumour areas, characterized by high hypoxia, harbour chemoresistant clones responsible by disease relapse, progression and dissemination. Preliminary findings from our group suggest that these areas express the STn antigen, which stems from a premature stop in protein glycosylation. This epitope has been found to favour disease dissemination and is also responsible by generating distinct protein signatures at the cell-surface, providing means to target aggressive cells. Nevertheless, the association between these STn expression and hypoxia remain yet to be elucidated.

In the present work, hypoxia and STn are the main targets explored, especially because changes in the glycosylation of cell-surface and growth under oxygen deficiency (hypoxia) are salient features of solid tumours that often correlate with advanced stages of malignancy such as invasion and metastasis. As shown by several studies, hypoxia induces a wide range of biological changes, such as decreased cell proliferation, increased expression of drug-resistance genes, selection of apoptosis-resistant clones, facilitation of tumour invasion and metastasis, reduced expression of DNA repair genes, and increased genomic instability. These mechanisms undoubtedly contribute to the evolution of malignant tumour cells. However, it remains to be fully understood why hypoxic tumour cells tend to be more aggressive and more resistant to treatment than non-hypoxic tumour cells within the same tumour, despite their similar genetic background. Furthermore, there is a lack of specific hypoxia-associated cell-surface biomarkers to guide therapeutics to these cell.

Based on this background, the present work aims to explore a possible association between STn expression and hypoxia, envisaging theragnostic biomarkers capable of improving bladder cancer overall survival.

The project experimental outline includes the characterization of the effect of hypoxia on three bladder cancer cell lines with different molecular and genetic profiles regarding the activation of stem-cell and/or epithelial-to-mesenchymal transition programs, degree of proliferation, invasion capacity and STn expression, followed by validation in human tumour samples. Studies including the hypoxia-mimetic Deferoxamine Mesilate that stabilizes HIF-1α through the inhibition of Prolyl Hydroxylases (PHDs) activity by the chelation of Fe^{2+}, will also be included to disclose HIF-1α-mediated alterations. The generated information is regarded of primary importance to expand the knowledge about the clinical relevance of the STn antigen in bladder cancer and create the rationale for a STn-based therapy.

III. Materials and Methods

3.1. Cell Lines

This study was conducted on three urinary bladder cancer cell models, namely T24, 5637 and HT1376 comprising the main genetic alterations commonly found in human urinary bladder cancer (UBC), which allows for the study of the most representative human bladder tumours. The accumulated data have shown that FGFR3 mutations are characteristic for low grade and low stage tumours whereas p53 and RB1 mutations are characteristic for invasive tumours. This has led to the suggestion that UBC develop through at least two molecular pathways, one related to FGFR3 and other related to p53 and RB1 alterations (162). The cell lines used have different histological origins, the 5637 cell line is derived from a grade II carcinoma, the HT1376 cell line from a grade III carcinoma and the T24 cell line from a transitional cell carcinoma (TCC). 5637 and HT1376 represent the E2F3/RB1 pathway with loss of one copy of *RB1* and mutation of the remaining copy. Additionally, HT1376 exhibit deletion of *PTEN* gene and no alteration of *PIK3CA*, which in combination with the inactivation of *p53* grants a more invasive and metastatic potential to this cell line. In contrast, the 5637 cell line does not present any loss of *PTEN* and loses *PIK3CA* gene, which gives it a less-invasive phenotype. The T24 cell line belongs to the alternative pathway of FGFR3/CCND1 by presenting a mutated *HRAS* and over-represented *CCND1* (cyclin D1) (163).

3.2. Cell culture conditions

Cells were cultured in RPMI Medium 1640 (1X) + GlutaMAX^{TM}-I (Gibco ®; Life Technologies) supplemented with 10% heat-inactivated FBS (Gibco ® Life Technologies) and 1% Pen Strep (10,000 Units/mL Penicilin, 10,000 µg/mL Streptomycin) (Gibco ® Life Technologies). Cell lines were cultured as a monolayer at 37 ºC in a 5% CO_2 humidified atmosphere (normoxia), and were routinely subcultured after trypsinization.

The cells were also grown under hypoxic atmosphere for 6, 24, 48 and 72 hours (as further indicated) in a BINDER C 150 incubator with 0.1% O_2, 5% CO_2 and the balance N_2.

Additionally, cells were exposed to 500 µM Deferoxamine Mesilate CRS (DFX, Sigma-Aldrich) to stabilize HIF-1α. All experiments were performed at 70-80% of cell confluence.

3.3. L-Lactate assay

A fluorometric L-Lactate assay kit (Abcam) was used to determine the concentration of the intermediary metabolism product L-Lactate in culture media. Lactate is produced in proliferating cells under anaerobic conditions, with L-lactate being the major stereo-isomer formed. In Abcam's L–Lactate Assay Kit, lactate is oxidized by lactate dehydrogenase to generate a product which interacts with a probe producing color (570 nm) and fluorescence (at Ex/Em= 535/587nm). The reaction product fluorescence was measured at Ex/Em=535/590nm in a microplate reader (SynergyTM Mx, BioTek).

3.4. Cell Proliferation Assay

A colorimetric BrdU cell proliferation ELISA kit (ab126556 Abcam) was used to estimate cell proliferation. The test allows a quantitative measurement of the incorporation of Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU), a synthetic nucleoside analogue to thymidine, into newly synthesized DNA of actively proliferating cells. Briefly, T24 ($0.35x10^5$ cells/mL), 5637 ($0.15x10^5$ cells/mL) and HT1376 ($0.25x10^5$ cells/mL) cells were cultured in 96 well plates and BrdU was added to the wells during the final 24 hours of culture. To enable antibody binding to incorporated BrdU, cells were fixed, permeabilized and the DNA denatured. This was all done in one step by treatment with a kit provided Fixing Solution. Subsequently, the anti-BrdU monoclonal antibody was pipetted into the wells and allowed to incubate for one hour, binding to any incorporated BrdU. Unbound antibody was washed and horseradish peroxidase-conjugated goat anti-mouse antibody was then added. The horseradish peroxidase catalysed the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) from a colourless solution to a blue solution (or yellow after the addition of stopping reagent), the intensity of which is proportional to the amount of incorporated BrdU in the cells. The coloured reaction product was quantified using a single absorbance read at 450 nm by a microplate reader (SynergyTM Mx,

BioTek) allowing the assessment of the population of cells which are actively synthesizing DNA.

3.5. Cell Invasion Assay

Invasion assays were performed using BD BioCoat Matrigel^{TM} invasion chambers, comprised by an 8 µm diameter pore size filter coated with a thin layer of Matrigel. Prior to each experiment, filters were re-hydrated in serum-free RPMI medium for 1 h at 37 ºC. After detachment of confluent cells by trypsinization, cells were suspended in complete culture medium, counted and seeded on the upper side of the matrigel-coated filter at a density of $5x10^4$ cells/ well. After incubation for 24h at 37 ºC under normoxic or hypoxic conditions, invasive cells were fixed with 4% Paraformaldehyde and non-invading cells, present on the upper side, were completely removed, to facilitate analysis. Cells that invaded the underside of the filters were mounted in Vectashield Mounting Medium with DAPI (Vectashiels®, Vector Laboratories), and visualized through a Leica DM2000 fluorescence microscope (Leica Microsystems). Invasive cells were scored in at least 12 microscopic fields (20x objective).

3.6. Flow Cytometry analysis

STn expression was determined by flow-cytometry. Approximately 10^6 cells/ml were seeded in 6-well plates and allowed to adhere for 24h. Subsequently, the medium was removed and the cells were repeatedly washed with PBS and dissociated into single-cell suspensions with Versene 1:5000 (1X) (Gibco) at 4 ºC, followed by filtration using a 70 µm Nylon cell strainer (BD Falcon). Detached cells were fixed with 4% Paraformaldehyde, suspended in 500 µL PBS and the anti-STn primary antibody was used according with table I indications. Finally, the cells were incubated with polyclonal rabbit anti-mouse Immunoglobulins/FITC (1:84) secondary antibody. After being washed, the cells were analysed by flow cytometry in a BeckMan Coulter FC500 Cytometer. Each independent experiment was performed in triplicate.

3.7. Neuraminidase treatments

Whole cells and protein extracts previously digested with α-neuraminidase were used as sialyl-Tn negative controls in flow cytometry and western blot analysis, respectively. Briefly, for flow cytometry analysis, cells were detached with Versene, filtered, suspended in RPMI Medium 1640 (1X) + GlutaMAXTM-I, and treated with 10 U/mL α-neuraminidase from *Clostridium perfringens* (Sigma) for 90 minutes at 37 ºC with frequent medium resuspension. For western blot, glycoproteins immobilized in nitrocellulose membranes were treated with 0.25 U/mL Neuraminidase for 2h at 37 ºC prior to probing with primary and secondary antibodies.

3.8. Gelatin Zymography

Gelatin zymography was performed to determine the activity of the matrix metalloproteinases MMP-2 and MMP-9. Proteins (15 µg/ lane) from conditioned media were separated on 10% polyacrylamide zymogram gels with 0.1% gelatin (MERCK) as a substrate. After electrophoresis, gels were incubated in 2% Triton X-100 (Sigma) in deionized water for protein renaturation, with gentle agitation for 30 minutes at room temperature. Subsequently, gels were incubated in MMP substrate buffer [50 mM Tris-HCl, pH 7.5; 10 mM $CaCl_2$] overnight with gentle shaking at 37 ºC. The gels were finally stained with filtered Coomassie blue solution (Sigma) for 30 minutes and then washed with deionized water until the adequate resolution was obtained. Gelatinolytic bands were observed as white areas against the blue background, and the intensity of the bands was evaluated using the Quantity One Software.

3.9. Western Blot

The expression of hypoxic biomarkers HIF-1α, CA IX and cancer-associated carbohydrate antigen STn were evaluated by western blot. Proteins were isolated from whole cells in cell lysis Rippa buffer (20mM Tris [pH=7.5], 150mM NaCl, 1% Triton-X100, 1% NP-40) containing protease and phosphatase inhibitor cocktail (Halt™ Protease & Phosphatase Inhibitor Cocktail; Thermo Scientific).The protein content was estimated

using DC Protein Assay (Bio-Rad) and then separated by SDS–PAGE under reducing conditions and transferred onto 0.45 µm nitrocellulose membranes (GE Healthcare). HIF-1α, CA IX, STn, TB-α and GAPDH membranes were blocked with Carbo-Free Blocking Solution 1X (Vector Laboratories) for 1h at room temperature and incubated according with table I. After a washing procedure, membranes were incubated with the respective secondary antibody according with table I. After final washing, the bound antibodies were revealed by chemiluminescence using either the WesterBright^{TM} Sirius Kit (advansta) or the Amersham ECL Prime Western Blotting Detection Reagent kit (GE Healthcare) or Amersham ECL Western Blotting Detection Reagents (GE Healthcare), depending on the amount of epitope available.

