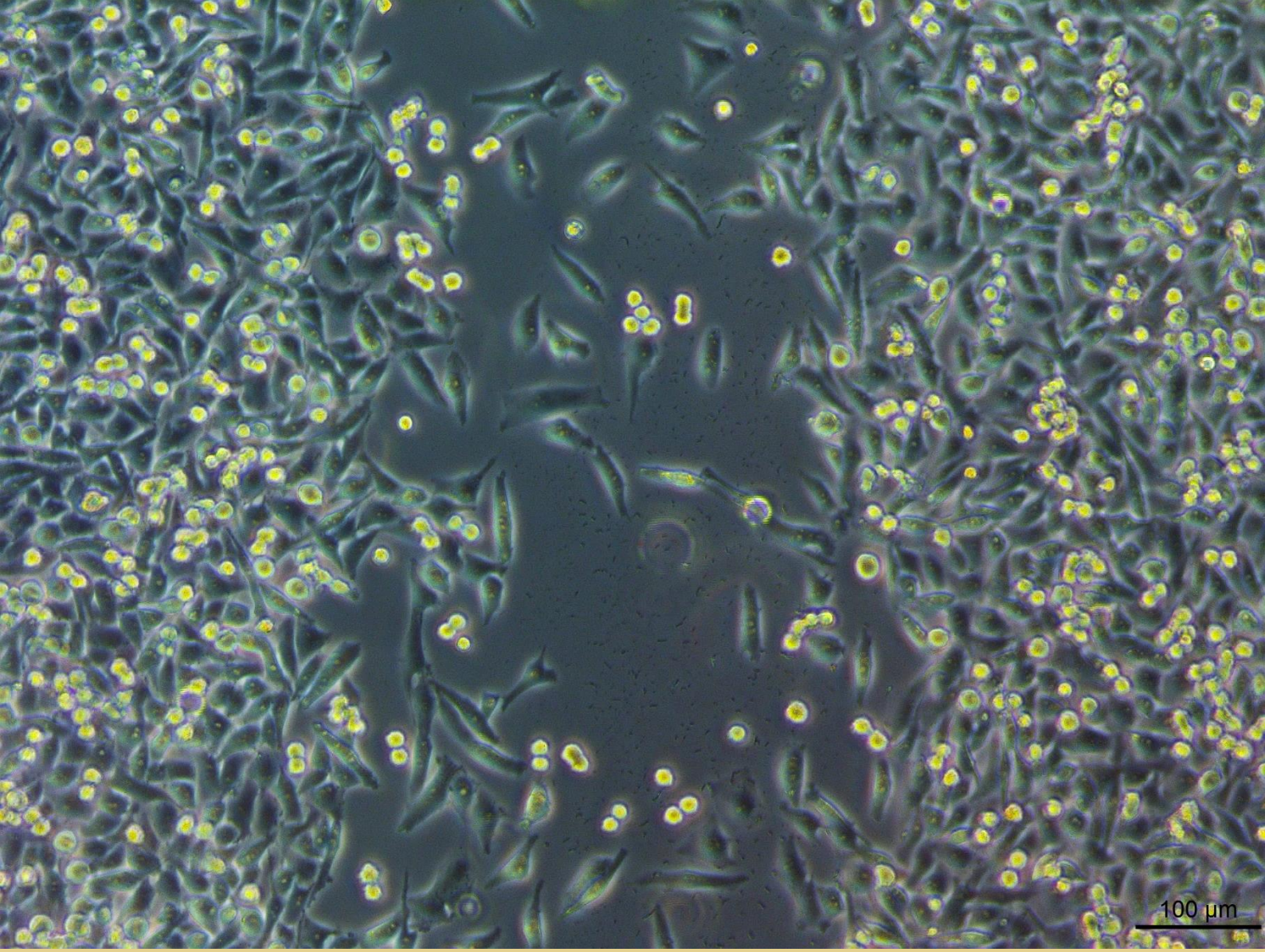

Diana Margarida de Almeida Rodrigues

ROLE OF THE EPITHELIAL-TO-MESENCHYMAL TRANSITION IN BLADDER CANCER AGGRESSIVENESS

Dissertation submitted to the Faculty of Sciences and Technology
of the University of Coimbra for obtaining the MSc degree
in Biomedical Engineering

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UNIVERSIDADE DE COIMBRA

INTEGRATED MASTER IN BIOMEDICAL ENGINEERING
FACULTY OF SCIENCES AND TECHNOLOGY
UNIVERSITY OF COIMBRA

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Supervisor:
Doctor Célia Gomes

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Abstract

Introduction: Bladder cancer (BC) is the most common urologic malignancy, being one of the main causes of death by cancer. There is increasing evidence supporting the role of epithelial-to-mesenchymal transition (EMT) in driving cancer progression, invasion and metastasis. The EMT is a reversible mechanism characterized by the loss of epithelial markers and gain of mesenchymal morphology with subsequent alterations in cell behaviour, decreased cell-cell recognition and adhesion, and rearrangement of cytoskeletal structures.

Aim: This thesis explored the role of the transforming growth factor beta (TGF-β) and of the epidermal growth factor (EGF) on promoting EMT in BC and how these growth factors influence the biological behaviour of BC cells regarding their stemness profile, drug resistance, proliferation rate, metabolic activity and invasive capacities.

Methods: Two human BC cell lines – HT-1376 and UM-UC3 – were treated with TGF-β (10 ng/mL) and EGF (50 ng/mL), alone or in combination, for 48 h. The expression of several EMT-related proteins and stemness markers was assessed by Western blot. Proliferation rates and chemosensitivity to cisplatin (CIS) were analysed by the MTT colorimetric assay. The metabolic activity was evaluated based on ^{18}F-FDG uptake, whereas the migration ability was assessed through a wound-healing scratch assay.

Results: TGF-β in combination with EGF significantly increased the expression of the mesenchymal marker vimentin in HT-1376 cells, but not in the UM-UC3 cell line. On the contrary, TGF-β *per se* had no significant effects on any of the proteins analysed in both cell lines. A tendency towards increased expression of ALDH2 and SOX2 was observed in TGF-β–treated HT-1376 and UM-UC3 cells, respectively. None of the growth factors induced significant alterations in the

proliferation rate and metabolic activity of BC cells, nor considerable effects on their chemosensitivity to CIS. In opposite, TGF-β, either alone or in combination with EGF, enhanced the migration ability of BC cells.

Conclusions: TGF-β alone or in combination with EGF increased the migration ability of BC cells without significant interference with cell proliferation and without promoting a complete EMT program.

Keywords: bladder cancer; epithelial-mesenchymal transition; transforming growth factor beta; epidermal growth factor; stemness; chemosensitivity; migration.

Resumo

Introdução: O carcinoma da bexiga (CB) representa a patologia mais comum do trato urinário, sendo uma das principais causas de morte devida ao cancro. Há cada vez mais evidências de que um processo designado transição epitelial-mesenquimal (TEM) desempenha um papel fundamental na progressão e metastização de tumores. A TEM é um mecanismo celular reversível que se caracteriza pela perda de marcadores epiteliais e aquisição de um fenótipo mesenquimal, resultando numa série de alterações ao nível do comportamento das células, fraca adesão intercelular e rearranjos na arquitetura do citoesqueleto.

Objetivo: Avaliar a importância do fator transformador de crescimento beta (TGF-β) e do fator de crescimento epidérmico (EGF) na ativação da TEM e tentar compreender de que forma é que estes influenciam o comportamento de células tumorais derivadas de CB, tendo em conta a expressão de marcadores de estaminalidade, resistência à terapêutica, capacidade de proliferação, actividade metabólica e propriedades invasivas.

Métodos: Duas linhas celulares de CB, HT-1376 e UM-UC3, foram incubadas com TGF-β (10 ng/ml) e EGF (50 ng/ml), aplicados isoladamente ou em combinação, durante 48 horas. A expressão de proteínas associadas à TEM foi determinada por Western blot. A taxa de proliferação e sensibilidade das células à cisplatina foram avaliadas através do ensaio colorimétrico de MTT. A atividade metabólica das células foi avaliada pela análise da captação de ^{18}F-FDG e a migração celular através de um ensaio de scratch.

Resultados: Os níveis de expressão da vimentina, um marcador mesenquimal, aumentaram significativamente após incubação com TGF-β e EGF em simultâneo nas células HT-1376, mas não na linha celular UM-UC3. Por outro lado, o fator de crescimento TGF-β *per se* demonstrou não ter efeitos significativos na expressão

de qualquer uma das proteínas analisadas, em ambas as linhas celulares. A expressão de ALDH2 e SOX2 estava tendencialmente aumentada em células HT-1376 e UM-UC3, respetivamente, após exposição ao TGF-β. Nenhum dos fatores de crescimento induziu alterações significativas na capacidade de proliferação ou na atividade metabólica das células, nem tampouco provocou efeitos consideráveis no que diz respeito à sua quimiossensibilidade à cisplatina. Pelo contrário, a incubação com TGF-β, isoladamente ou em combinação com EGF, aumentou a motilidade das células de ambas as linhas celulares estudadas.

Conclusão: A exposição isolada ao TGF-β, bem como aos dois fatores de crescimento em combinação, aumentou a capacidade migratória das células, sem interferir significativamente com a sua taxa de proliferação e sem que as células tenho sofrido uma TEM por completo.

Palavras-chave: carcinoma da bexiga; transição epitelial-mesenquimal; fator transformador de crescimento beta; fator de crescimento epidérmico; estaminalidade; quimiossensibilidade; migração.

Contents

List of acronyms

^{18}F-FDG	2-deoxy-2-(^{18}F)fluoro-D-glucose
AKT	protein kinase B
BC	bladder cancer
BCG	Bacillus Calmette-Guérin
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
BSA	bovine serum albumin
CARis	carcinoma *in situ*
CSC	cancer stem cell
CT	computed tomography
DTT	dithiotreitol
Dvl	Dishevelled
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
FOX	forkhead box
GLI	glioma
GSK-3β	glycogen synthase kinase 3 beta
GSTM	glutathione S-transferase mu
HCC	hepatocellular carcinoma
HDI	Human Development Index

HH	hedgegog
HIF1α	hypoxia-inducible factor 1-alpha
HMGA	high mobility group AT-hook
IL	Interleukin
JNK	c-Jun N-terminal kinase
LEF	lymphoid enhancer binding factor
MAPK	mitogen-activated protein kinase
MET	mesenchymal–epithelial transition
MIBC	muscle-invasive bladder cancer
MMP	matrix metalloproteinase
MR	magnetic resonance
mTORC	mammalian TOR complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAT	N-acetyltransferase
NF-κB	nuclear factor kappa B
NICD	Notch intracellular domain
NMIBC	non-muscle-invasive bladder cancer
PAR	partitioning-defective protein
PBS	phosphate-buffered saline
PET	positron emission tomography
PI3K	phosphoinositide 3-kinase
PTCH	patched homolog
PVDF	polyvinylidene difluoride
RC	radical cystectomy
RT	room temperature
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulphate
SOX	sex-determining region Y box
TCC	transitional cell carcinoma

TGF-β	transforming growth factor beta
TNM	tumour, node, metastasis
TUR	transurethral resection
TβRI	TGF-β family receptor type I
TβRII	TGF-β family receptor type II
WB	Western blot
ZEB	zinc finger E-box-binding homeobox
ZO	zonula occludens

Chapter 1

Introduction

1.1 Bladder cancer

1.1.1 Epidemiology

Bladder cancer (BC) has become a common urothelial malignant disease worldwide, arising from cells that start to grow uncontrollably in the epithelial lining of the bladder. It ranks as the ninth most frequently diagnosed cancer around the world and it globally represents the thirteenth cause of death by cancer, with an estimated 430 000 new cases diagnosed and 165 000 BC deaths in 2012[1].