Table I.- Primary and secondary antibody specification

Method	***Primary Antibody***	***Secundary antibody***	***Incubation Conditions***
western Blot	Mouse monoclonal [16H4L13] to HIF-1-alpha (invitrogen)	Polyclonal anti-Mouse whole IgG affinity-Purified secondary antibody (Jackson ImmunoResearch)	Primary Ab: Dilution 1:250, 4 ^{0}C overnigh
			Secunday Ab: Dlilution 1:70.000, Room Temperature, 30 minutes
	Mouse monoclonal [2D3] to Carbonic Anhydrase IX (abcam)	Polyclonal anti-Mouse whole IgG affinity-Purified secondary antibody (Jackson ImmunoResearch)	Primary Ab: Dilution, 1:2000 Room Temperature, 30 minutes
			Secundary Ab: Dilution 1: 70.000, Room Temperature, 30 minutes
	Mouse monoclonal [B-5-1-2] to alpha-Tubulin (Sigma)	Polyclonal anti-Mouse whole IgG affinity-Purified secondary antibody (Jackson ImmunoResearch)	Primary Ab:Dilution 1: 15.000, Room Temperature, 30 minutes
			Secundary Ab: Dilution 1: 70.000, Room Temperature, 30 minutes
	Rabbit Polyclonal [FL-335] to GAPDH (Santa Cruz Biotechnology)	Polyclonal Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) secondary antibody (Jackson ImmunoResearch)	Primary Ab: Dilution 1:1000, Room temperature, 30 minutes
			Secundary Ab: Dilution 1:130.000, Room Temperature, 30 minutes
	Mouse Monoclonal [TKH2] to STn in culture supernatant	Polyclonal anti-Mouse whole IgG affinity-Purified secondary antibody (Jackson ImmunoResearch)	Primary Ab: Dilution 1:5, Room Temperature, 30 minutes
			Secundary Ab: Dilution 1:130.000, Room Temperature, 30 minutes
Flow Cytometry	Mouse Monoclonal [TKH2] to STn in culture supernatant	Polyclonal Rabbit Anti-Mouse Immunoglobulins/FITC (1:84)	Primary Ab: 30 µl/10^{6} cells, Room Temperature, 30 minutes
			Secondary Ab: 6 µl/10^{6} cells, Room temperature in the dark, 15 minutes

3.10. Gene expression measurements

Total RNA from cultured cells was isolated using TriPure isolation Reagent (Roche). The RNA quantity and its purity were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc). The cDNA was obtained through reverse transcriptase reaction using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Reverse transcriptase reaction contained a total volume of 60 µL reaction mix with: 30 µL of master mix containing 1x RT Buffer, 4 mM of total dNTPs, 150 U MultiScribe Reverse Transcriptase Enzyme (Applied Biosystems, Foster City, CA, USA) and 30 µL (200 ng/ µl) of RNA sample. The amplification conditions were the following: 25 ºC for 10 minutes (min), 37 ºC for 120 min. and RT inactivation at 85 ºC for 5 min. All reverse transcriptase reactions included two no-template controls (double distilled water replacing template RNA was used as negative controls). The products were amplified in a Mycycler Thermal cycler (Bio-Rad).

The expression levels of a panel of 21 genes, including 3 reference genes were assessed by quantitative polymerase chain reaction (qPCR) on an StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with a 10 µL final volume mixture containing 1x TaqMan® Universal PCR Master Mix II (Applied Biosystems, Foster City, CA, USA), TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) (Table II) and cDNA. Thermal cycling conditions were: 10 min. at 95 °C followed by 45 cycles of 15 seconds (sec.) at 95 °C and 1 min. at 60 °C. All reactions were run in duplicate. During the cDNA exponential amplification the product formation was proportional to the fluorescence emission resulting from the TaqMan probe degradation (van der Velden et al., 2003). The mRNA levels were normalized for the expression of *GAPDH*, which was taken as a suitable endogenous control, once it was the most stable of the 3 evaluated reference genes. The relative mRNA levels were calculated by adapting the $2^{-\Delta\Delta Ct}$ formula, according to the method described by Livak (164).

Table II - TaqMan Gene Expression Assays references used to assess transcript levels for the 24 *selected* genes

Cell function	***Gene***	***TaqMan Gene Expression Assay reference***
Self-renewal and stemness	NANOG	Hs04260366_g1
	LIN28A	Hs01552405_g1
	OCT-4 (POU5F1)	Hs04260367_g1
	KLF9	Hs00230918_m1
	KLF4	Hs00358837_g1
	SOX2	Hs01053049_s1
Epithelial phenotype	CDH1	Hs01023894_m1
	DSP	Hs00950591_m1
	EpCAM	Hs00901885_m1
Mesenchymal phenotype	FN1	Hs00365052_m1
	CDH2	Hs00983056_m1
	VIM	Hs00185584_m1
Extracellular matrix synthesis and promotion of changes to cell shape	SPARC	Hs00234160_m1
EMT	SNAI1	Hs00195591_m1
	SNAI2	Hs00950344_m1
	TWIST1	Hs01675818_s1
	TWIST2	Hs00382379_m1
	ZEB1	Hs01566410_m1
	ZEB2	Hs00207691_m1
	RUNX1	Hs01021971_m1
	RUNX2	Hs01047973_m1
Housekeeping genes	GAPDH	Hs03929097_g1
	HPRT	Hs99999909_m1
	ActB	Hs99999903_m1

3.11. Analysis of HIF-1α, CA IX, STn in bladder tumours

Formalin-fixed, paraffin embedded (FFPE) tissue sections were screened for HIF-1α, CA IX and STn by immunohistochemistry using the avidin/biotin peroxidase method. Briefly, 3 μm sections were deparaffinised with xylene, rehydrated with graded ethanol series, microwaved for 15 min in Antigen Unmasking Solution, high pH (Vector

Laboratories) and exposed to 3% hydrogen peroxide for 20 min. The expression of STn was then evaluated using anti-STn mouse monoclonal antibody, clone TKH2, 1:5 overnight at 4 ºC, that identifies both single and clustered STn residues, whereas CA IX was identified using monoclonal mouse anti-human CA IX antibody (abcam),clone 2D3, 1:100 1h at room temperature. The expression of HIF-1α was evaluated using monoclonal rabbit anti-human HIF-1α (Invitrogen), clone 16H4L13, 1:75 overnight at 37 ºC. After blockage with BSA (5% in PBS), the antigens were identified with Vectastain Elite ABC peroxidase kit (Vector Laboratories) followed by incubation with 3,3-diaminobenzidine tetrahydrochloride (DAB, Dako). Finally, the slides were counterstained with haematoxylin for 1 min. A semi-quantitative approach was established to score the immunohistochemical labelling based on the intensity of staining and the percentage of cells that stained positively. Both the percentage of positive tumour cells and the staining intensity were taken into account. The percentage of positive cells was rated as follows: 0 points, no expression; 1 point, < 10% positive tumour cells; 2 points, 11–50% positive cells; 3 points, 51–80% positive cells; and 4 points, > 81% positive cells. The staining intensity was rated as follows: 1 point, weak intensity; 2 points, moderate intensity; and 3 points, strong intensity. According to the overall score resulting from the arithmetic sum of these variables, the tumours were classified into four groups: absent nuclear immunostaining (0 points), < 10% positive cells, regardless of intensity (1-2); weak nuclear immunostaining (3); moderate nuclear immunostaining (4-5); and strong nuclear immunostaining (6-7). For statistical reasons, tumours were then scored according to a two-scale system: low reactivity denoting tumours with absent or weak nuclear immunostaining (0-3 points) and high reactivity denoting tumours with moderate to strong nuclear reactivity (4-7 points). According to the criteria defined by Ferreira et al., the tumours were considered positive for the STn antigen whenever reactivity was observed (15).

3.12. Statistical Analysis

Statistical analysis was performed with IBM Statistical Package for Social Sciences-SPSS for Windows (versions 20.0). T-student test was used to compare mean values with a 95% confidence interval. Chi-square analysis was used to compare categorical variables.

IV. Results and Discussion

Muscle invasive bladder cancer is a highly aggressive malignancy characterized by decreased overall survival and poor response to chemotherapy, urging the identification of novel biomarkers with theragnostic potential. Preliminary observations in clinical samples (Ferreira et al., unpublished results) suggested that hypoxic conditions may promote the overexpression of proteins presenting the cancer-associated carbohydrate antigen sialyl-Tn at the cell-surface, which has been found to enhance cell invasion capability and immune tolerance *in vitro*. Furthermore, the overexpression of specific cancer-related glycoproteins associated with aberrant glycosylation by STn has been shown to generate unique protein signatures at the cell-surface, providing means to selectively target aggressive cells (15,120,165). Despite these observations, the association between hypoxia and STn warrants validation.

In the present work we assessed how hypoxia modulates proliferation, invasion, proteolytic capacity, as well as STn expression and the activation of stem-cell and/or epithelial-to-mesenchymal transition genetic programs in three genetically and molecularly distinct bladder cancer cell lines. HIF-1α, CA IX and STn expression was further validated in clinical samples. Moreover, HIF-1α-mediated alterations was disclosed using the hypoxia-mimetic DFX that stabilizes HIF-1α through the inhibition of Prolyl Hydroxylases (PHDs) activity by the chelation of Fe^{2+}.

4.1. Hypoxic response

The first part of the study was devoted to assessing the hypoxic biomarkers HIF-1α and CA IX in the three cell lines over time, under hypoxic conditions (0.1% O_2) and using the hypoxia mimetic DFX (500 µM), envisaging the optimization of the exposure time for further analysis. As previously described, HIF-1α is ubiquitously expressed by human cells and is an essential transcription factor for the transcription of *CA9* gene and expression of CA IX protein. In experiments conducted in normoxia in the presence of DFX, the compound stabilizes HIF-1α through the inhibition of Prolyl Hydroxylases (PHDs) activity by the chelation of Fe^{2+}, which is required for enzymatic activity, thereby mimicking hypoxic conditions. The inhibition of this enzyme, predominantly localized in the cytoplasm

where it plays a pivotal role in targeting HIF-1α for proteasomal degradation, was used in an attempt to highlight HIF-1α-mediated events.

Western blot data showed HIF-1α protein has two bands at approximately 92 and 110 kDa (Figure 5A). The two bands for HIF-1α may result from post-translational modifications, splice variants or isorforms or multimer formation. Recent studies suggest that phosphorylation of HIF-1α by mitogen-activated protein (MAP) kinases is a common modification that increases its transcriptional activity independently of the effects of hypoxia, but the site(s) of phosphorylation and the mechanism by which transcriptional activity is enhanced have not been established yet (166). Additionally, splice variants of HIF-1α created by alternative splicing of the same gene, lacking several exons or displaying different exons than the wild type protein have already been reported and also may account for the two bands pattern for HIF-1α protein (167). Some of these isoforms encode cytoplasmic HIF-1α protein or proteins with altered transcriptional activity compared to the wild type protein. Regarding the N-terminal domain, two HIF-1α isoforms with different first exons have been identified: HIF1α1.2, a protein with a different first and second exons which is 59 amino acids shorter than wild type HIF-1α and encodes a cytoplasmic protein specifically expressed in the human testis (168); HIF1α1.3, which is present in activated T-lymphocytes and encodes a functional protein with weaker transcriptional activity that the wild type protein (169). At the C-terminal domain, isoforms lacking either exon 12 (170) or exons 11 and 12 (171) have been reported. Both isoforms have been shown to be stable cytoplasmic proteins and inhibit the function of full length HIF-1α. Another shorter HIF-1α isoform lacking exon 14 has also been reported (172). This isoform was shown to be 3-fold less active than full-length HIF1α. Furthermore, multimers may also contribute to this feature. Although multimers are usually prevented in reducing conditions, strong interactions can result in the appearance of higher bands. Altogether, post-translation modifications, splice variants and multimer formation contribute to the two bands pattern, and for quantification purposes the sum of the two consecutive bands was taken into account. The identification of the proteins in these bands by mass spectrometry should, in the future, allow confirming these hypotheses. The CA IX and TB-α proteins appear in a single band pattern and have observed band sizes of 55 kDa and 50 kDa, respectively (Figure 5A).

Figure 5B shows that for T24 and 5637 cell lines, HIF-1α expression is higher at 24h, while for HT1376 cell line it is significantly increased at 6h in hypoxia and DFX conditions in relation to normoxia. The CA IX proteins follow the same tendency as HIF-1α (Figure

5B), in accordance with the fact that the transcription of this protein is strongly regulated by HIF-1α. An increase in the exposure time of T24 and 5637 cells to hypoxia and DFX (48 and 72h) results in a decrease in HIF-1α and CA IX protein levels. Such differences may reflect a response shift from acute hypoxia (≤ 24h) to longer-term or chronic hypoxia (> 24h), which has been described to affect the transcription and translation of several proteins that might indirectly influence the expression of these markers (22). In addition, miRNAs may also directly influence HIF-1α responses to prolonged hypoxia. As part of the RNA-induced silencing complex (RISC), miRNAs negatively regulate gene transcription by annealing to the 3′ untranslated region of specific mRNA targets to repress translation, enhance mRNA degradation, or both (26,173). Hypoxia initially induces increased expression of HIF-1α, but during sustained hypoxia, the amount of HIF-1α mRNA is reduced. Instead, there is considerable overexpression of the HIF-1α natural antisense transcript (aHIF), which encodes the antisense template of the 3'-untranslated region of HIF-1α mRNA. This represents a negative feedback loop, in which aHIF inhibits the translation of HIF-1α mRNA after chronic hypoxia, clarifying the pattern observed (174,175). Furthermore, hypoxia has been shown to induce the expression of a number of other miRNAs, which have been termed "hypoxamirs" that regulate HIF-1α expression (17,21,105,106,176)

Finally, the molecular differences observed between cell lines overtime might be explained by different genetic characteristics of each cell line. In tumour cells, loss of p53 activity results in increased HIF-1α expression and increased transcription of downstream target genes such as CA IX. Furthermore, PTEN negatively regulates the PI3K pathway and, therefore, loss of PTEN activity leads to increased HIF-1α expression. So, PTEN mutations might promote tumour growth by synergistically promoting HIF-mediated responses (20). In HT1376 cells, PTEN gene is deleted which in combination with the inactivation of p53 potentiates expression of HIF-1α. This particular features may explain the reason why HT1376 cell line shows significant levels of HIF-1α protein after only 6h of exposure to hypoxic conditions in comparison with T24 and 5637 under the same microenvironmental challenge.

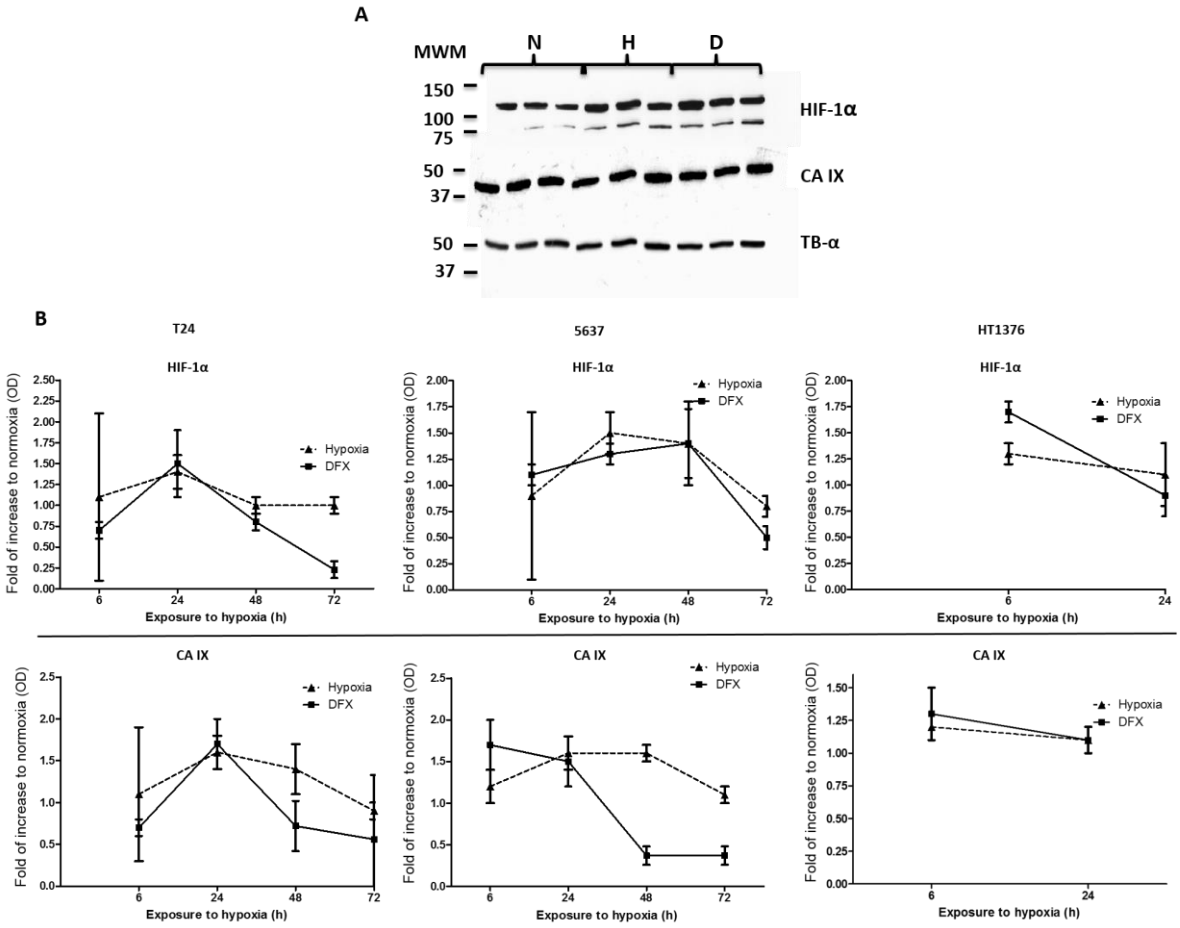


Figure 5. Urinary bladder cancer cell lines exposed to hypoxia and hypoxia mimetic DFX show differential expression of hypoxia markers over time. All cell lines were cultures for 24h before exposure to any experimental conditions. A. Western Blot analysis of HIF-1α and CA IX proteins, 24h (T24 and 5637 cell lines) and 6h (HT1376 cell line) after treatment. TB-α was used as loading control. Molecular weight markers are shown to the right and are expressed in kDa. The western blot samples appear in the following order: T24, 5637 and HT1376 Normoxic conditions (N); T24, 5637 and HT1376 Hypoxic conditions (H); T24, 5637 and HT1376 DFX treatment (D); B. Protein expression time course of HIF-1α and CA IX was measured by western blot analysis. Graphs represent average value of at least three independent experiments, flags correspond to SD and ***p < 0.001; **p < 0.01; *p < 0.05

The metabolic shift of the cells from aerobic to anaerobic metabolism, a critical event underlying hypoxia, was also confirmed by increased lactate levels in hypoxic cells culture mediums (Figure 6). Similar behaviour was also observed upon DFX exposure, which suggests that HIF-1α may be the key mediator for these changes. Hypoxic tumour cells primarily use glucose for glycolytic energy production and release lactic acid, creating a lactate gradient that mirrors the oxygen gradient in the tumour. Although lactate is generally considered a waste product, Sonveaux et al. showed that it is a prominent

substrate that fuels the oxidative metabolism of oxygenated tumour cells (177). There is therefore a symbiosis in which glycolytic and oxidative tumour cells mutually regulate their access to energy metabolites. The same authors further identified monocarboxylate transporter 1 (MCT1) as the prominent path for lactate uptake by a human cervix squamous carcinoma cell line that preferentially utilized lactate for oxidative metabolism (177). The expression of MCT1 will also be assessed in the future in the bladder cancer cell models. Interestingly, HT1376 cell line shows significantly elevated basal levels of lactate in normoxic conditions in comparison to the other cell lines (Figure 6). A number of studies have shown that malignant transformation is associated with an increase in glycolytic flux and in anaerobic and aerobic cellular lactate excretion, which is consistent to the basal levels of lactate in normoxic conditions for all cell lines (178). Nevertheless, other mechanisms could account for the HT1376 particular case. The HT1376 elevated basal levels of lactate may correlate with elevated basal levels of HIF-1α, since lactate also stimulate the accumulation of HIF-1α, independently of hypoxia (27). In various tumour types investigated, high concentrations of lactate were correlated with a high incidence of distant metastasis already in an early stage of the disease. Moreover, recent reports show various biological activities of lactate that can enhance the malignant behaviour of cancer cells. These mechanisms include the activation of hyaluronan synthesis by tumour-associated fibroblasts, up-regulation of vascular endothelial growth factor and HIF-1α itself, and direct enhancement of cellular motility that generates favourable conditions for metastatic spread. Thus, lactate accumulation not only mirrors but also actively enhances the degree of tumour malignancy, which correlate to the characteristics of grade III carcinoma HT1376 cells.

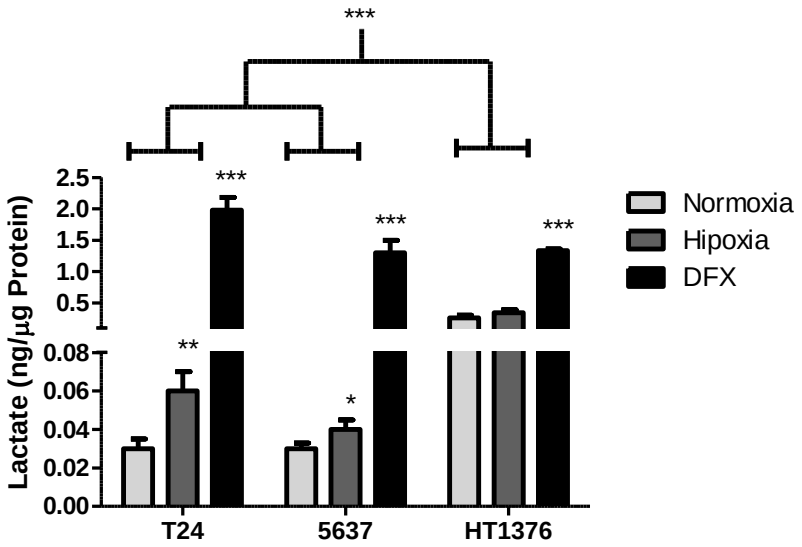


Figure 6. When exposed to hypoxic conditions all UBC cell lines actively switch to an anaerobic metabolism. All cell lines were cultures for 24h before exposure to any experimental conditions. After a hypoxia (0.1% O_2) or DFX (500μM) exposure period of 24h (T24 and 5637) and 6h (HT1376), lactate levels of the cultures conditioned mediums were measured. Graph represents average value of three replicates, flags correspond to SD and ***p < 0.001; **p < 0.01; *p < 0.05.