The burden of this type of cancer is three to four times greater in men than in women. This association between gender and propensity for BC has been widely investigated and numerous explanations for this discrepancy have been offered[2]. For example, it has been suggested that gender-related variations in a panel of hepatic pathways could lead to differences in metabolic detoxification of carcinogens, causing a differential exposure of the urothelium to these foreign substances[3–6]. Therefore, according to this hypothesis, identical exposure to the carcinogens would result in differential gender-specific incidence rates. It has also been speculated that the differential distribution of urinary microorganisms between men and women could also be implicated in the process of tumorigenesis and partially explain these gender disparities[7]. Another factor thought to contribute to this gender imbalance is the sex steroid hormone pathway, which may differentially impact BC at various carcinogenesis steps[8–10]. On the other hand, women usually have more advanced tumours at the time of BC diagnosis and have less favourable outcomes after treatment, exhibiting higher risk of disease recurrence, progression and mortality. The former fact ensues from gender disparities in the opportuneness and thoroughness of initial diagnostic evaluation of haematuria, with women experiencing a remarkable delay in urologic referral and undergoing guideline-concordant examination less frequently, and the latter is probably due to not only the delayed diagnosis but also to differences in treatment efficacy and disease biology[11–22].

Additionally, substantial geographic variations can be noticed in the incidence rates of the disease, with the highest values observed in men living in very high Human Development Index (HDI) countries in Southern and Western Europe and Northern America, but also in a few regions of Northern Africa and Western Asia, although likely for different reasons (e.g., prevalence of urinary schistosomiasis)[1].

Age-standardized incidence rates therefore vary according to sex and HDI, with the highest rates being found in men from very high HDI countries and the lowest in women from low and medium HDI countries[1]. Age-standardized BC incidence for several countries around the world are represented in Figure 1.1.

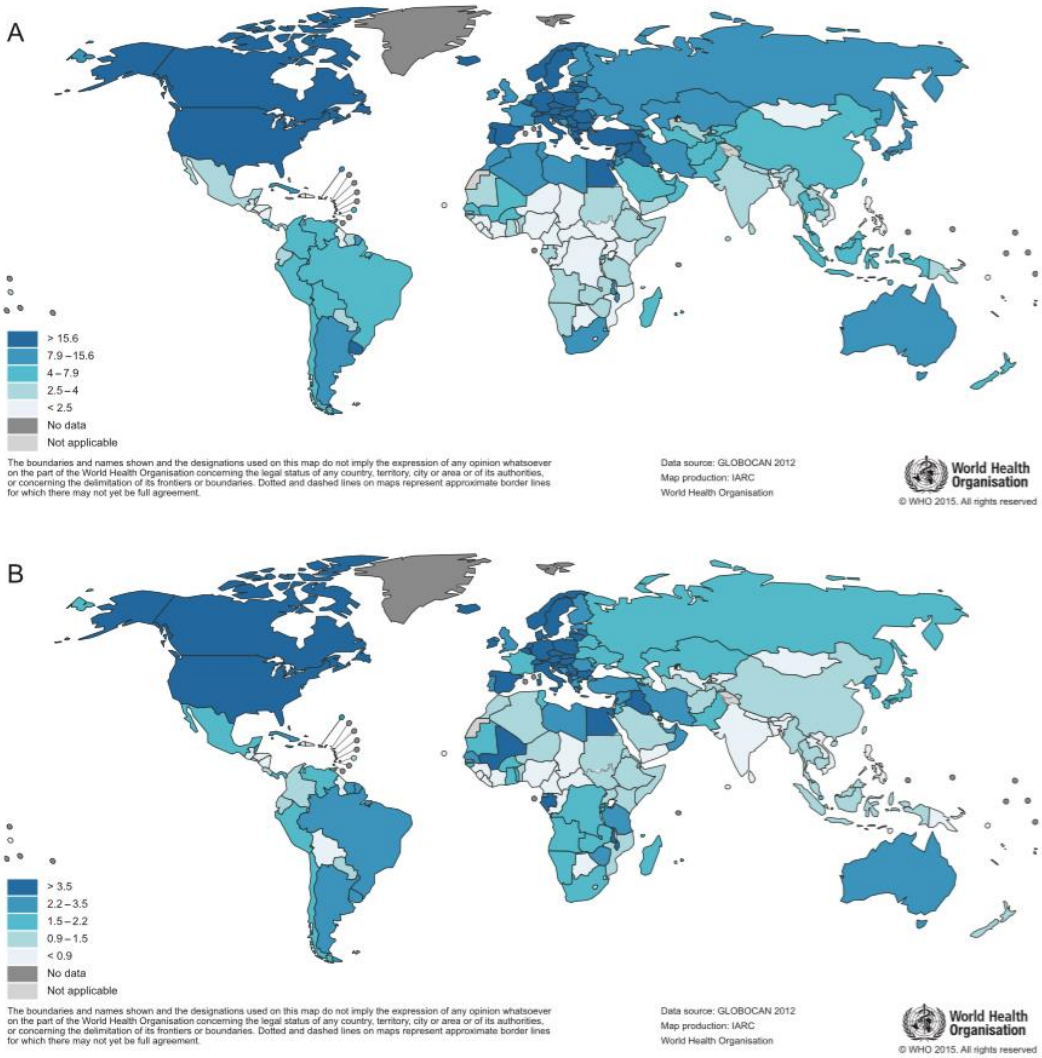

Figure 1.1 – Estimated age-standardized incidence BC rates for 184 countries of the world in 2012 for A | men and B | women. Data source: GLOBOCAN 2012.

Specifically, Europe has among the highest incidence rates of BC in the world, namely in some countries within Southern, Western and Northern Europe. Moreover, it also has the highest mortality rates recorded worldwide, mainly in Eastern and Southern Europe and in the Baltic countries[1].

In Portugal, BC is the sixth most commonly diagnosed type of cancer within the overall population, with approximately 3 000 new cases reckoned in the year of 2012. Among those, 2 400 were detected in men (fourth most frequent cancer), and the remaining 600 in women (eleventh most frequent cancer), following the global tendency mentioned earlier. Additionally, it ranks as the sixth and thirteenth leading cause of death by cancer in male and female individuals, respectively, which together constitute the eighth most significant cause of death by cancer in the whole country[23].

The chance of developing BC sharply increases with age, thus establishing the elderly as the most significant risk group. In fact, about 75% of all new cases diagnosed between 2010 and 2014 in the United States refer to people older than 65 years old[24]. This malignancy is also more prevalent in the White population when compared to Black, Asian, American Indian or Hispanic and Non-Hispanic populations[24].

1.1.2 Risk factors

The connection between BC and potential risk factors for tumour development has been the subject of a large number of epidemiologic studies[25–28]. Nevertheless, the presence of a single or multiple risk factors is not imperative for BC emergence, as not all the risk-group individuals develop the disease, whereas it is possible that some individuals with this type of cancer have few or none risk factors associated.

Apart from age, gender and ethnicity, cigarette smoking represents the primary risk factor for BC[29,30], accounting for 23% of all female and 50% of all male occurrences[31]. Tobacco smoke contains aromatic amines and polycyclic aromatic hydrocarbons, which are excreted through the renal pathway and known

to exert a carcinogenic effect on the entire urinary system. In fact, compared to people who have never smoked, regular cigarette smokers have two to fourfold higher chances of contracting the disease than non-smokers. Moreover, the risk rises even more for heavy smokers (20 cigarettes per day and/or over 40 years of smoking), as these have up to a fivefold greater risk of developing BC[6,29,32–35]. Furthermore, users of unfiltered, high-tar or black tobacco cigarettes are considered to be more susceptible than those smokers of filtered, low-tar or blond tobacco[32]. Pipe, cigar, and environmental smoking have also been reported as risk factors for BC, although epidemiologic data on these forms of smoking is not fairly conclusive[36,37]. For former smokers, the risk of contracting the malignancy appears to decrease by about 30-40% in the first four years after quitting with further decline over time, but it remains higher than that of never-smokers for at least 25 years after cessation[34,35].

Following cigarette smoking, occupational exposure to carcinogens, such as aromatic amines, and polycyclic aromatic and chlorinated hydrocarbons, is viewed as the second most important risk factor for BC. These carcinogenic agents can be found in the products of the chemical, dye, paint, rubber, and metal industries as well as in petroleum derivates, plastics, cigarette smoke, and motor vehicle exhaust. Individuals with occupational exposure to hair dyes – hairdressers and barbers – experience enhanced risk of BC too, although it is not clear whether other lifestyle characteristics are involved[38]. The risk associated with the personal use of hair dye has also been reported, although results are controversial.

Furthermore, increasing evidence suggests a significant influence of genetic predisposition on BC incidence. Indeed, several studies have demonstrated that first-degree relatives of patients who have been diagnosed with BC have 50-100% increased chances of also developing the disease[39,40], and the scenario can get even worse if the proband is diagnosed before 60 years of age[39].

Inherited genetic factors, such as the *NAT2* (N-acetyltransferase 2) slow acetylator or *GSTM1* (glutathione S-transferase mu 1) null genotypes, have been established as promoters of bladder carcinogenesis[41]. As a matter of fact, overall BC risk among NAT2 slow acetylators is about 1.3 to 1.5 times higher than in rapid

acetylators[42] and the *GSTM1* null status is also associated with a modest increase in risk[43,44]. These genes encode crucial enzymes involved in detoxification of environmental carcinogens. Such proteins though exhibit a widely genetic polymorphism[45], playing an important role in individual cancer susceptibility.

Many more gene polymorphisms have been correlated with predisposition for this malignancy, but the existent information is not fully enlightening yet.

Similarly, medical conditions may influence individual susceptibility to bladder tumorigenesis. For instance, urinary schistosomiasis has been consistently reported to be strongly associated with BC[46]. This condition is caused by infection with the trematode Schistosoma haematobium, which leads to chronic irritation of the bladder epithelium. Accordingly, BC – mostly squamous cell carcinomas – is quite common in areas of the world where schistosomiasis is endemic, like some regions of Northern Africa[47,48]. Other infections or the presence of stones in the urinary tract might cause chronic irritation of the urothelium as well, increasing the risk of developing BC[49,50].

Drinking water contaminated with chlorination by-products[51,52], arsenic[53,54] or nitrates[55] has been recognized as a cause of BC incidence. High consumption of coffee[56], dietary intake of animal fats and eggs[57], grilled, salted, canned and processed meat[58,59], and soy-based products[60] as well as the exposure to certain drugs (e.g., cyclophosphamide used in chemotherapy)[61] are likewise additional risk factors for BC.

Protection from BC related to high fluid intake was observed, both for water and for other fluids[62]. This might be explained by a presumptive higher dilution of metabolites in urine and increased frequency of voiding, thus reducing the contact between carcinogens and the bladder epithelium. However, total daily fluid intake depends on many factors and epidemiological findings are largely inconsistent[62–64]; moreover, difficulties in measuring total fluid intake result in undetermined BC risk[65]. Consumption of fresh fruits and vegetables[66], tea[64], skimmed milk, yogurt or other fermented milk products (containing Lactobacillus casei Shirota)[67],

carotenoids[68] and selenium-rich products[69] may also be important protective factors against BC occurrence.

1.1.3 Urothelial carcinogenesis

BC is a morphologically heterogeneous malignancy. About 90% of the newly diagnosed cases are transitional cell carcinomas (TCCs), which are presumed to arise from mutations within the bladder urothelium[70]. Other uncommon types of BC include squamous cell carcinomas (highly associated with schistosomiasis infections), adenocarcinomas, small cell carcinomas and sarcomas.

It is currently believed that TCCs may emerge from two distinct carcinogenic pathways: either from the papillary non-invasive pathway or from the non-papillary invasive pathway[71].

Approximately 80% of the diagnosed TCCs correspond to papillary injuries – non-muscle-invasive BCs (NMIBCs) – which develop from low-grade hyperplastic precursor lesions towards the bladder lumen[72]. Patients with NMIBC usually have a favourable outcome, as proven by the 5-year survival rate of about 90%. However, 50-70% of the cases will recur within 5 years, and 10-30% will progress to muscle-invasive disease[72], meaning that BC patients will need lifetime surveillance, which entails significant economic expenses[73].

Carcinomas deriving from the non-papillary muscle-invasive pathway – muscle-invasive BCs (MIBCs) – account for the remaining 20% of TCCs. These normally originate from high-grade dysplastic precursor lesions, or from the aggressive poorly differentiated high-grade anaplastic carcinoma *in situ* (CARis) tumours[74]. Patients diagnosed with MIBC present 5-year survival rates between 30-60%. Unfortunately, these kind of tumours have high propensity for metastatic progression, leading to a 5-year survival rate drop to just 10%[75]. MIBCs are characterized by tumour growth into either the lamina propria or the muscle layer of the bladder, or even deeper.

The extent of BC dissemination into the urothelium layers defines its staging. According to the TNM (tumour, node, metastasis) staging system, Ta, T1

and Tis (CARis) are conventionally categorized as NMIBCs or papillary bladder tumours limited to the mucosal (Ta and CARis) or submucosal layer (T1) (Figure 1.2). Ta, T1 and CARis account for 60, 30 and 10% of NMIBCs, respectively. MIBCs are all high-grade tumours and are further divided in T2-T4 stages, depending on the depth of invasion[76,77] (Figure 1.2). This stratification is of great importance for an early selection of the most appropriate therapeutic approach.

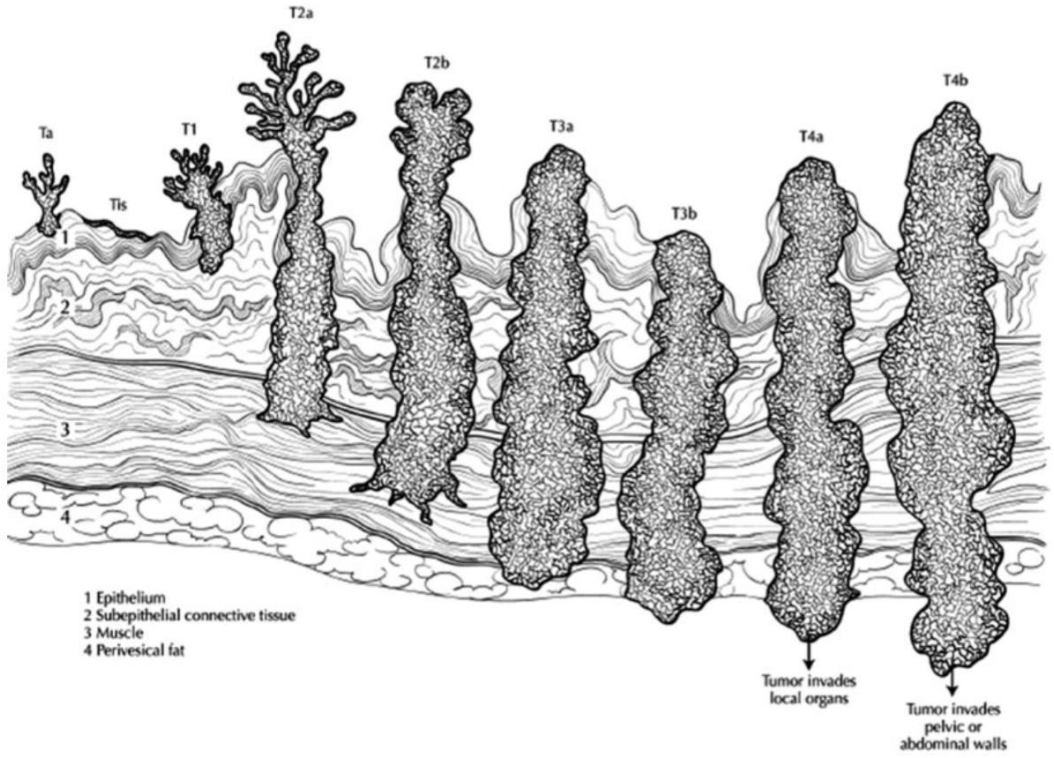

Figure 1.2 – Extent of primary bladder cancer. Bladder cancer staging, considering the TNM classification system. Low-grade tumours correspond to superficial non-muscle-invasive carcinomas, while high-grade tumours represent invasive carcinomas. (Adapted from Jacobs *et al.*, 2010)

1.1.4 Clinical presentation and diagnosis

The most frequent symptom related to BC is haematuria (macro or microscopic presence of blood in urine), occurring in about 85-90% of the cases at initial presentation[78]. The remaining portion of newly diagnosed patients probably presents signs commonly associated with other urological benign conditions, such as urinary tract infections and/or lower urinary tract symptoms, including irritative bladder symptoms (e.g., increased voiding frequency and urgency) and dysuria (i.e.,

painful or difficult urination)[79]. Patients with advanced disease may also exhibit pelvic or bony pain, lower-extremity edema, or flank pain[80].

Microscopic urinalysis for haematuria and urine cytology are the most widely adopted strategies for primary investigation on BC[81].

Urine cytology is a simple and non-invasive procedure based on microscopic examination of the urinary sediment for tracking of malignant urothelial cells., It exhibits good sensitivity for the detection of high-grade tumours, but a poor one for detecting low-grade malignancies[82]. Patients with positive results are referred for further evaluation, which typically comprises cystoscopy (i.e., endoscopic examination of the lower urinary tract), followed by histological examination of the material obtained by biopsy, if necessary.

In an effort to reduce discomfort and pain experienced with cystoscopy[83], the scientific community has investigated less invasive urine biomarkers (Table 1.1). However, their performance remains subpar as compared to the standard urine cytology and cystoscopy, making their use still controversial and debatable. With the same goal, innovative techniques of virtual cystoscopy have emerged, such as computed tomography (CT) virtual cystoscopy[84] and magnetic resonance (MR) cystoscopy[85].

Table 1.1 – Non-invasive bladder tumour markers. (Adapted from Sun & Trinh, 2015)

	Marker	Sensitivity (%)	Specificity (%)
Cytology	Tumour cells sloughed into urine	7-17 for low-grade 53-90 for high-grade	90-98
NMP-22	Nuclear protein released during apoptosis	44-50 for non-muscle invasive BC 90 for muscle-invasive BC	87
BTA Stat and BTA TRAK	Detects urothelial basement membrane	50-80	50-75
ImmonoCyt (DiagnoCure, Inc., Québec, Canada)	Immunofluorescence-3 monoclonal antibodies	50-74	62-73
UroVysion	FISH with probes to Chr 3, 7, 17, 9p21	68-86	40-93

As cystoscopy only inspects the lower tracts, imaging of the upper urinary tract is also recommended. Diagnostic imaging modalities available for this purpose include CT urography[86], intravenous urography[87], ultrasonography[87] and MR urography[88].

1.1.5 Treatment

The choice of an appropriate treatment for BC relies on the stage of the disease at the time of diagnosis.

For NMIBC, the first-line treatment is the transurethral resection (TUR) of all visible lesions in the bladder wall. For improved results, i.e., to reduce the risk of recurrence and/or progression, TUR is often combined with intravesical chemotherapy or immunotherapy[89]. Bacillus Calmette-Guérin (BCG) represents the standard intravesical immunotherapeutic approach for treating early-stage or high-risk superficial BC[90]. In turn, high-grade tumours display increased risk of recurrence and/or progression to more aggressive stages, possibly requiring an additional TUR along with adjuvant intravesical therapy with BCG or chemotherapeutic agents, like mitomycin C (MMC)[91]. In most severe cases, patients with recurrent NMIBC are submitted to radical cystectomy (RC), which consists in the removal of the entire bladder. This procedure is likewise adopted when BC is classified as invasive. In such cases, removal of nearby lymph nodes, part of the urethra, and close organs possibly containing cancer cells might also be necessary[92,93].

In the case of MIBCs, more assertive treatments are needed. Currently, preoperative chemotherapy with cisplatin (CIS) followed by the previously mentioned RC is the most widely accepted therapeutic option. Nevertheless, 30-40% of MIBC patients will recur after RC, presenting a 5-year survival rate of about 50%[94]. For some patients with MIBC or suspected to be at high risk of recurrence, adjuvant combined chemotherapy with gemcitabine and CIS, or a MVAC multidrug CIS-based regimen are viable choices[75].

Although significant improvements in care and prognosis of BC patients have been achieved by some advances in the available therapeutic tools, they have not proven to be sufficient for permanently eradicating the tumour. Therefore, further research is mandatory in order to identify new therapeutic targets for the development of more efficient drugs, with the ultimate purpose of improving the efficiency of the current therapies and thus prevent relapse and/or progression into more aggressive and frequently untreatable stages of BC.

1.2 Epithelial-mesenchymal transition

The epithelial-mesenchymal transition (EMT) is a reversible biological process that implies a phenotypical cell switch from an epithelial-like state to a mesenchymal one. It plays an essential role in normal physiological conditions, occurring during embryogenesis, tissue morphogenesis and wound healing, but it is also linked to pathological conditions namely fibrosis and cancer progression.

This mechanism involves several biochemical changes, such as downregulation of epithelial and upregulation of mesenchymal markers, loss of cell-cell adhesions and apical-basal polarity, matrix remodelling, a dramatic reorganization of the cytoskeleton architecture, and changes in cell shape (Figure 1.3), finally leading to enhanced migratory and invasive capacities, and elevated resistance to senescence and apoptosis.

Three types of EMT with very distinct functional consequences can be discerned, depending on the physiological context (Figure 1.4).

Type 1 EMT is related with implantation, embryogenesis and organ development. Its main purpose is to generate diverse cell types that share the same mesenchymal phenotypes (primary mesenchyme) and have the potential to subsequently undergo the reverse process known as mesenchymal-epithelial transition (MET) in order to generate secondary epithelia. This class of EMT neither causes fibrosis nor induces an invasive phenotype leading to systemic spread through bloodstream[95].