Altogether, bladder cancer cell lines T24 and 5637 overexpress hypoxia biomarkers HIF-1α and CA IX after 24h of exposure to oxygen deprivation, while for HT1376 this effect was already observable at 6h. Concomitantly, these cells shifted towards an anaerobic metabolism. The stabilization of HIF-1α with DFX resulted in similar behaviours suggesting that this transcription factor might regulate these events.

4.2. Morphological characterization of urinary bladder cancer cells

In normoxic conditions T24 cells show a heterogeneous appearance, i.e. cells on the periphery of the islands mostly present a polyhedral morphology but peripheral elongated cells are also present. T24 cells are pleomorphic and have more centroid nucleus than the other cell lines (Figure 7A). The growth of T24 cells in tissue culture was characterized by a disorderly pattern of growth in one or more layers and by mixed epithelioid-fibroblastoid morphology. These observations are consistent with the original characterization of T24 cell line (128,129). In the course of hypoxic treatment, cells acquire a more elongated semblance as well as a larger intercellular space between them. These morphological

changes are more pronounced during DFX treatment, suggesting this might be an HIF-1α-mediated phenomena.

Concerning the 5637 cell line, in normoxic conditions 5637 cells present polyhedral morphology with the nucleus in a lateral position. Even in the inner cells of the island the nucleus is not the centroid of the cell. Peripheral normoxic cells also present extensive areas of membrane ruffling (MR). In hypoxic conditions polyhedral and elongated cells are present as well as membrane ruffling. This morphology can be considered as intermediate between normoxic and DFX conditions. When subjected to DFX treatment, cells do not form cohesive but dispersed islands with hook-shaped cells (HSC) in the extremities, which along with increased intercellular distances suggests possible cell scattering (Figure 7B). The 5637 cell line scattering has already been reported by other authors in other conditions than hypoxia (130).

Normoxic HT1376 cells present polyhedral morphology with peripheral nucleus and grows in islands. The existence of giant cells, large vacuoles and cytoplasmatic granules could be noticed in cultured cells. These cells have an extensive membrane area without membrane ruffling. Intercellular spaces are evident and emphasized with higher amplification, highlighting the cell junctions (CJ) (Figure 7C). When subjected to hypoxia and DFX treatment, HT1376 cells have a very similar appearance to that observed in normoxic conditions, suggesting these cells retain their original morphology under oxygen privation or that 6h treatments may not be enough to see morphological changes. The epithelial morphology of HT1376 cell line and the presence of citoplasmatic vacuoles were also reported by other authors (131).

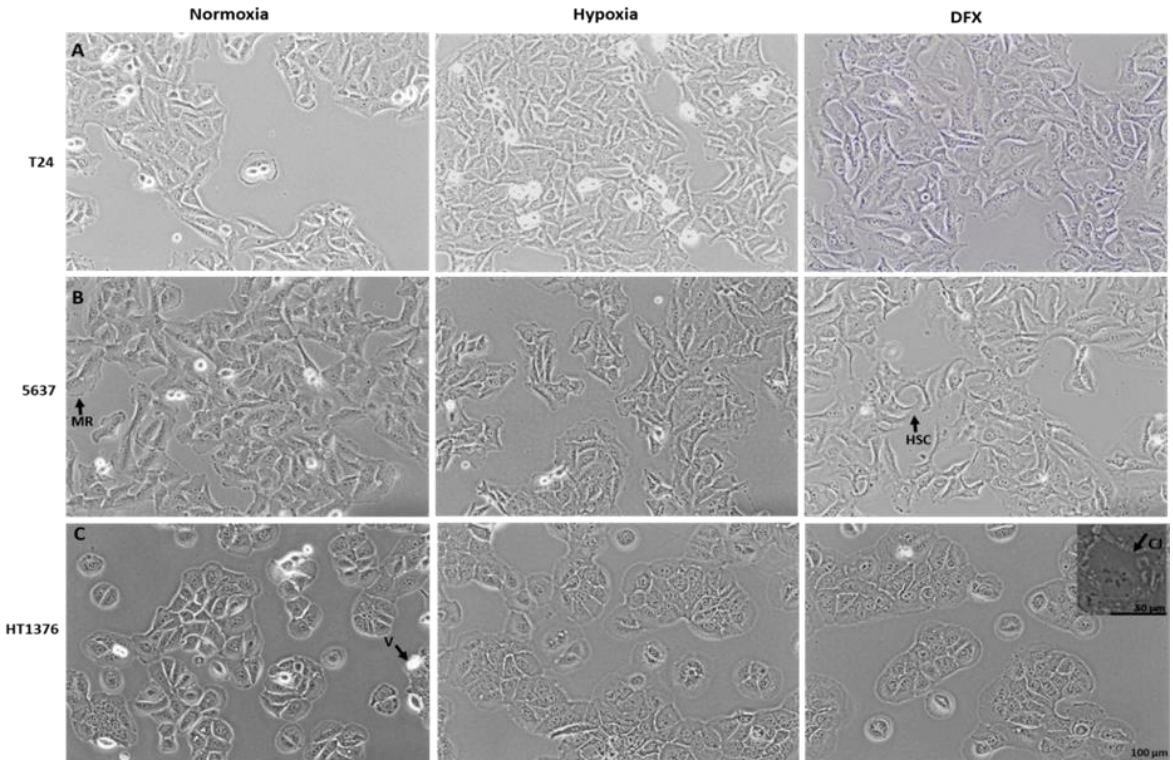


Figure 7. Hypoxic conditions modify morphological traits of urinary bladder cancer cell lines. All cell lines were cultured for 24h before exposure to experimental conditions. T24 (A) and 5637 (B) cells were then exposed to 500 µM DFX or 0.1% O2 for 24h, while HT1376 (C) cells were exposed to the same conditions for 6h. Images were acquired using a high-resolution inverted microscope. CJ, cell junctions, HSC, hook-shaped cells, MR, membrane ruffling; V, vacuole. Scale bars correspond to 100 and 50 µm.

4.3. Hypoxic modulation of EMT and Stemness

As previously described, the epithelial-to-mesenchymal transition (EMT) is a process by which epithelial cells lose their polarity and are converted to a mesenchymal phenotype, which is regarded as a critical event in morphogenetic changes during embryonic development, wound healing and cancer metastasis (82).The hypoxic microenvironment emerges as an important factor in the induction of pathological EMT, which is a key link in cancer progression (83). According with this observations, to explore the events mentioned above in UBC models, the cells were further characterized in relation to the expression of panel of 21 genes associated with stem cell (*NANOG, LIN28A, POU5F1, KLF9, KLF4, SOX2),* epithelial (*CDH1, DSP, EPCAM*), epithelial-to-mesenchymal transition (*SNAI1, SNAI2, TWIST1, TWIST2, ZEB1, ZEB2, RUNX1, RUNX2*), and mesenchymal (*FN1, CDH2, VIM, SPARC*) programs. The *GAPDH* gene was

selected as reference gene, since it was expressed at constant levels in all experimental condition and all cell lines.

According to the map in Figure 8A, under normoxia the T24 and 5637 cells presented a significant down-regulation of epithelial markers (*CDH1, EPCAM* and *DSP*) accompanied by an up-regulation of mesenchymal genes (*CDH2, FN1, SPARC*, and *VIM*) when compared to HT1376. The up-regulation of mesenchymal markers is particularly evident in the 5637 cell line whereas the T24 cell line showed a more marked stem cell character. A comparison between these two cell lines highlighted a significant ($p \leq 0.005$) down-regulation of *KLF4*, *CDH1, DSP* and *CDH2* and up-regulation of *NANOG, LIN28A, TWIST1* and *ZEB2*. An integrative analysis of these differences using ClueGo and CluePedia showed that T24 cell line presents a significant up-regulation of pathways involved in stem cell development and also a profound deregulation of cell-cell adhesion, translated by a negative regulation of cell-cell contact and adherent junction organization, suggesting a higher migration potential. In resume, gene expression analysis highlights that both T24 and 5637 present a pronounced mesenchymal properties, with T24 exhibiting a more pronounced stem nature. On the other hand, the HT1376 cell line showed a marked epithelial nature which was not found in the other two cell lines. Noteworthy, is that 5637 cells are close related with HT1376 from the genetic point of view (163); however this cell line was found more similar to T24 based on the studied gene expression patterns.

Cells were then grown under hypoxia and in the presence of DFX to determine the influence of oxygen and HIF-1α on the gene expression pattern. According to Figure 8B, T24 and 5637 presented similar variations in terms of genes expression from hypoxia to normoxia, while HT1376 had a distinct gene expression pattern, like previously observed in normoxia. The most string difference between the cell lines was the up-regulation of epithelial-to-mesenchymal and mesenchymal markers (6/11 studied genes) in HT1376, which was not evident in the other cells that already presented this phenotype in normoxia. However, both similarities and differences could be observed between the cell lines based on the most significant gene variations ($p \leq 0.005$) as highlighted by the Venn diagram in Figure 8C. In particular, at the 95% confidence level, T24 and 5637 cells significantly down-regulated *SPARC* and *SNAI1* while overexpressing *SNAI2* and *RUNX1*. On the other hand, T24 and HT1376 cells shared the up-regulation of *FN1* and *KLF4*, while 5637 and HT1376 both up-regulated *ZEB2*. The T24 cells distinguished from the other cell lines by also down-regulating *KLF9* while up-regulating *RUNX2*. An increase in *POUF1* was

only observed in 5637 and *TWIST1* overexpression was only found in HT1376. The integrative analysis of these profiles using ClueGo and CluePedia (Figure 9) showed that T24, 5637 and HT1376 cells in hypoxia presented a positive regulation of stem cell development, i.e. up-regulation of any process that increases the rate, frequency or extent of stem cell maturation, which does not include the steps involved in committing a cell to a specific fate. The T24 cell line also showed up-regulated stem cell differentiation processes, which comprehends events that drive relatively unspecialized cells to acquire specialized features of a stem cell. In addition, 5637 and HT1376 cells presented an up-regulation of biological pathways committed to mesenchymal development. Altogether, this data suggests that hypoxia drives bladder cancer stem cell establishment and mesenchymal phenotypes.