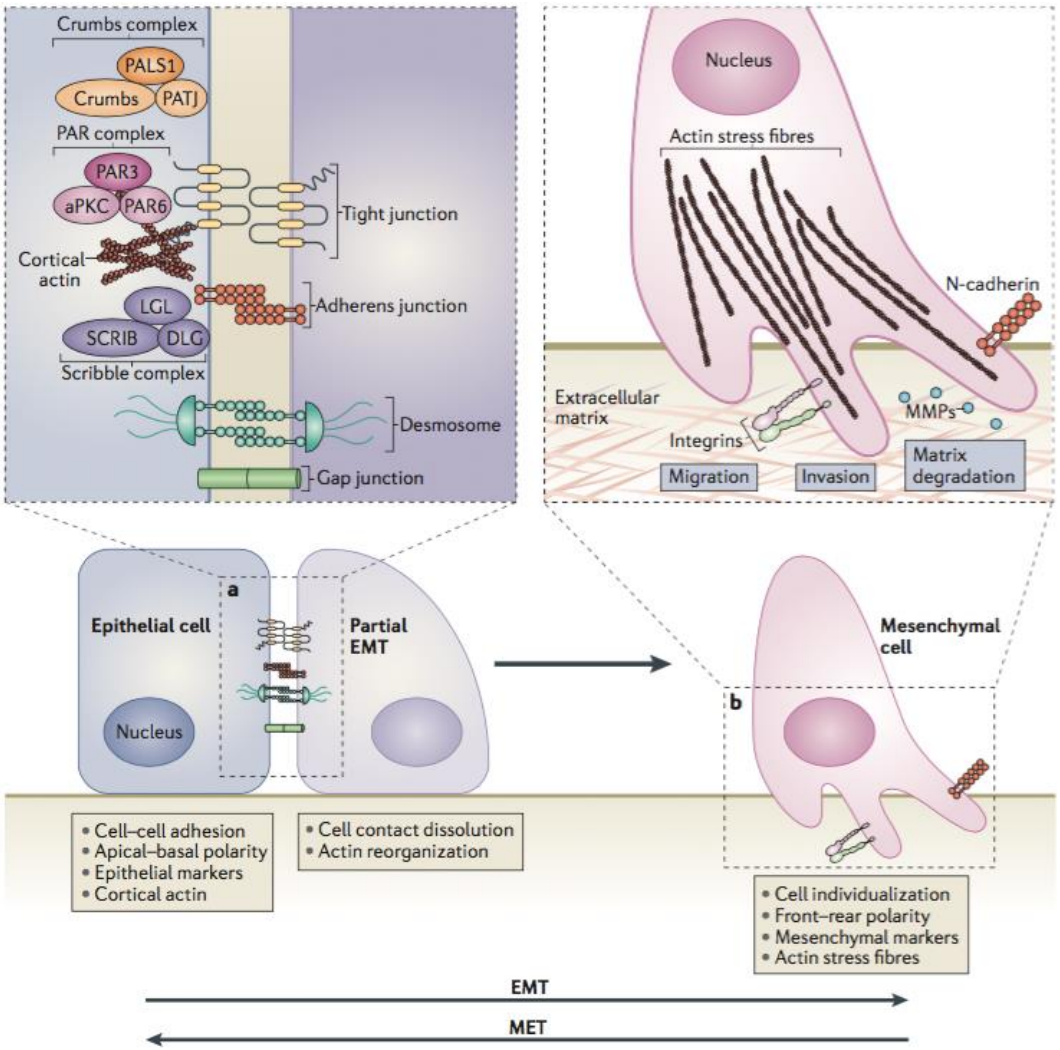

Figure 1.3 – Cellular events during EMT. a | The first steps of EMT are the disassembly of epithelial cell-cell contacts – that is, tight junctions, adherens junctions, desmosomes and gap junctions – and the loss of cell polarity through the disruption of the Crumbs, PAR and SCRIB polarity complexes. The expression of epithelial genes is repressed, concomitantly with the activation of mesenchymal gene expression. **b** | Next, the epithelial actin architecture reorganizes, and cells acquire motility and invasive capacities by forming membrane protrusions, and by expressing MMPs that can degrade ECM proteins. The process of MET enables the cells that have undergone EMT to revert to the epithelial state. (Adapted from Lamouille *et al.*, 2014)

In turn, type 2 EMT is important for wound healing, tissue regeneration, and organ fibrosis. Accordingly, in these situations EMT is actually activated as part of a repair-associated event, which normally generates fibroblasts and other related cells to reconstruct tissues following trauma or inflammatory injury[95].

Finally, type 3 EMT occurs in neoplastic cells that have previously gone through genetic and epigenetic changes, specifically in genes that favour the outgrowth of localized tumours. In fact, this kind of EMT grants carcinoma cells the capability of invasion and metastization, and thereby promotes the final, life-threatening manifestations of cancer progression[95].

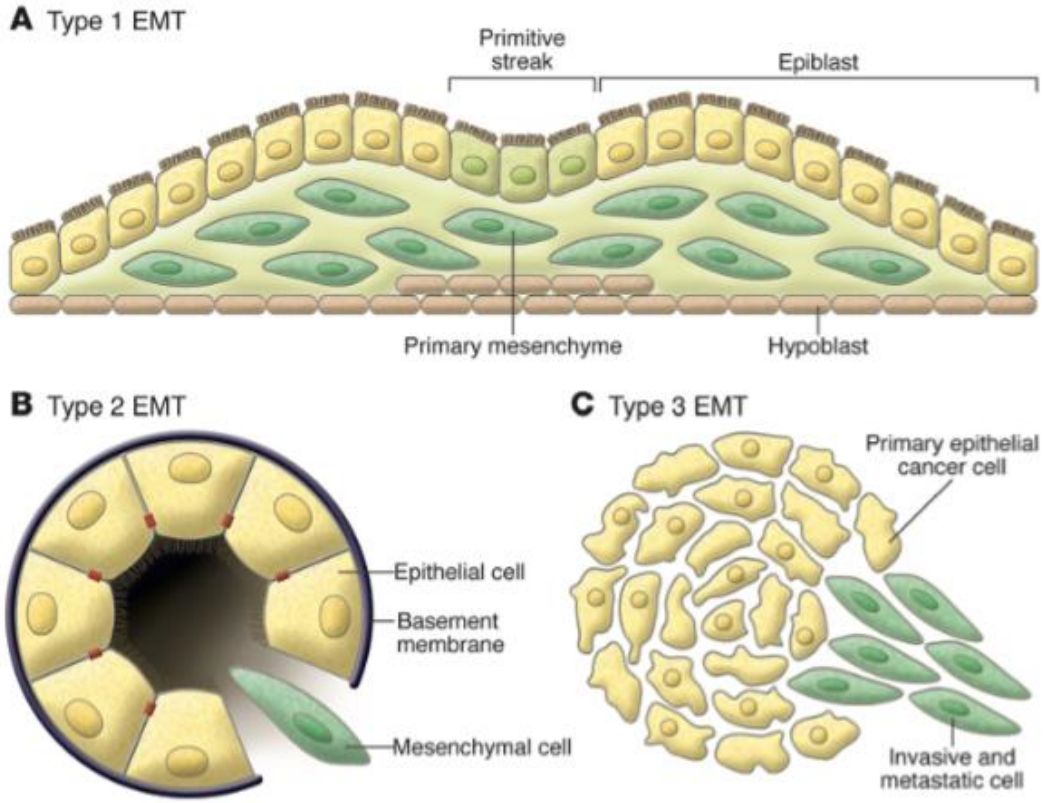

Figure 1.4 – Different types of EMT. A | Type 1 EMT is associated with implantation and embryonic gastrulation and gives rise to the mesoderm and endoderm and to mobile neural crest cells. The primitive epithelium, specifically the epiblast, gives rise to primary mesenchyme via an EMT. This primary mesenchyme can be re-induced to form secondary epithelia by a MET. It is speculated that such secondary epithelia may further differentiate to form other types of epithelial tissues and undergo subsequent EMT to generate the cells of connective tissue, including astrocytes, adipocytes, chondrocytes, osteoblasts, and muscle-cells. **B** | EMTs are re-engaged in the context of inflammation and fibrosis and represent the type 2 EMTs. Unlike the type 1 EMT, the type 2 EMT is expressed over extended periods of time and can eventually destroy an affected organ, if the primary inflammatory insult is not removed or attenuated. **C** | Finally, the secondary epithelia associated with many organs can transform into cancer cells that later undergo the EMTs that enable invasion and metastasis, thereby representing type 3 EMTs. (Adapted from Kallury *et al.*, 2009)

1.2.1 Type 3 EMT: cancer progression and metastasis

Uncontrolled cell proliferation and angiogenesis point out the onset of primary epithelial cancers[96]. Further acquisition of invasive properties by tumour cells, initially manifested by their ability to cross over the basement membrane, is thought to be the trigger for the later stages of the many-sided process that eventually culminates with metastatic dissemination, entailing serious consequences for patients. The genetic control and biochemical processes responsible for the development of this invasive phenotype and consequent systemic spread of malignant cells, ultimately resulting in macroscopic metastases formation, have been intensively studied, with the EMT emerging as the key-step underlying such events[97]. However, from an histopathological point of view, the secondary tumour colonies at faraway sites resemble the primary tumour from which they arose which means that EMT-derived migratory cancer cells must shed their mesenchymal phenotype via MET in the meantime of secondary tumour genesis[98]. The propensity of disseminated cancer cells to return to their initial epithelial status likely reflects the local microenvironment they are exposed to, after extravasation into the parenchyma of a distant organ[97].

These mechanisms concerning the progress of carcinomas to a metastatic stage are illustrated in Figure 1.5.

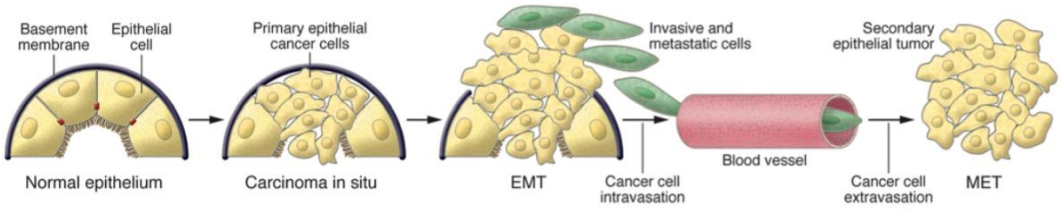

Figure 1.5 – Contribution of EMT to cancer progression. Progression from normal epithelium to invasive carcinoma goes through several stages. The invasive carcinoma stage involves epithelial cells losing their polarity and detaching from the basement membrane. The composition of the basement membrane also changes, altering cell-ECM interactions and signalling networks. The next step involves EMT and an angiogenic switch, facilitating the malignant phase of tumour growth. Progression from this stage to metastatic cancer also involves EMTs, enabling cancer cells to enter the circulation and exit the bloodstream at a remote site, where they may form micro- and macro-metastases, which may involve METs and thus a reversion to an epithelial phenotype. (Adapted from Kallury *et al.*, 2009)

1.2.1.1 Cellular and molecular events during EMT

Upon EMT activation, specialized cell surface protein complexes, which constitute essential cell-cell junctions for epithelial integrity, are dismantled and junction proteins are relocalized and/or degraded (Figure 1.3). Accordingly, the deconstruction of tight junctions during EMT is concomitant with a decrease in the expression of claudin and occludin – the two main components of tight junctions – and with the dispersal of zonula occludens 1 (ZO1) from intercellular contacts[99]. In addition, the dissolution of adherens junctions results in E-cadherin degradation[100], preventing its interaction with β-catenin, which is either degraded or not (as in the case of response to Wnt signalling), so that it can operate in transcription[101]. EMT also disrupts desmosomes[99,100] and compromises gap junctions' integrity by reducing connexin levels[102].

The destabilization of epithelial junctions further implies the loss of apical-basal polarity displayed by epithelial cells[99], which is sustained by polarity complexes, namely the partitioning-defective protein (PAR), Crumbs, and Scribble complexes, that are physically and functionally comprised along the cell junction architecture (Figure 1.3). As a matter of fact, reduced E-cadherin expression hampers SCRIB (a protein belonging to the Scribble complex) interaction with the lateral plasma membrane[103], and lower expression levels of E-cadherin or SCRIB impairs adhesion and enhances cell motility[104].

EMT also prompts a cortical actin cytoskeleton reorganization, inducing changes that will allow cell elongation and directional motility[100,105] , such as the development of new actin-rich membrane projections (Figure 1.3), including lamelipodia, filopodia, and invadopodia, which favour cell movement and operate as sensory extensions of the cytoskeleton, aside from nurturing cell invasion through extracellular matrix (ECM) degradation[106,107]. Moreover, EMT is likewise marked by improved cell contractility and actin stress fibre formation. These dynamic rearrangements in actin organization are managed by RHO GTPases[108–110], which also exert a role in the commutation from apical-basal to front-rear polarity by interplaying with proteins involved in the maintenance of apical-basal polarity[111,112].