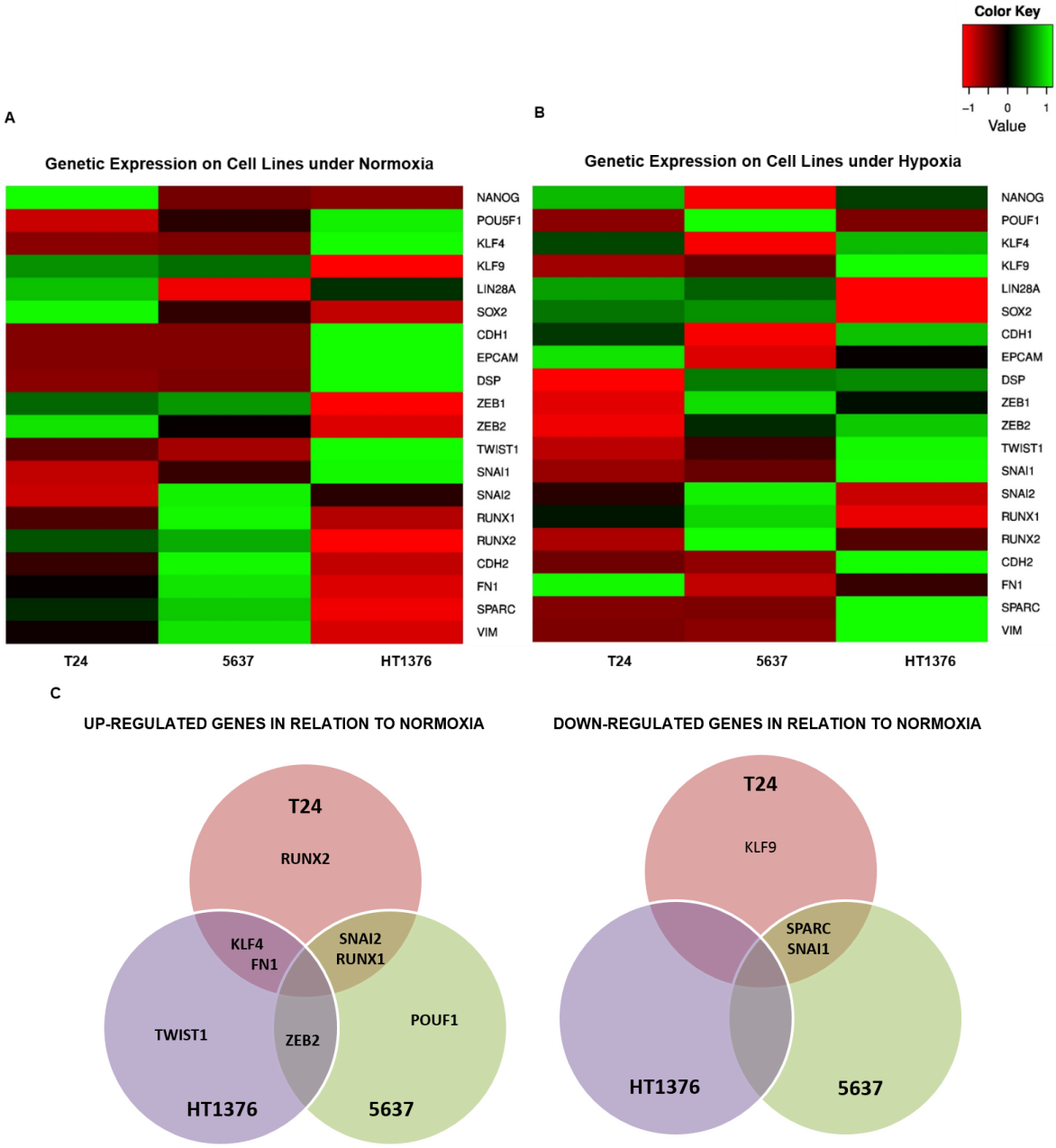


Figure 8. Heatmaps showing normoxic (A) and hypoxic (B) modulation of the transcript levels of a small group of EMT, Stem and mesenchymal markers. All cell lines were cultured for 24h before exposure to any experimental conditions. After hypoxia (0.1% O_2) or DFX (500µM) exposure period of 24h (T24 and 5637) or 6h (HT1376), the transcript levels of a 21 genes tailored panel were quantified by qRT-PCR C. Venn diagrams showing statistical significant data, focusing on overlapping patterns of gene expression in each cell line.

Figure 9.Unified conceptual framework integrating genetic expression data and non-redundant biological functions. The functional interaction network was obtained using Cytoscape Software Version 3.1.1.CluePedia and ClueGo plugins for

single cluster analysis and comparison of gene clusters in order to explore cellular processes and their dynamics. ***p < 0.001; **p < 0.01; *p < 0.05.

The cells were then compared based on variations in gene expression under hypoxia in relation to normoxia (conditions HN) and growth with DFX in relation to normoxia (conditions DN). Figure 10 allows the determination of a set of genes which are under- or overexpressed at any given significance level. At the 95% confidence level, some genes are overexpressed at both HN and DN: *FN1* (HT1376), *KLF4* (T24 and HT376) and *SNAI2* (T24 and 5637). More interestingly, *ZEB2* (HT376) and *RUNX1* (T24) would also be in this category, for a confidence level of 90% (Figure 10 top-right insert). Also a 95% confidence level, *SPARC* (T24 and 5637) and *KLF9* (T24) were under-expressed both in DN and in HN (Figure 10, bottom-center insert). *SNAI1* was under-expressed in HN conditions in both T24 and 5637 cells, but over-expressed, at a 95% confidence level, when DN conditions are considered (Figure 10, bottom-right insert). At a 90% confidence level, *RUNX1* and *POUF1* appeared to be over-expressed in 5637 cells in HN conditions, but under-expressed in DN (Figure 10, middle-right insert). Overall, Figure 10 shows that the weighted under- and over- expression of the tested genes in HT376 cells under hypoxia and under DFX treatment are moderately correlated. This allows the hypothesis that these cells have similar behaviour in hypoxia and in DFX, from the point of view of the activation of the tested genes. This behaviour is not observed for 5637 and T24 cells, again in accordance with previous observations of similarities between the two cell lines. Noteworthy, is that the *SNAI2*, *RUNX2* and *KLF4* genes that drive, together with HIF-1α, the stem cell character of T24 cells in hypoxia presented equal behaviour with DFX treatment. Likewise, *SNAI2*, which together with HIF-1α contributes to stem cell development in 5637 cells, is also modulated by DFX. Finally, *KLF4* and *ZEB2* that contribute to stem cell development in HT1376 are also suggested to be driven by HIF-1α. Altogether, these observations reinforce the key role played by HIF-1α in driving the stem cell character of bladder cancer cells.

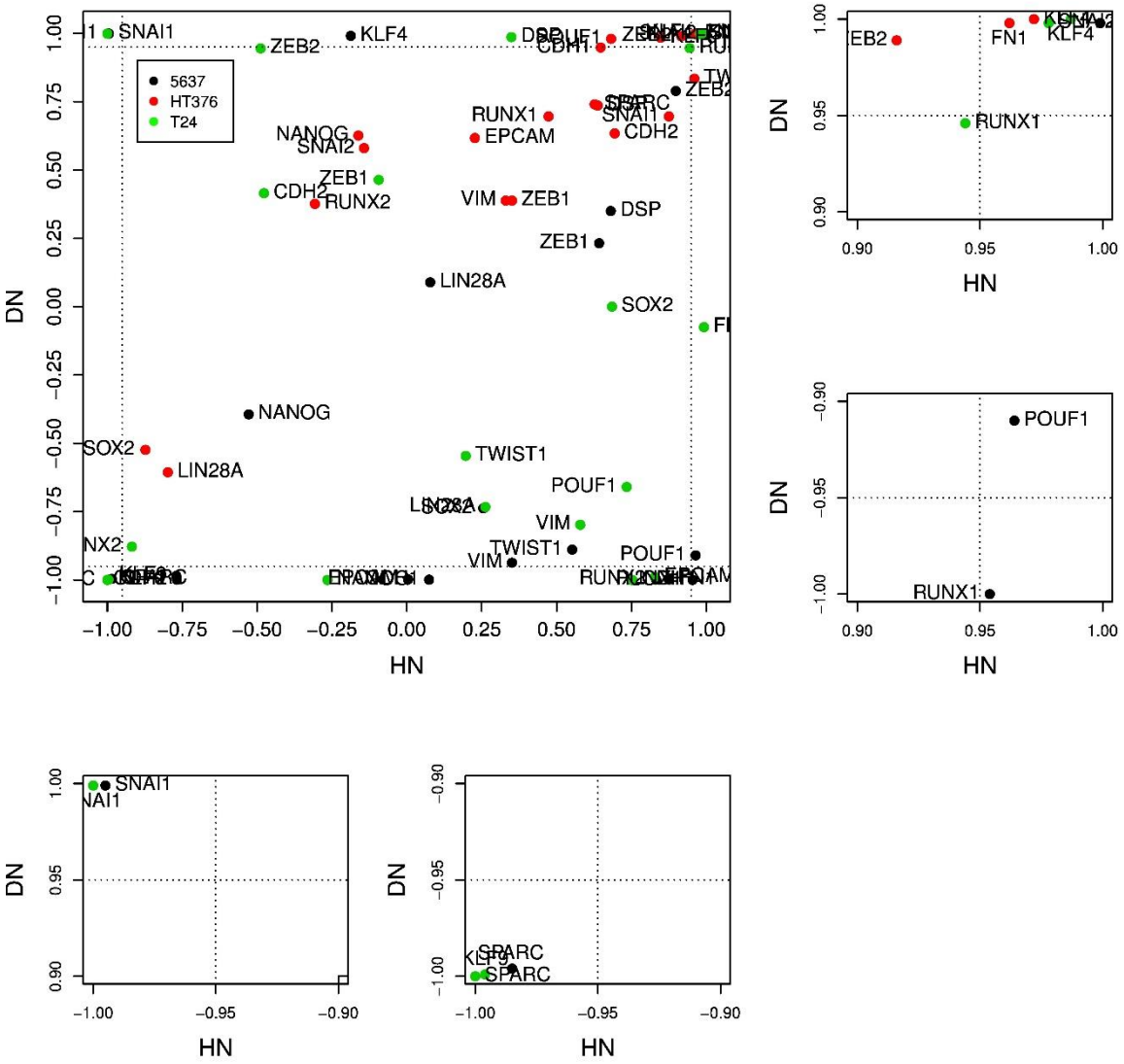


Figure 10. Scatterplot analysis of gene expression data in O_2 deprivation conditions (conditions HN) or under DFX treatment (conditions DN) in relation to normoxia.

4.4. Expression of STn and ST6GalNAc.I in bladder cancer cells

Little is known on the expression of the tumour-associated carbohydrate antigen sialyl-Tn (STn) in bladder cancer. However, the cancer-specific nature of this antigen was already been demonstrated and the presence of STn has been strongly associated with the overexpression of *ST6GalNAc.I* in several human malignancies. The expression of STn in the evaluated cell lines was determined by flow cytometry and the results resumed in Figure 11. As shown in Figure 11A, STn is expressed by all cell lines in normoxic

conditions and has significant expression increase in hypoxic conditions as well as in the presence of DFX. The presence of STn was validated by the decrease in fluorescence in the FACS to levels similar to the cells auto-fluorescence after neuraminidase treatment (Figure 11B).