Changes in gene expression during EMT are evident and depend on the degree of progression from the epithelial to the mesenchymal phenotype. As an example, the downregulation of genes encoding epithelial cell junction proteins is accompanied by the upregulation of genes encoding proteins that promote mesenchymal adhesion. Specifically, the decrease in E-cadherin levels is balanced by an increase in N-cadherin expression[100,113], with transitioning cells losing their connection with epithelial cells and acquiring affinity for mesenchymal cells, precisely through N-cadherin interactions, which are weaker than E-cadherin interactions and support cell invasion and migration[114]. Genes encoding cytoskeletal and polarity complex proteins also suffer modifications within their expression profile[99,115]. The composition of the intermediate filament changes, enabling cell motility through activation of the protein vimentin and its interactions with motor proteins[116]. Similarly, EMT results in downregulation of epithelial integrins and activation of others with key roles in EMT progression, given that transitioning cells do not interact with a basement membrane, but communicate with a distinct ECM instead[100]. It is important to note that integrin complexes are crucial for cells to receive signals from the ECM[100,117]. Concomitant with the changes in the integrin repertoire, increased expression of proteases, such as the matrix metalloproteinases MMP2, MMP3 and MMP9, also correlates with EMT, leading to ECM protein degradation and invasion, or increased levels of cellular reactive oxygen species (ROS), which further results in the upregulation of Snail1[100,107,118].

1.2.1.2 Transcription factors involved in EMT

Several transcription factors are known to participate in the process of EMT induction and mediate the changes in gene expression that are required for the epithelial-to-mesenchymal phenotypic switch. These include the zinc finger transcription factors Snail1 and Snail2, basic helix-loop-helix (bHLH) factors such as Twist, and zinc finger E-box-binding homeobox 1 and 2 (ZEB1 ZEB2)[119,120] (Figure 1.6).

1.2.1.2.1 Snail transcription factors

The Snail family of transcription factors plays a crucial role in the regulation of EMT. Snail1 and Snail2 are both critical for the inhibition of E-cadherin expression, precluding the transcription of the *CDH1* gene, which ultimately leads to tumour metastasis[121,122]. In fact, accumulation of Snail1 in the nucleus correlates with a significant decrease of E-cadherin expression and is associated with the induction of metastatic phenotypes in breast cancer[123]. Furthermore, circulating tumour cells isolated from metastatic hepatocellular carcinomas (HCCs) were found to have a 20-fold increase in Snail1 abundance as compared to cells deriving from non-metastatic HCCs[124]. Depending on the physiological context, multiple signalling cascades may be responsible for promoting Snail1 expression, such as those activated by transforming growth factor beta (TGF-β), Wnt, Notch, and growth factors that act through receptor tyrosine kinases (RTKs)[125]. Accordingly, Snail1 – as well as Snail2 – cooperates with distinct transcription regulators to control gene expression. For example, it works jointly with SMAD3–SMAD4 complex to enable the repression of E-cadherin and occludin expression mediated by TGF-β[126], and cooperates with mitogen-activated protein kinase (MAPK) downstream elements to promote MMPs expression[127]. Snail2 is also important for mediating EMT in the course of disease progression and metastization[128,129].

Post-translational modifications controlled by some signalling pathways might likewise dictate the localization and/or degradation of Snail1, and therefore influence its activity[125]. Hereupon, phosphorylation of Snail1 by glycogen synthase kinase 3 beta (GSK-3β) facilitates its translocation to the cytoplasm where it is again phosphorylated and subsequently marked for ubiquitin-mediated degradation[130]. The Wnt and the phosphoinositide 3-kinase–protein kinase B (PI3K-AKT) signalling pathways prevent Snail1 phosphorylation by GSK-3β[123], and Notch and nuclear factor kappa B (NF-κB) activity unsettles the interaction between GSK-3β and Snail1[131,132], enhancing Snail1 stability. Additionally, polycystin 1 (PKD1) also phosphorylates Snail1 to facilitate its nuclear export[133], whilst small C-terminal domain phosphatase 1 (SCP1) counterweights the GSK-3β–mediated phosphorylation and retains Snail1 in the nucleus[134]. On the other

hand, Snail1 phosphorylation by p21-activated kinase 1 (PAK1) or large tumour suppressor 2 (LATS2) increases its nuclear retention as well, promoting EMT[135,136]. Conversely, the p53 tumour suppressor recruits Snail2 for degradation, thus supressing cancer invasion[137].

1.2.1.2.2 bHLH transcription factors

Twist1 and Twist2 belong to the bHLH family of transcription factors and are essential for the development of cancer metastasis[120]. In human mammary cells, Twist1 stimulates the expression of Snail2, inducing EMT. This is in agreement with previous reports which revealed an increase in Twist1 in metastatic mammary tumours, when compared to their less metastatic counterparts[120,138]. Twist1 also favours *CDH1* repression[139] and several microRNAs expression which, in turn, mediates the inhibition of numerous target genes[140] involved, for instance, in cytoskeletal reorganization and cancer metastasis[141]. Apart from its cooperation with Snail, Twist1 is thought to repress E-cadherin and promote N-cadherin expression through interaction with other proteins[142,143]. Hypoxia-inducible factor 1-alpha (HIF1α) is responsible for activating Twist expression under hypoxic conditions, thereby contributing to EMT advancement and consequent tumour cell dissemination[143].

Similarly to what happens with Snail, the activity of Twist1 is conditioned by its MAPK-mediated phosphorylation, which grants protection against degradation by ubiquitin[144].

1.2.1.2.3 ZEB transcription factors

The ZEB family of transcription factors has attracted notable interest as its members are thought to regulate cancer progression[145]. Indeed, both ZEB1 and ZEB2 indirectly repress E-cadherin expression[146,147] , and the upregulation of these proteins in mammary epithelial cells induces the dissociation of adherens junctions, critical for maintaining the epithelial phenotype[147,148]. In addition, they are also implicated in some matrix remodelling mechanisms related to EMT, as ZEB1 and ZEB2 increase the expression of genes encoding important MMPs[149]. Twist1 interacts with Snail1 in order to activate ZEB1 expression[150], in response to TGF-β and Wnt proteins, as well as the expression of growth factors accountable for

triggering RAS–MAPK signalling pathways[142].

ZEB expression is post-transcriptionally supressed by microRNAs, and ZEB2 post-transcriptional sumoylation by PRC2 drives its nuclear export, which mitigates ZEB2-controlled gene expression/repression[151].

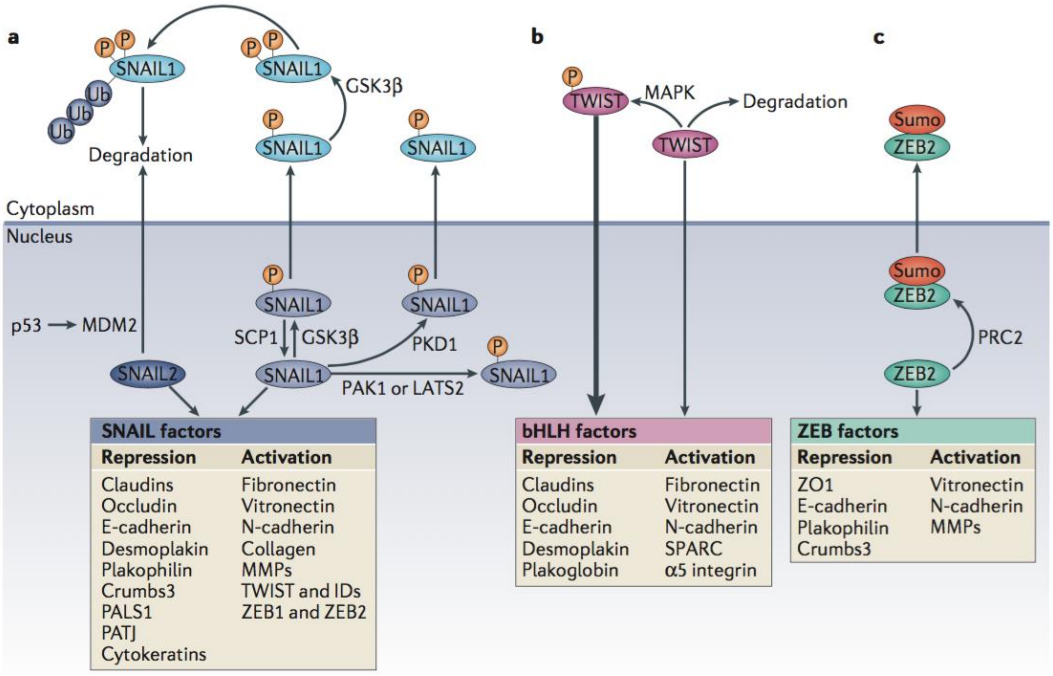

Figure 1.6 – Roles and regulation of major EMT transcription factors. EMT is driven by **a** | Snail, **b** | bHLH and **c** | ZEB transcription factors that repress epithelial marker genes and activate genes associated with the mesenchymal phenotype. Post-translational modifications regulate their activities, subcellular localization and stability. (Adapted from Lamouille *et al.*, 2014)

1.2.1.2.4 Novel transcription factors regulating EMT

Other transcription factors have been recently implicated on the induction or regulation of EMT. Several of these are defined by a DNA-binding forkhead domain, therefore referred to as forkhead box (FOX) transcription factors[152], while others are characterized by a DNA-binding dual zinc-finger module and belong to the GATA family[153], and the sex-determining region Y box (SOX) transcription factors[154] cooperate with Snail proteins, driving EMT and cell invasion[155]. The lymphoid enhancer binding factor-1 (LEF-1), member of the T cell factor (TCF) transcription factors family, is also capable of directly inducing EMT by repressing E-cadherin expression[156]. Additionally, overexpression of LEF-1 promotes EMT in

colon carcinoma cell lines via Wnt/β-catenin signalling pathway[157], whereas its inhibition prevents EMT in many systems[158,159].

A detailed list of transcription factors implied in EMT, along with their direct targets and the signalling pathways responsible for inducing their activity, is presented in Table 1.2.

Table 1.2 – EMT transcription factors, their direct targets and the signalling pathways that induce their activity. (Adapted from Lamouille *et al.*, 2014)

	Downregulated expression during EMT	**Upregulated expression during EMT**	**Regulatory signalling pathways**
Snail1 and Snail2	E-cadherin, claudins, occludin, Crumbs3, PALS1, PATJ, cytokeratins, desmoplakin and plakophilin	Fibronectin, N-cadherin, collagen, MMP15, MMP2, MMP9, Twist, ID1, ID2, ZEB1 and ZEB2	TGF-β–SMAD3, Wnt–β-catenin, Notch, PI3K–AKT, NF-κB, EGF and FGF
Twist1	E-cadherin, claudins, occludin, desmoplakin and plakoglobin	Fibronectin, N-cadherin and α5 integrin	MAPK
ZEB1 and ZEB2	E-cadherin, ZO1, Crumbs3 and plakophilin	N-cadherin and MMPs	TGF-β–SMAD3, Wnt–β-catenin and RAS–MAPK
FOXD3	Unknown	Unknown	β1 integrin and laminin
FOXC2	E-cadherin	Fibronectin, vimentin, N-cadherin and αSMA	TGF-β–SMAD3
FOXF1	E-cadherin, claudin1, occludin, desmoglein1β, desmoglein2, desmocollin2 and desmoplakin	Fibronectin and N-cadherin	Unknown
FOXQ1	E-cadherin	Fibronectin, N-cadherin and vimentin	Unknown
FOXO3A	E-cadherin	Snail1	AKT

FOXA1	E-cadherin	Fibronectin, vimentin and Snail1	TGF-β, HGF and AKT
FOXA2	E-cadherin and ZO1	Fibronectin, vimentin, N-cadherin, Snail1 and Snail2	TGF-β, HGF and AKT
Serpent, GATA4 and GATA6	E-cadherin, Crumbs and claudins	N-cadherin and MMP1	Unknown
HMGA2	E-cadherin	Snail1, Snail2 and Twist	TGF-β–SMAD3
SOX9	Unknown	Snail2	BMPs and PKA
KLF8	E-cadherin	MMP9	Unknown
CBFA-KAP1	Unknown	FSP1	Unknown
ZNF703 (also known as Zeppo1 in mice)	E-cadherin	Vimentin, N-cadherin, Snail1 and cytokeratin	RHO–GTPase
PRX1	E-cadherin	Vimentin and laminin	BMP2 and TGF-β

The repression of E-cadherin by the various transcription factors mentioned above seems to be the critical event during EMT. This idea is supported by several facts[160–162], including long-standing data demonstrating that loss of E-cadherin is an indicator of carcinoma progression and can be used as a poor prognosis predictor in a variety of tumours[160].

Growing evidence suggests the existence of synergistic mechanisms between the EMT-inducing transcription factors, although some studies confirm that inhibiting a single transcription factor is sufficient to prevent EMT[163]. In any case, it is clearly evident that the adjustments in gene expression that occur during EMT are managed by diverse transcription factors, even though some of these represent master regulators, while others have more restricted functions.

1.2.1.3 miRNA-mediated control of EMT

Besides the direct effects of EMT-related transcription factors on gene expression, changes at the RNA level are also determinant factors controlling EMT progression. Besides the alternative splicing of many mRNAs, which extensively occurs in EMT and gives rise to multiple protein isoforms[164–169], EMT is also

guided by non-coding microRNAs that selectively bind mRNAs, subsequently mediating their degradation or simply inhibiting their translation[170]. Some of these mechanisms regulate the expression of master transcription factors involved in EMT, whereas others target characteristic gene transcripts of either the epithelial or the mesenchymal phenotypes, as those encoding polarity and adhesion junction complex proteins, and signalling intermediators.

In the first case, for example, miR29b and miR30a restrain Snail1 expression, reversing EMT and reducing cell invasion[171,172]. Furthermore, miR1 and miR200-b interact with Snail2 in a double-negative feedback loop[173], similar to that between miR34 and Snail1[174], and miR203 and Snail1[175]. Additionally, members of the miR-200 family and miR205 prevent the translation of *ZEB1* and *ZEB2* mRNAs[176], and miR-200 and ZEB expression is further controlled by a double-negative feedback mechanism as well[177]. Therefore, a reduction in miR-200 expression leads to an increment in ZEB levels and EMT development. On the contrary, upregulation of miR-200 and miR-192 represses EMT in liver carcinoma by minimizing ZEB1 and ZEB2 expression[178], and Snail1 and Twist expression is indirectly activated by miRNA let-7 in a pancreatic carcinoma model[179] and by miR-365 in lung adenocarcinoma cells[180].

On the other hand, augmented miR-9 expression instigates mammary carcinoma cells to manifest a mesenchymal phenotype, accompanied by increased cell migration and invasion, by supressing E-cadherin expression[181]. In addition, the expression of miR194, which attenuates N-cadherin levels, is diminished in advanced gastric cancer[182]. Accordingly, enhanced miR194 expression contributes to the downregulation of N-cadherin, as well as to the reduction of cell migration and invasiveness, in mesenchymal liver cancer cells[183]. miR-491-5p inhibits PAR3 expression in response to TGF-β, thus destabilizing tight junctions[184], and miR661 is involved in epithelial cell junction disassembly and cell-cell adhesion impairments in Snail-expressing tumour cells[185]. Various microRNAs, such as miR-155[186], miR-24[187], miR-31[188], and miR-124[189] also regulate EMT by adjusting the activity and/or expression of RHOA, which affects actin organization and tight junction stability.

Assuredly, the contribution of microRNAs is crucial for the management of EMT, as they represent an extensive regulatory network which plays a critical role on adjusting gene expression.

1.2.1.4 Signalling pathways involved in EMT

EMT can be induced by various growth and differentiation factors, such as protein members of the TGF-β superfamily and growth factors that act through receptor tyrosine kinases. Among these, TGF-β has received particular attention as a major inducer of EMT.

Protein members of the TGF-β superfamily – TGFs-β, bone morphogenetic proteins (BMPs), activins, and growth and differentiation factors – operate through binary combinations of transmembrane dual specificity kinase receptors (acting as Ser/Thr and Tyr kinases), and play important roles in embryonal development and in the control of tissue homeostasis as well as in the induction of EMT in pathological contexts, more specifically in cancer progression and metastasis (Figure 1.7).

During carcinogenesis, increased expression and activation of TGF-β1 instigates an epithelial response, that eventually culminates in EMT, which is a crucial mechanism enabling cancer cell invasion and dissemination[95,129,190].

BMPs also regulate EMT and MET[98,191]. For example, BMP2, BMP4 and BMP7 promote EMT and subsequent invasiveness in pancreatic cancer cells, which is illustrated by the loss of E-cadherin expression and upregulation of MMP2[191].

TGF-β signalling occurs through the formation of a heterotetrameric receptor complex composed of type I and type II TGF-β family receptors (TβRIs and TβRIIs, respectively). Upon ligand binding, TβRIIs phosphorylate and activate TβRIs. TβRI subsequently mediates the phosphorylation of the intracellular signalling effectors SMAD2 and/or SMAD3, which combine with SMAD4 to assemble trimeric SMAD complexes. These assembled SMAD complexes are then translocated into the cell nucleus where they exert its function by activating or repressing gene transcription, through interaction with DNA-binding transcription factors at regulatory gene sequences[192,193]. As a matter of fact, inhibition of the

kinase activity of TβRIs blocks TGF-β–induced EMT in many cell types[194–196], whereas a constitutive activation of TβRIs initiates EMT[197]. The implication of SMADs in this process has also been demonstrated in several studies[195,198,199].

In response to TGF-β, SMAD complexes not only activate the expression, but also increase the activity of EMT transcription factors. TGF-β induces Snail1 and Snail2 expression in a SMAD3-dependent way[200,201], and the SMAD3–SMAD4 complex also cooperates with Snail1 in response to TGF-β, reinforcing the repression of the E-cadherin and occludin encoding genes[126]. TGF-β also potentiates the expression of ZEB1, which is additionally regulated by MAPK signalling[202]. SMAD3–SMAD4 complex is further implicated in TGF-β–derived gene expression, by interaction with ZEB1 and ZEB2[202,203], as well as in the activation of Twist[204] and high mobility group AT-hook 2 (HMGA2) [205] expression.

Additional changes in gene expression without direct need of EMT-related transcription factors may also occur during EMT thanks to the TGF-β–activated SMADs[206,207], which are able to directly mediate the expression of some mesenchymal genes, such as those encoding fibronectin, vimentin and collagen αI.

The role of TGF-β in EMT induction is farther relevant as it can also induce signalling by activating RHO-like GTPases, PI3K and MAPK pathways[142,198] .

Activation of RHOA, RAC and CDC42 GTPases results in actin reorganization along with lamellipodia and filopodia development[106]. Consequent stimulation of RHO-associated protein kinase (ROCK) and LIM domain kinase (LIMK) following TGF-β–induced RHOA activation constitutes an important factor enabling EMT[208,209]. In turn, PAR6, previously phosphorylated by TβRIIs upon binding of TGF-β, mediates RHOA ubiquitination and degradation by the E3 ubiquitin ligase SMAD ubiquitylation regulatory factor 1 (SMURF1), leading to disruption of tight junctions[210] .

Exposure to TGF-β may also instigate the activation of AKT through PI3K, subsequently activating mammalian TOR complex 1 (mTORC1) and mTORC2, both essential for the transition from an epithelial to a mesenchymal phenotype,

thereby contributing to an increased cell size, protein synthesis, motility and invasion[196,211]. Pharmacological inhibition of this pathway prevents TGF-β–induced EMT, as it reduces Snail1 expression, attenuating the repression of E-cadherin and the activation of MMP9, and preventing invasive behaviour[211]. Conversely, AKT-mediated inhibition of GSK-3β stabilizes Snail1 activity to regulate the expression of several EMT-related genes[130,212]. AKT also favours EMT by phosphorylation of the heterogeneous nuclear ribonucleoprotein E1 (hnRNPE1) [213].

TGF-β proteins also promote EMT via extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) MAPK pathways[214,215], implying that inhibition of these MAPK kinases activity will cease EMT[163,216]. ERK–MAPK signalling is induced by the adaptor protein SRC homology 2 domain-containing-transforming A (SHCA), which is phosphorylated by TβRIs, providing a docking site for growth factor receptor-bound protein 2 (GRB2) and son of sevenless (SOS), while initiating the RAS–RAF–MEK–ERK MAPK pathway[217]. This leads to an increment in transcription, further repressing E-cadherin and increasing N-cadherin and MMP expression[218]. Besides, similarly to AKT, ERK5 MAPK inhibits GSK-3β, therefore increasing Snail1 activity[219]. On the other hand, activation of p38 and JNK signalling pathways is dependent on the interaction between the ubiquitin ligase TNF receptor-associated factor 6 (TRAF6) and the TGF-β receptor complex, driving TGF-β–activated kinase 1 (TAK1) activation[220,221]. p38 and JNK cooperate with SMAD3–SMAD4, hence mediating TGF-β–induced transcription and gene expression[222,223].

Apart from TGF-β, a number of growth factors may induce EMT through downstream signalling after binding to RTKs. Since TGF-β receptors also have kinase activity, many of the signalling pathways triggered by TGF-β are likewise induced by RTKs activation in response to growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), and platelet-derived growth factor (PDGF)[224–227]. Many of these ligands potentiate cell proliferation and contribute to partial EMT[228].

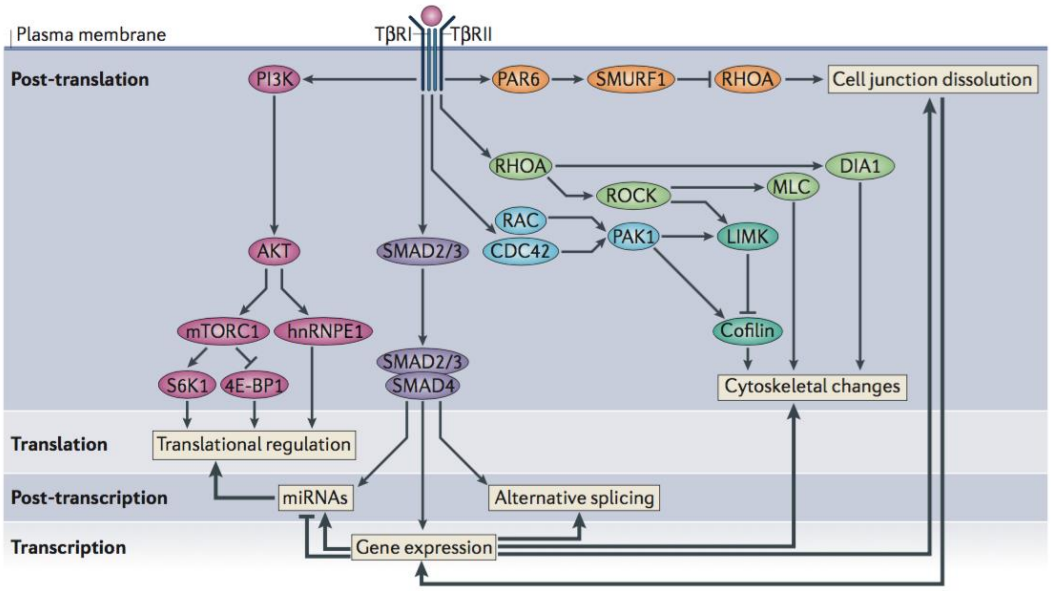

Figure 1.7 – Molecular mechanisms of TGF-β–induced EMT. The initiation of, and progression through, EMT are regulated at the transcriptional, post-transcriptional, translational and post-translational levels. TGF-β induces EMT by acting at several of these levels, in a SMAD-dependent and non-SMAD-dependent manner. (Adapted from Lamouille *et al.*, 2014)

With particular interest for this research project, EGF is known to stimulate cell motility, in an alpha 2 integrin-dependent manner in mammary epithelial cells[229]. Moreover, EGF inactivates focal adhesion kinase (FAK), a signalling mediator which interacts with integrin transmembrane complexes, promoting cell detachment from the ECM to enable cell motility[230]. In addition to favouring the development of a migratory phenotype, EGF embrocates E-cadherin internalization, reducing cell-cell adhesion and impairing the epithelial layer[231], and E-cadherin underexpression by stimulating Snail1 and Twist activity[232] . Consistent with these observations, EGF-related Cripto 1 has been demonstrated to increase the levels of the mesenchymal markers N-cadherin and vimentin[233], and Snail1 expression[233], besides allowing motility and invasion of tumour cells and cancer progression[234]. However, Cripto 1 is thought to rely on its ability to interact with Nodal and facilitate Wnt signalling, rather than acting as a ligand[235,236]. EGF effects further extend to a raise in the production and secretion of MMP2 and MMP9, and to the switch-on of ERK and integrin-linked kinase (ILK) pathways, thereby contributing to changes in cellular morphology and promoting cell migration[237].