The *ST6GalNAc.I, C1GALT, and GCNT1* genes expression were quantified by the real-time RT-PCR method. The enzymes mRNA levels were normalized for the expression of *GAPDH*, which was taken as a suitable endogenous control for bladder cancer cells out of three housekeeping genes tested. Contrasting with the overexpression of STn (Figure 11A), the mRNA levels of ST6GalNAc.I do not follow this tendency, suggesting that in bladder cancer models the overexpression of *ST6GalNAc.I* may not be the factor accounting for STn overexpression (Figure 11C). The reasons leading to over-expression of this truncated mucin-type O-glycan are multiple. Namely, in breast cancer cell lines, ST6GalNAc.I can override the core 1/core 2 pathway by being present throughout the Golgi allowing the production of large quantities of STn expressing proteins (117). Additionally, somatic mutations in *Cosmc* gene may also lead to STn overexpression, since loss of *Cosmc* results in loss of T-synthase in the Golgi apparatus and accumulation of Tn antigen, which can be converted to STn by the existing ST6GalNAc.I enzyme (179). Furthermore, increased activity of ST6GalNAc.I and/or down-regulation/decreased activity of several other competing glycosyltransferases could also be responsible for the results (94,158,165,180,181). In this particular case, a dramatic down-regulation of the *C1GALT*, and *GCNT1* genes, encoding for core 1 Gal-T and core 2 GlcNAc-, responsible by further elongation of O-glycans (Figure 11C), could be observed for all cell lines under hypoxia and DFX treatment.

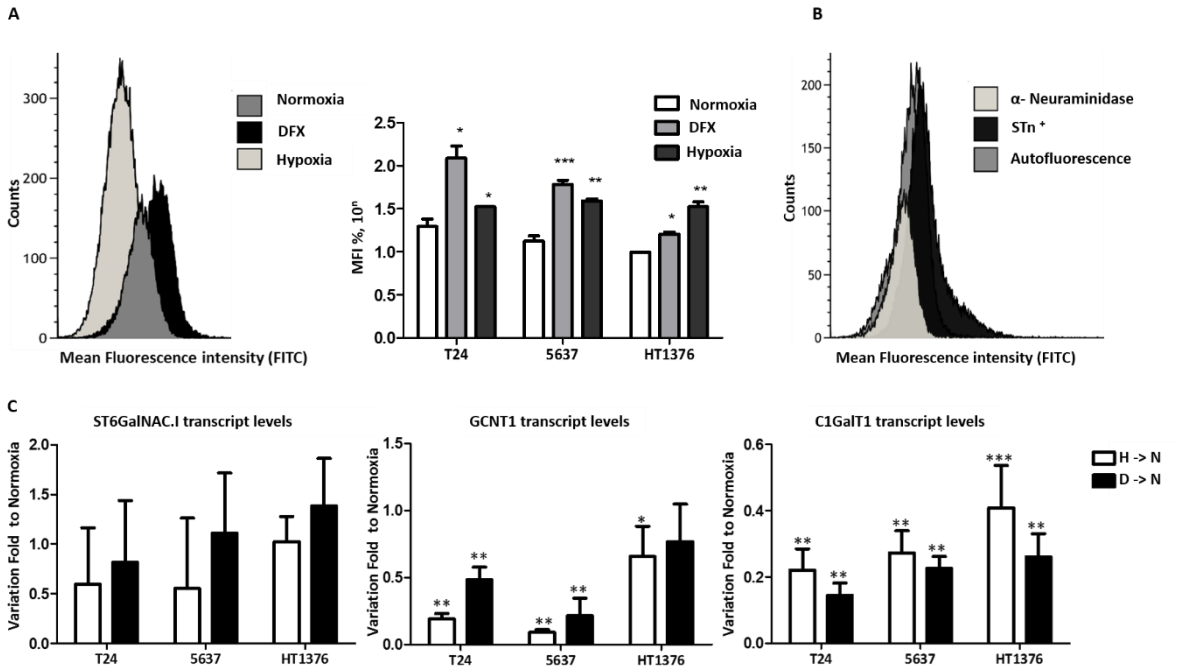


Figure 11. Bladder cancer cell lines overexpress STn in hypoxic conditions in a ST6GalNAc.I independent manner. A. Flow cytometry histogram overlays show stronger STn expression upon O_2 deprivation and DFX treatment; Bar graph showing Mean fluorescence intensity (MFI) of STn signal in normoxic conditions (21 % O_2) versus hypoxic conditions (0.1% O_2; 500 µM DFX). T24 and 5637 were submitted to normoxic or hypoxic condition for 24h while HT1376 cell line was submitted to the same condition for 6h. B. The decay of signal with neuraminidase treatments, confirms signal specificity. C. Bar graphs showing the ST6GalNAc.I, GCNT1 and C1GalT1 transcript levels upon hypoxia and DFX treatment. Graphs represent average value of three different experiment, flags correspond to SD and ***p < 0.001; **p < 0.01; *p < 0.05.

Altogether, it is described for the first time that hypoxia induces the expression of STn in bladder cancer cells, in what appears to be a *ST6GalNAc.I* overexpression-independent HIF-1α-mediated mechanism. The fact that three bladder cancer cell lines with different genetic and molecular backgrounds overexpress, under hypoxic conditions, the STn antigen strongly reinforces that its presence in bladder tumours may be, at least in part, governed by the microenvironment. This data also strongly suggests that STn overexpression in hypoxia stems from an HIF-1α-mediated down-regulation of the glycosyltransferases involved in the elongation of O-chains.

4.5. Cellular proliferation

Hypoxic microenvironment requires cellular adaptation to stress, which includes decreased cell proliferation (28,74). Based on these observations, the three cell lines were characterized in relation to their proliferation in hypoxia and in the presence of DFX. The most proliferative cell line for all conditions is T24, followed by 5637, whereas the proliferation rate of HT1367 is the lowest (Figure 12). Furthermore, the proliferation rate of all tumour cells, under hypoxic conditions, is lower than the proliferation rate of the same tumour cells under normoxic conditions. Moreover, DFX shows a growth-inhibitory activity even more prominent than hypoxia itself, suggesting a key role for HIF-1α in the modulation of bladder cancer cell proliferation (Figure 12).

Hypoxic conditions have been found to significantly reduce the population doubling time in melanoma and squamous cell carcinoma cell lines, and it was described that exposure of several solid tumour cell lines (A549, HCT 15, and NCI H460), T-lymphoma cells and ovarian cancer cells to extreme hypoxia induced cell cycle arrest, mostly in a HIF-1α-dependent manner (28,182,183). Furthermore, under conditions of severe hypoxia (0.1% O_2), cultured cells may exhibit increased apoptotic rates (30,184). DFX shows a more prominent growth-inhibitory activity than the oxygen deprivation conditions, probably because the iron chelation by DFX has intrinsic anti-proliferative effects, beyond the consequent effects of stabilization of HIF-1α. It is well known that iron is required for the growth of all cells, and the capacity of DFX to inhibit DNA synthesis has been demonstrated in several mammalian cells. Ribonucleotide reductase, a rate-controlling enzyme in DNA synthesis, is one possible target for the action of the drug. Indeed, ribonucleotide reductase, which catalyses the enzymatic formation of deoxyribonucleotides, contains a free tyrosyl radical as part of its polypeptide structure that requires the continuous presence of oxygen and iron during regeneration. It has been proposed that iron is removed passively from the medium by DFX and other iron chelators, thus preventing regeneration of the iron radical centre. Therefore, the iron chelation by DFX, which results in less tyrosyl free-radical formation, could be involved in the inhibition of ribonucleotide reductase activity in our bladder cancer models as well (185–188). Moreover, DFX treatment has apoptotic effects, which might also account for this observations. For instance, in human lymphocytes, DFX has genotoxic effects and induces p53 accumulation and p53-mediated damage response, ultimately leading to apoptosis

(189). On the other hand, DFX-induced apoptosis is also mediated by the p38 pathway and a caspase-8-dependent Bid-Bax pathway (190).

Viable but hypoxic and non-proliferating tumour cells are of particular interest, because it is presumed that they comprise a substantial fraction of the cells in solid tumours and could be the major source of chemotherapy resistant clones.

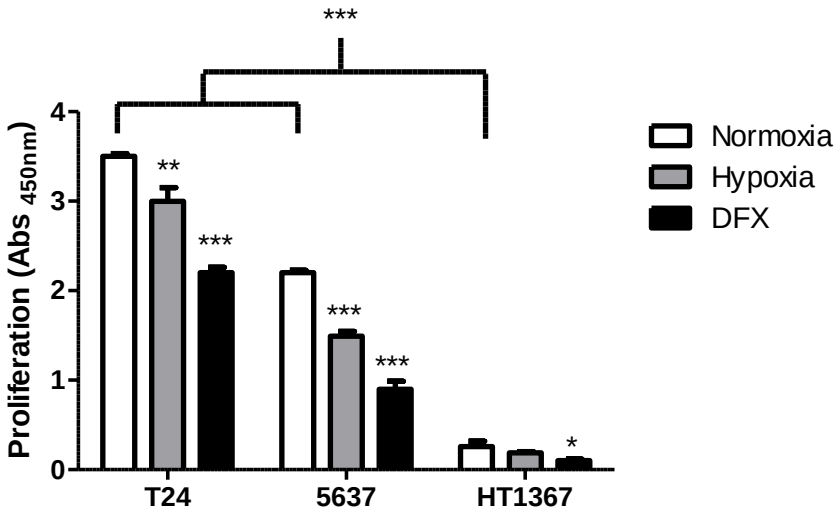


Figure 12. Oxygen deprivation and DFX treatment have *in vitro* growth-inhibitory effects on bladder cancer cells. All cell lines were cultures for 24h before exposure to any experimental conditions. BrdU was added to the cultured cells medium and after a hypoxia (0.1% O_2) or DFX (500µM) exposure period of 24h (T24 and 5637) and 6h (HT1376), the BrdU incorporation was assessed. Graph represents average value of three replicates, flags correspond to SD and ***p < 0.001; **p < 0.01; *p < 0.05.

4.6. Invasion and proteolytic activity of urinary bladder cancer models

As previously described, a critical event in tumour cell invasion is degradation of the extracellular matrix (ECM), a complex network of extracellular macromolecules such as collagen, proteoglycans, fibronectin, laminin and many other glycoproteins that act as a barrier to the invasion of cancer cells. Although several different proteases are implicated in ECM degradation, a special group of metalloproteinases, matrix metalloproteinases (MMPs), a family of zinc and calcium-dependent proteolytic enzymes, have been given special attention in this work, mostly because of their important roles in several cancer-supporting cellular processes besides extracellular matrix (ECM) remodelling, such as

angiogenesis, apoptosis, epithelial-to-mesenchymal transition and cell proliferation (148). Moreover, hypoxia has been described to potentiate the migration/invasion of cancer cells, which is a critical step for disease progression and dissemination. Based on these observations, the three bladder cancer cell lines were first characterized in relation to their migration/invasive potential on matrigel. In normoxia T24 and 5637 cells presented higher invasion than HT1376; however exposure to hypoxia increased this property in all cell lines in comparison to normoxia (Figure 13A). Conversely, growth under oxygen deprivation in the presence of an anti-STn antibody significantly lowered invasion, reinforcing our previous observations supporting a role for STn in bladder cancer invasion (Figure 13B) (15,120).

A gelatin zymography assay (Figure 13C) was then used to evaluate if these observations stemmed from higher MMPs activity or were a consequence of the higher cell motility presented by cells in hypoxia. First it was observed that MMPs activity was similar between the three cell lines grown under normoxia (Figure 13D), leading to conclude that T24 and 5637 cells crossed matrigel due to their higher migratory capacity. Such observations further reinforce the mesenchymal character of these cell lines previously suggested by morphological and gene expression analyses. Exposure to hypoxia did not promote a statistically significant increase in MMPs activity, again reinforcing a main role for motility in the context of bladder cancer hypoxic cells dissemination (Figure 13D).

Altogether, these findings show that hypoxia enhances cell motility irrespectively of their morphological and genetic background in, what appears to be, a STn-expression dependent process. The ClueGo and CluePedia analysis, revealed in all cell lines a decreased rate, frequency or extent of cell adhesion molecule production by *GCNT1* down-regulation, further reinforcing that these events contribute to negatively regulate cell adhesion.

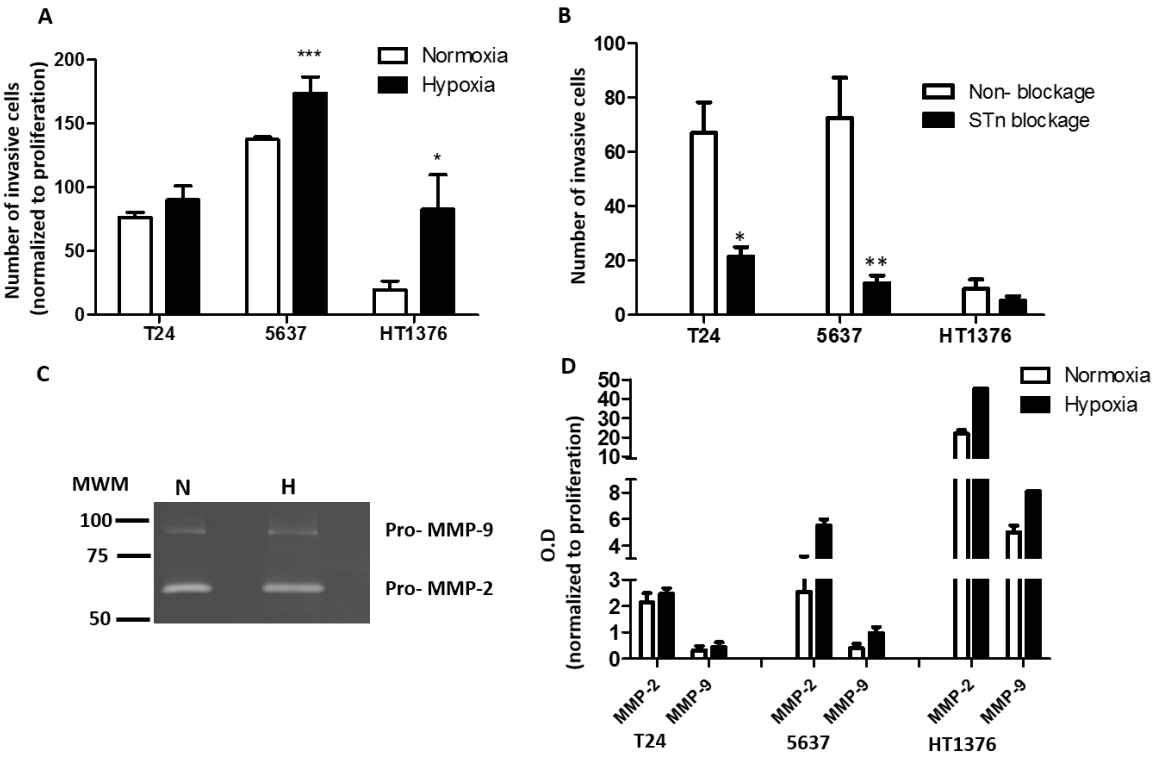


Figure 13. Hypoxia influences invasive ability but not MMP proteolytic activity in urinary bladder cancer cells. A. The invasive cells from 24 h hypoxic cultures and aerobic control cultures were visualized through a Leica DM2000 fluorescence microscope and scored in at least 12 microscopic fields (20x objective) when DAPI-counterstained nuclei passed through the filter pores. B. A 24h invasion assay was conducted under oxygen deprivation conditions in the presence of the anti-STn antibody TKH2 and the number of invasive cells visualized as previously described. C. Representative Gelatinase zymogram of bladder cancer cells show two bands, one corresponding to pro-MMP-2 (72 kDa) and other corresponding to pro-MMP-9 (92 kDa). MWM are shown to the left and are expressed in kDa. D. Bar graph showing greater expression of pro-MMP-2 over pro-MMP-9 in all cell lines with no significant changes between normoxic and hypoxic conditions. Graphs represent average value of three different experiment, flags correspond to SD and ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

4.7. STn glycoproteomic profilling

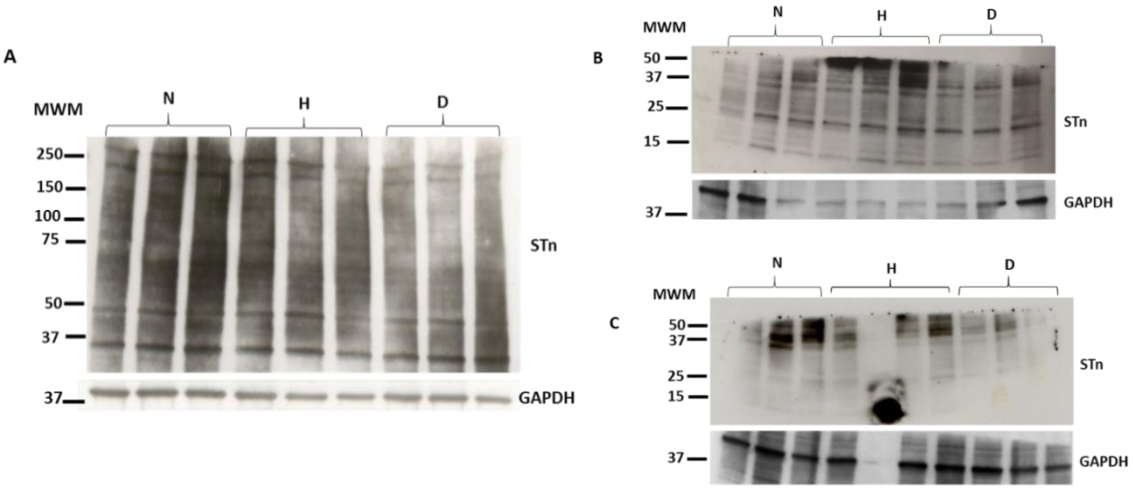
The STn antigen is a post-translational modification that may be putatively expressed by all glycoproteins at the cell surface (105) as translated by the smear in the western blots presented in Figure 14. However, two high-molecular weight bands at approximately 250 and 200 kDa and four low molecular weight bands at 50, 37, 25 and 15 kDa are distinguishable for all the cell lines and experimental conditions (normoxia, hypoxia, DFX; Figure 14A and 14B). The specificity of STn expression was confirmed by the disappearance of the bands upon treatment with a α-neuraminidase (Figure 14C). The relative quantification of the main bands highlighted a common profile for 5637 and

HT1376 cell lines in hypoxia, characterized by an increased expression of all evaluated proteins in relation to normoxia (Figure 14D). Similar behaviour was observed in the T24 cell line, but only for low MW bands. The exposure to DFX appears to modulate the overexpression of STn glycoproteins in T24, specifically for low molecular weight bands. However, for 5637 and HT1376, DFX only promoted the overexpression of high MW bands observed, again denoting a common behaviour at the protein level for these cell lines. The different profiles presented by the cells in hypoxia and DFX also suggest that not all glycoprotein STn expression is mediated by HIF-1α; however these findings warrant more in depth evaluation. Altogether, these findings demonstrate that exposure to hypoxia translates into an alteration in the glycoprofile of bladder cancer cells, that may be explored to selectively target these cells. More studies are necessary to identify the STn-expressing proteins and to determine if the alterations found by flow cytometry and western blot are influenced solely by alterations in glycosylation pathways and/or by the relative abundance of the glycoproteins carrying this antigen. This information is expected to translate into further understanding on the role of glycosylation on the biological and clinical behaviour of bladder cancer.

Nevertheless, based on previous findings, the high MW bands are concordant with the presence of mucins. Namely, MUC1, a high-molecular weight transmembrane protein previously reported to be heavily O-glycosylated and one of the main carriers of STn antigen, which lead us to hypothesize that the intense staining band at 250 kDa present in the blot refers to MUC1 mucin (127,191,192). Other mucins were reported to have a roll in bladder cancer such as MUC2, MUC4 and MUC7, which may account for more than one high MW bands (193,194). MUC1 is expressed on the apical surface or in umbrella cells of the normal non-neoplastic bladder urothelium and strong expression of MUC1 was also observed in urothelial carcinoma. Moreover, according to the literature, it is suggested that high levels of expression of sialylated MUC1 are associated with an aggressive phenotype, which together with its hypoxia-mediated overexpression may contribute to a more malignant phenotype of 5637 and HT1376 cell lines (195). Since, the decrease in high MW proteins is accompanied by an increase in low MW proteins in T24 cell line, one can hypothesise that the low MW species observed could result from the proteolysis of high MW proteins/ mucins. This hypothesis lines with the identification of a pathway of EGF (epidermal growth factor)-dependent metastasis that requires a Src (tyrosine-protein kinase)-mediated MUC1 proteolysis (196,197). The authors report that EGF stimulation induces MUC1 cleavage, leading the MUC1 cytoplasmic domain transmembrane subunit

(MUC1.CD) (15kDa) to translocate to the nucleus, where it promotes the expression of a metastatic gene signature associated with epithelial-to-mesenchymal transition. Those results showed that EGFR and Src activity, and consequently proteolytic cleavage of MUC1, contribute to tumour metastasis. Nevertheless, these observations need to be validated in a large number of patients and efforts should be conducted to identify these glycoproteins and disclose their contribution to malignancy. Other works correlate STn expression with invasion and metastatic potential in bladder cancer which associated with this observations could suggest an important role of sialylated MUC1 in the metastatic potential of our models as well (15).The other low MW bands might be a result of similar cleavage processes of high MW mucins (198,199).