Additional signalling pathways are involved in the activation or regulation of EMT, although their *modus operandi* is less well defined (Figure 1.8).

The binding of Wnt ligands to Frizzled receptors results in phosphorylation of low-density lipoprotein receptor-related protein 6 (LRP6) by GSK-3β with subsequent recruitment of dishevelled (Dvl) and axin to the plasma membrane, precluding GSK-3β to form a complex with axin and phosphorylate β-catenin, which therefore prevents β-catenin of being ubiquitinated and degraded, enabling it to translocate to the nucleus and regulate gene expression[101]. In the absence of signalling, β-catenin is sequestered in the cytoplasm and marked for proteasomal degradation through phosphorylation by the GSK-3β-axin complex[238]. Wnt signalling has been found to be inappropriately active in numerous cancers[239], directly promoting Snail stability. Wnt-mediated induction of EMT through Snail2 is concordant with supplementary evidence of decreased E-cadherin and increased fibronectin expression following accumulation of nuclear β-catenin[240]. Moreover, Wnt is thought to boost Twist expression, as it has been found to do so within mammary epithelial cells[241]. Upregulation of Wnt/β-catenin signalling pathway also seems to be crucial for cancer progression and metastization[240].

The Notch signalling pathway is likewise involved in EMT during cancer progression. Binding of Delta-like or Jagged ligands to Notch receptors causes the Notch intracellular domain (NICD) to be released, facilitating its translocation to the nucleus where it regulates gene expression[242]. In fact, NICD binds to transcription repressor complexes to switch on the expression of genes encoding fundamental proteins for tumour development. Notch signalling can control the expression of Snail1 both directly[243] and indirectly[131], whereas Snail2 is essential for Notch-mediated repression of E-cadherin and β-catenin activation. Accordingly, inhibition of Notch 1 in lung adenocarcinoma cells reduces their invasive behaviour and partially reverts EMT[244]. Complementarily to its direct effects, Notch indirectly regulates EMT via several signalling pathways, and through the action of diverse regulatory miRNAs. In pancreatic cancer cell lines, for example, inhibition of Notch leads to a decrease in the expression of MMP9, allowing the extravasation of malignant cells[245].

In hedgegog (HH) signalling, ligands bind to patched homolog 1 (PTCH1) and PTCH2, releasing Smoothened and activating an intracellular cascade[246], with subsequent activation of glioma (GLI) family transcription factors responsible for the transcription of several target genes, such as those encoding PTCHs, Wnt, and Snail[239]. In fact, ectopic expression of GLI1 in kidney epithelium induces Snail1

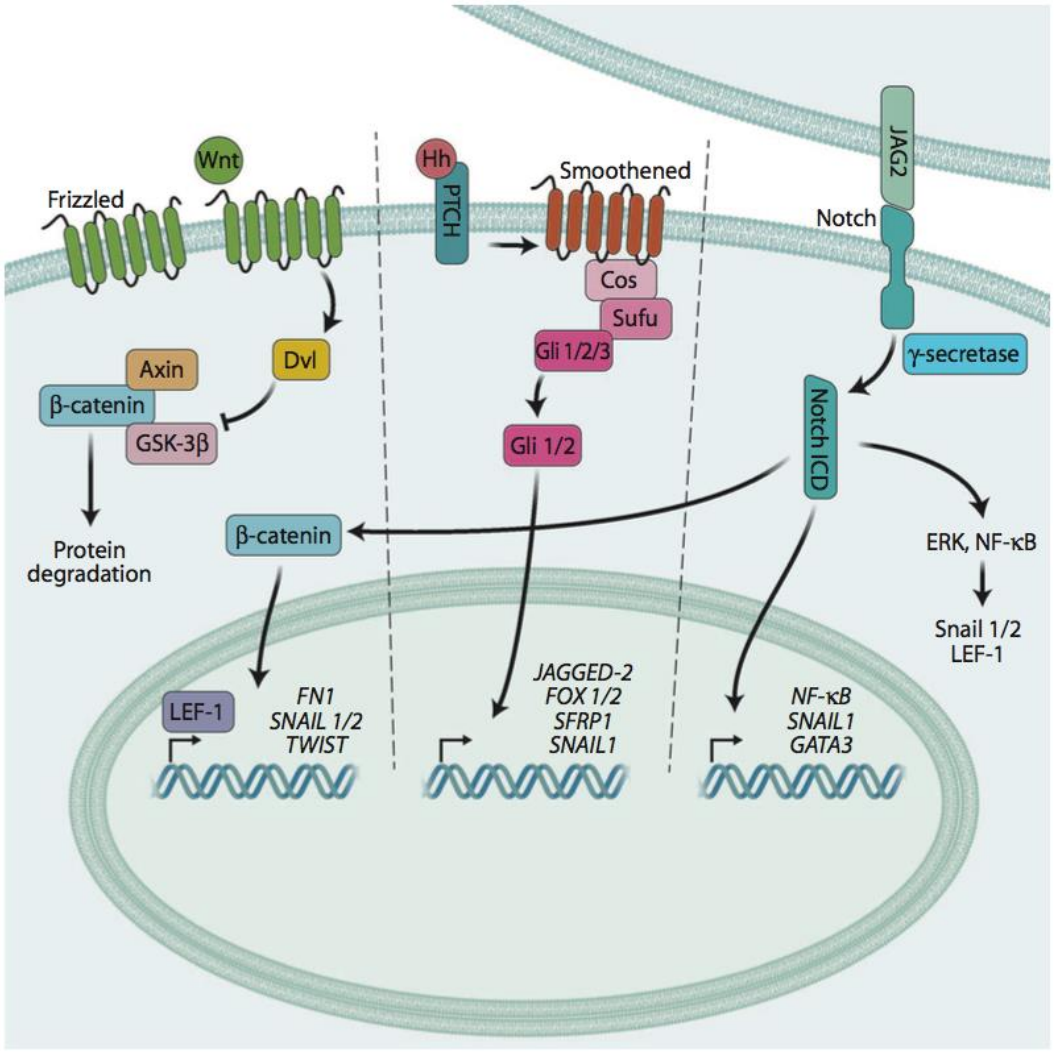

Figure 1.8 – Wnt, Notch, and HH signalling in EMT. Wnt ligands bind and activate Frizzled receptors, which promote Dvl-dependent inhibition of GSK-3β, a kinase that causes degradation of cytoplasmic β-catenin. This enables the accumulation and nuclear localization of β-catenin to activate the LEF-1 transcription factor, which promotes the expression of various EMT-associated genes. The intercellular interaction between JAG2 and its receptor Notch induces the γ-secretase–mediated cleavage and release of the NICD, which can directly activate target genes related to EMT signalling. The NICD can also stabilize cytoplasmic β-catenin and activate other pathways, like ERK and NF-κB, that activate Snail1/2 and LEF-1 transcription factors. HH signalling induces EMT-associated gene expression through activating GLI transcription factors. (Adapted from Gonzalez *et al.*, 2014)

expression and loss of E-cadherin, as well as an increase in sonic HH expression and signalling associated with increased Snail1 expression in epithelial cancers, including at the invasive front of neuroendocrine cancers[247]. Furthermore, HH signalling induces TGF-β1 secretion for increased motility[248] and JAG2 expression, resulting in cleavage of the NICD[131]. Other possible targets for HH may be Frizzled-related protein 1 (SFRP1)[249], known to modulate Wnt signalling, and both FOX1 and FOX2, two mesenchymal transcription factors that control the intracellular accumulation of β-catenin[250].

Additional factors within the tissue or tumour microenvironment may trigger EMT. For example, hypoxia facilitates EMT by mediating the expression of the transcription factor HIF1α, which leads to an increase in TGF-β levels and activates Twist and lysyl oxidase (LOX) expression[251]. HIF1α also induces Snail1 expression in ovarian carcinoma cells, resulting in the loss of E-cadherin[252].

EMT might also have its origin in an inflammatory stimulus, such as the release of inflammatory cytokines by immune and endothelial cells, and cancer-associated fibroblasts. As an example, interleukin-6 (IL-6) promotes EMT in breast cancer cells, which correlates with decreased E-cadherin levels and increased N-cadherin, vimentin, Snail1 and Twist expression, as well as with enhanced cell invasion capacities[253]. Ectopic Twist expression in these cells instigates IL-6 expression and activates the transcription factor signal transducer and activator of transcription 3 (STAT3), which afterwards promotes Snail1 expression. IL-8 has also been implicated in EMT initiation[254].

Integrin-induced EMT relies on its cooperation with NF-κB, which inhibits GSK-3β and stabilizes Snail, LEF-1, and β-catenin expression in order to promote EMT[255].

1.2.1.5 EMT and the acquisition of stem-like properties

Apart from the required adaptation of cancer cells to the microenvironment of a foreign tissue during colonization (i.e., the formation of micro- and macrometastases), the trait of self-renewal seems to be equally essential for tumour dissemination and metastization. Accordingly, such self-renewing cells, termed

cancer stem cells (CSCs), have been identified in several neoplasias. The extent of this subpopulations of CSCs greatly varies within distinct tumours depending on several factors, including the tissue from which the tumour arises, genetic and epigenetic changes accumulated during tumour progression, and the signals coming from the specific microenvironment in which the tumour is located[256].

CSCs are generally more quiescent, can reside in CSC niches, making them difficult to reach, express ATP-binding cassette (ABC) transporters, enabling drug efflux, and are equipped with reinforced mechanisms of DNA repair. All these properties help CSCs to stand safe against the currently used radio- and chemotherapeutic approaches for BC treatment, which cause lethal DNA damage. In other words, available techniques for fighting BC only target transit-amplifying and more differentiated cells, indeed causing shrinkage of the tumour. However, as CSCs are not affected, tumours will recur (Figure 1.9). In order to efficiently treat cancer, targeting of CSCs is pivotal, ideally in combination with therapies targeting the bulk of the tumour[256–258].