As previously described, 5637 and HT1376 cell lines belong to the same molecular pathway of bladder cancer carcinogenesis which may account for the similarities in STn pattern even in response to hypoxia. On the other hand, T24 cell line follows another molecular pathway of carcinogenesis and has a somewhat different STn pattern. In addition, the differential STn pattern between cell lines and experimental conditions vouch the importance of glycosylation as biomarker reflecting not only genetic differences but environmental pressures as well.



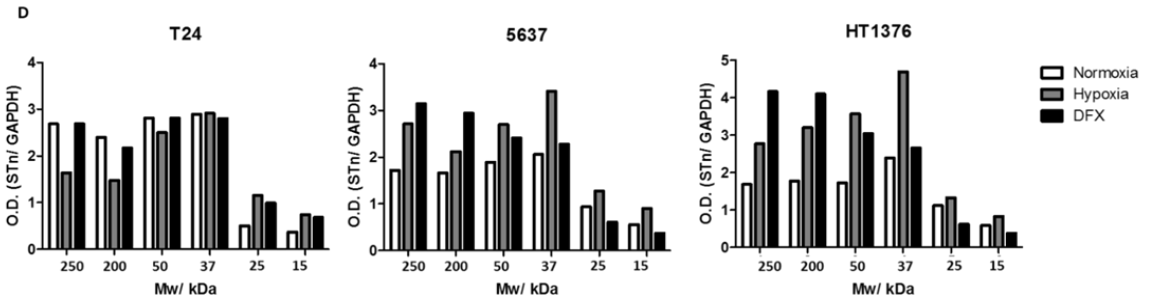


Figure 14. STn expression pattern in urinary bladder cancer cells, as analysed by western blotting. A. Using a 10µg protein input, two intense bands at approximately 250 and 200 kDa are present in all cell lines and experimental conditions, suggesting an association between STn expression, mucins and malignancy. The molecular weights/ kDa are shown on the left. All cell lines and all conditions presented low-molecular weight bands between 50 kDa and 15kDa. The optical density (OD) of the bands was estimated and normalized in relation to GAPDH, previously observed to be a stable housekeeping gene in this bladder cancer cell lines. The bands were then classified in relation to their intensity for comparison purposes. B. To better analyse the STn profile regarding the low molecular weight bands, a new WB using a 20µg protein input was carried out. C. To determine the specificity of the low-molecular weight bands a Neuraminidase enzyme treatment was applied. Because Neuraminidase cleaves the glycosidic linkages of neuraminic/sialic acids, the STn structure is disrupted preventing TKH2 linkage. The western blot samples appear in the following order: T24, 5637 and HT1376 Normoxic conditions (N); T24, 5637 and HT1376 Hypoxic conditions (H); T24, 5637 and HT1376 DFX treatment (D). D. Bar graph showing differences in optical densities of the bands between experimental conditions.

4.8. HIF-1α, CA IX and STn expression in bladder tumours

The expression of hypoxia markers HIF-1α and CA IX and cancer-associated glycan STn was evaluated by immunohistochemistry in a series including 30 NMIBC tumours and 43 MIBC tumours, randomly selected, representing all stages of the disease (15 pTa, 15 pT1, 13 pT2, 15 pT3, 15 pT4), to disclose associations between altered glycosylation and hypoxia.

All studied tumours were positive for HIF-1α and approximately 70% presented an extensive expression (> 20% of the tumour area), irrespectively of their stage. HIF-1α was predominantly detected in the tumour cells cytoplasm but also in the nucleus, where it acts has a transcription factor. However, 43% of NMIBC (13/30) and 85% of MIBC (37/43), presented both cytoplasmic and nuclear HIF-1α expressions, demonstrating an association between the presence of the protein in the nucleus and advanced stage bladder cancer ($p < 0.005$). The nuclear HIF-1α positive tumours were further re-classified according to the degree of STn expression (Table IV). Table III shows that high nuclear expression was present in only 43% of the NMIBC (pTa and pT1) and in 72.1% of the MIBC (pT2, pT3 and pT4), reinforcing previous association of nuclear HIF-1α expression with muscle invasion ($p < 0.05$). Altogether, these findings strongly suggest an association

between high hypoxia levels and muscle invasive bladder cancer. The CA IX, a cell-surface protein up-regulated by HIF-1α under hypoxic conditions, was highly expressed in all tumours (> 30% of the tissue). However, as expected, CA IX expression was significantly overexpressed (> 50%) in tumours presenting high levels of nuclear HIF-1α.

Regarding the evaluation of STn antigen, table IV shows the frequency of STn presence along the different stages of the disease. Higher STn expression was observed for MIBC (60%) when compared to NMIBC (30%), demonstrating an association of the antigen with muscle invasion (p= 0.03), in accordance with previous observations. The expression of HIF-1α and STn was further evaluated and it was found that all the tumour areas in which STn is expressed presented HIF-1α nuclear expression. Moreover, high expression of STn antigen co-localized with high nuclear HIF-1α expression in 75% of the cases, irrespectively of their stage.

Table III – Correlation between tumour stage and nuclear HIF-1α expression

Tumour stage	***High HIF-1α nuclear expression***		
Ta	4/15 (26.7%)	NMIBC 13/30 (43%)	P<0.05
T1	9/15 (60%)		
T2	9/13 (69%)	MIBC 31/43 (72.1%)	
T3	10/15 (66.7%)		
T4	12/15 (80%)		
Total	44/73 (60.3%)		

Table IV – Correlation between STn and HIF-1α expression

Tumour stage	***STn*** $^{+}$		***Tumours with STn/ HIF-1α nuclear positive areas***
Ta	3/15 (20%)	NMIBC 9/30 (30%)	3/4 (75%)
T1	6/15 (40%)		4/6 (67%)
T2	6/12 (50%)	MIBC 25/42 (60%)	3/4 (75%)
T3	10/15 (67%)		7/9 (78%)
T4	9/15 (60%)		2/3 (67%)
Total	33/72 (47%)	P=0.03	19/26 (73%)

V. Concluding remarks and Future perspectives

The STn antigen is considered a pancarcinoma antigen, based on the fact that it is expressed by the majority of advanced stage solid tumours, while absent from the corresponding healthy tissues. It is also far from being an innocent bystander in the disease outcome, since it was found to be a promoter of invasion and disease dissemination in many models, including stomach, pancreas and breast (120,165) carcinomas. In agreement with these observations our group has recently demonstrated that STn is expressed by over 70% of advanced stage bladder cancers, in poorly proliferative tumour areas and invasion fronts. Moreover, we found that STn expression enhanced the motility and invasive potential of cancer cells *in vitro* and are now detecting the antigen in lymph node and distant bladder cancer metastasis (unpublished data). Subsequent studies concluded that STn-expressing cancer cells impair DC maturation and promote a tolerogenic function, limiting their capacity to trigger protective anti-tumour T cell responses (156). Altogether these findings suggest that STn positive cells are endowed with the capability to invade and disseminate throughout the organ and to distant locations, while avoiding immune surveillance. Based on these observations we envisage that STn antigens and, in particular, STn expressing glycoproteins are potential targets to circumventing tumour-induced tolerogenic mechanisms and to avoid disease progression.

Nevertheless, and despite the key role of STn expression in cancer, the events responsible by this premature stop in elongation of *O*-glycans by sialylation of the Tn antigen are largely unknown. To this date two main mechanisms have been described: i) the overexpression of *ST6GalNAc.I*, which is commonly observed; ii) loss-of-function mutations in *C1GALT1* chaperone Cosmc, described by few reports. This work now demonstrates, for the first time, that hypoxia, a salient feature of solid tumours, is the main promoter of STn overexpression in bladder cancer.

We started by submitting three bladder cancer cell lines showing different genetic backgrounds to hypoxia and observed an overexpression of HIF-1α, CA IX and increased lactate levels in the culture media, denoting a shift to anaerobic metabolism. All cells responded similarly, by showing altered cellular morphology, characterized by increased intercellular spaces, impaired proliferation and enhanced cell migration. This was also true for cells growing the presence of DFX, highlighting these are HIF-1α-mediated events.

However, a gene expression analysis of genes associated with stem cell, epithelial cell, epithelial-to-mesenchymal and mesenchymal cell programs highlighted significant similarities between T24 and 5637 in normoxia as well as hypoxia; interestingly 5637 cells were regarded as genetically related to HT1376. Based on these observations we concluded that T24 and 5637 cells present a more mesenchymal nature, with T24 being markedly more immature, whereas HT1376 presents a more epithelial nature. Moreover, hypoxia up-regulated *SNAI2*, *KLF4* and *FN1* genes in a HIF-1α- mediated process, thus activating EMT and stem gene programs in all cell lines. Concomitantly, and despite morphological, genetic and molecular differences, all cells overexpressed the STn antigen in hypoxia, which translated in higher cell migration/invasion mediated by this antigen. More interestingly, STn overexpression was not linked to an overexpression of *ST6GalNAc.I* but to a striking down-regulation of the enzymes that participate in the downstream elongation of O-glycans. This highlights a new mechanism by which bladder cancer cells, probably of stem cell nature, acquire migration capacity in response to the hypoxic challenge. The effect was reverted by reoxygenation of the cells, denoting an on-off switch which endows bladder cancer cells with the capability to escape hypoxic niches and ultimately colonize distant locations. These observations may acquaint for the focal expression of STn in bladder tumours and in the metastasis (unpublished data). Cells grown in the presence of DFX also overexpressed the STn antigen, suggesting that HIF-1α may play an active role in the modulation of the glycophenotype by direct or indirect modulation of *O*-glycosyltransferases transcription. Studies on a retrospective series of bladder tumours comprehending all stages of the disease has confirmed the associations between hypoxia, translated by the nuclear overexpression of HIF-1α, and elevated STn. The high STn and nuclear HIF-1α phenotypes was predominantly observed in MIBC, also in accordance with *in vitro* studies. In previous studies we also reported that STn positive cells are mainly present in non-proliferative niches, which present high resistance to cisplatin-based regimens used in bladder cancer treatment. Here we observed that STn overexpression under hypoxia was accompanied by a decrease in cell proliferation. Imagiological studies by imagestream flow cytometry are ongoing to determine the cell cycle phase of STn positive cells envisaging understanding its susceptibility to chemotherapy. Furthermore, we aim to also determine if the presence of this antigen contributes positively to the mitigation of cell proliferation. It would also be important to confirm the capability of STn positive cells to form metastasis and recapitulate tumour heterogeneity in distant locations. Given the pancarcinoma nature of the STn antigen,

studies should be conducted to understand if hypoxia promotes similar alterations in cell lines from other organs. Finally, a preliminary glycoproteomic study has showed that bladder cancer cells under hypoxia overexpress low molecular weight STn-positive proteins (< 50 kDa). The future identification of the proteins responsible by this distinct pattern by Mass Spectrometry-based proteomics are expected to provide highly specific theranostic biomarkers to improve bladder cancer management. Furthermore, this will bring more insights about the biological significance of STn expression in bladder cancer but also other advanced stage tumours that also overexpress this antigen.

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