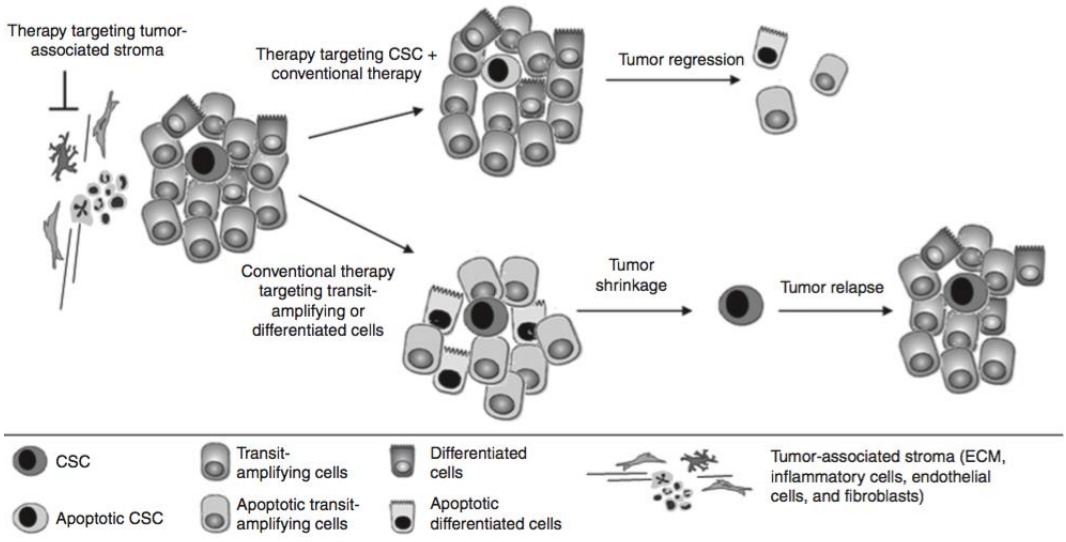

Figure 1.9 – Effective tumour therapies. Conventional therapies target cell populations within the bulk of the tumour, leading to tumour shrinkage. However, as CSCs can resist conventional therapy, tumour relapse will occur. For effective therapies, both transit-amplifying cells and differentiated cancer cells, and CSCs should be targeted (for example, by combining conventional therapy and induction of CSC differentiation), thereby removing not only the bulk of the tumour but also the source of the cancer. In addition, targeting of the tumour stroma is likewise a promising approach. (Adapted from van der Horst *et al.*, 2012)

As mentioned before, the ability to generate an unlimited number of progeny is mainly a feature ascribed to stem cells, which are capable of not only self-renewing but also of differentiating into cells with no self-renewing capabilities[256,259]. Actually, the mechanisms underlying the development of an entire tumour following experimental implantation of a single cancer stem cell and the macroscopic outgrowth after metastatic dissemination seem to be very similar, corroborating that CSCs may be the main responsible for metastasis formation[260]. In fact, the activation of EMT programs has been associated with the acquisition of stemness traits by both normal and neoplastic cells[261–263], providing a ready source of CSCs by enabling the dedifferentiation of the epithelial cells of carcinomas.

Accumulating evidence starts to uncover the molecular connections between the EMT program and the stem-cell phenotype. The transcription factor ZEB1 has been shown to negatively modulate the expression of microRNAs known to suppress stemness, namely members of the miR-200 family[264,265]. In turn, these inhibit the expression of Bmi-1[266], a polycomb protein essential for the maintenance of the stem-cell state in cancer cells, as well as Suz12[267], a histone-modifying enzyme required for the transcriptional repression of the gene encoding the protein E-cadherin by ZEB1 and Snail[268]. At the same time, Wnt–β-catenin signalling has been shown to be active in CSCs in a wide spectrum of cancers (particularly in breast[269], colon[270] and liver carcinomas[271]) and other malignancies[272,273], clarifying the reason why this signalling cascade has been proposed as a major therapeutic target for the obliteration of CSCs[274]. Additionally, CSCs benefit from several Twist-mediated mechanisms for enhanced resistance to apoptosis[275], which makes them the perfect candidates for metastization, due to their increased survival during early steps of metastasis and during the attempts to settle in distant, potentially inhospitable, tissue microenvironments.

Considering the emerging links between EMT and stemness properties, the observation that populations of carcinoma cells newly formed after various cytotoxic treatments express elevated levels of mesenchymal markers as compared to initially treated cells[276,277] holds the further implication that these therapy-resistant survivors are greatly enriched in CSCs, which are able to proceed and then

regenerate entirely new tumours, therefore driving clinical relapse.

1.2.1.6 EMT and its importance on BC

As previously mentioned, the switch from an epithelial-like expression of E-cadherin to an expression of the mesenchymal-phenotype characteristic protein N-cadherin is a markedly significant event triggering EMT. Hence, the role of these proteins in BC progression has been extensively investigated. After analysing several reports on cadherin expression in BC, Bryan and Tselepis concluded that the normal urothelium strongly expresses E-cadherin, while some NMIBCs show reduced expression of this protein. In the case of MIBCs, the majority of them present a significant decrease in E-cadherin expression or do not express this protein at all. However, N-cadherin is absent in the normal urothelium and most NMIBCs also lack N-cadherin expression, whilst a great number of MIBCs express this mesenchymal marker[278]. The presence of the protein vimentin has been suggested to contribute for EMT as well, being somehow linked to BC grade and stage[279,280].

Various EMT-related transcription factors are also differentially expressed in BC. For instance, increased levels of Twist correlate with a decline in E-cadherin expression and are notably associated with tumour progression and metastization[281,282]. Moreover, Twist expression was also found to be related to smoking[281], which represents the major risk factor for BC as outlined before, and to variables linked to poor prognostics[283]. Similarly to the observations for Twist, Snail2 expression is increased in BC, which likewise promotes cancer progression and metastasis formation, as opposed to what happens with Snail1, whose expression levels seem to be diminished[282]. Nonetheless, data regarding the role of the EMT-associated Snail1 in BC is not consistent. For example, Bruyere et al. reported that high Snail1 expression in NMIBC might be a reliable predictor of tumour recurrence[284]. Additionally, despite the flimsy results, Kenny et al. stated that the expression of ZEB transcription factors is a prognostic tool indicative of BC progression[285].

The contribution of microRNAs for the regulation of EMT mechanisms in BC is also relevant. Indeed, it has been suggested that members of the miR-200

family are upregulated in epithelial BC cell lines and downregulated in mesenchymal BC cell lines, while increased expression of miR-200 reverts EMT, as illustrated by higher levels of E-cadherin, morphologic changes and reduced migratory capacity, by directly inhibiting ZEB1 and ZEB2 expression[286]. Besides, miR-200 and miR-205 loci have been shown to be silenced in both MIBCs and BC cell lines through hypermethylation of the corresponding promoters, and Twist1 has been found capable of directly binding to miR-200 and miR-205 promoters, possibly operating as a repressor of the mentioned microRNAs[287]. More recently, raised levels of miR-205 expression were reported to correlate with unfavourable clinical outcomes in patients with MIBC[288]. In bladder carcinoma, members of the miR-34 family are also frequently inactivated[289].

Given that bladder tumorigenic tissues are bathed by urine, urinary microRNAs are of particular interest in BC research. In fact, lower urinary miR-200a levels associates with greater risk of disease recurrence[290]. Accordingly, the expression of miR-200a along with that of many other microRNAs, such as miR-200b, miR-200c, miR-141, miR-429, miR-205, and miR-192, has been found to be significantly decreased in patients with BC[291].

The existence of cells presenting with stemness properties has also been observed in BC. With such discovery, it has been postulated that cells with this stem/progenitor-like phenotype are the ones responsible for the establishment of premalignant cell fields over the epithelial line of the bladder. This results in multifocal heterogenic tumour outgrowth, given the additional accumulation of genetic mutations, epigenetic alterations, and interaction with the tumour-specific surrounding environment, the so-called stroma[292]. The urothelial CSCs likely originate from cells located in the basal urothelial cell layer, the innermost layer of cells at bladder lumen, which is attached to the basement membrane and is thought to enclose the normal urothelial stem cells. In line with the previous hypothesis, recently gathered data testifies the resemblances between urothelial CSCs and basal urothelial stem cells. For example, bladder CSCs have been characterized for several molecular markers which, in fact, correspond to well-known basal markers[293–295]. Moreover, as urothelial stem cells already possess self-renewal

capacity, they are noteworthy candidates to further convert into malignant cells[296].

Several studies have been focused on the identification of CSCs in BC, resorting to certain extracellular surface markers[293] and/or intracellular proteins[297–299]. These stemness indicators correlate with enhanced motile and invasive properties, asymmetric proliferation and self-renewing capacity, as well as with drug resistance, which turns them useful for diagnostic purposes and makes them interesting drug targets. Additionally, functional assays have also been used for urothelial CSC isolation and phenotype confirmation, including some approaches based on the characteristic multidrug resistance of CSCs, sphere-forming assays, or xenotransplantation[287,288,290,300].

1.3 Objectives

Increasing evidence suggest that the activation of EMT programs in cancer cells facilitates tumour progression and metastatic dissemination. This process is closely regulated by a variety of signalling pathways and growth factors, such as TGF-β and EGF, which exert profound effects in the induction of EMT in both physiological and pathological conditions. In this study, we aimed to explore the role of TGF-β and EGF on promoting EMT in BC and how these growth factors influence the biological behaviour of BC cells regarding their stemness, drug resistance, proliferation rate, metabolic activity and migratory ability.

To address these questions, we proposed to:

- Evaluate the role of TGF-β and EGF in promoting EMT in two human BC cells lines derived from high-grade tumours, HT-1376 and UM-UC3, by measuring the expression of EMT-related proteins;
- Investigate whether TGF-β and EGF provides an enrichment of a stem-like phenotype in BC cells, by analysing the expression of two stemness-related markers (SOX2 and ALDH2);
- Evaluate how TGF-β and EGF influence the chemosensitivity profile of BC cells to CIS;

- Assess the effects of TGF-β and EGF in the proliferation rate and metabolic activity of BC cells;
- Analyse the role of TGF-β and EGF in BC cell migration by performing a scratch assay.

Chapter 2

Methods

2.1 Cell culture

Human HT-1376 and UM-UC3 BC cell lines (ATCC, VA, USA) derive from high grade transitional cell carcinomas and grow in monolayer. These cells were routinely cultured in RPMI-1640 medium (R4130, Sigma-Aldrich, MO, USA), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; 10270106, Gibco, CA, USA) and 1% (v/v) antibiotic/antimycotic containing 0.25 µg/mL amphotericin B, 100 units/mL penicillin and 100 µg/mL streptomycin (A5955, Sigma-Aldrich, MO, USA). Cells were maintained in a humidified atmosphere at 37ºC within an incubator with 5% CO_2 until they reached 80-90% confluence. They were then detached with trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA; T4049, Sigma-Aldrich, MO, USA) and subcultivated at an appropriate ratio. Every procedure involving manipulation of cells was performed in sterile conditions within a laminar flow chamber.

2.1.1 Cell counting

Prior to the experiments, the total number of viable cells was determined using the trypan blue exclusion assay. Trypan blue is a stain used to selectively colour dead cells, which will turn blue once their cell membranes are compromised. Conversely, viable cells with intact membranes will not be stained and appear brilliant and clear to microscope. To execute this technique, equal volumes (20 µL) of cell suspension and trypan blue solution 0.4% (93595, Sigma-Aldrich, MO, USA) were mixed together and transferred into a Neubauer chamber. Immediately afterwards, cells were observed and counted using an inverted microscope (Nikon, Eclipse TS 100). Both viable and non-viable cells were counted in the four corner quadrants. Cell viability was calculated as the percentage of viable cells relative to the total number of cells. Only cells with viability higher than 90% were used in all experiments. The number of cells was calculated by applying the following expression:

$$Number\ of\ cells = cell\ average\ \times\ dilution\ factor\ \times\ 10^4$$

2.1.2 Epithelial-to-mesenchymal phenotype induction

The EMT was induced in BC cells through an inflammatory stimulus by exposing them to TGF-β (10 ng/mL) and EGF (50 ng/mL), individually or in combination, during 48 h. Stock solution of TGF-β (R&D Systems, McKinley, MN, USA) was reconstituted in sterile 4 mM HCl containing 1 mg/mL bovine serum albumin (BSA; A9418, Sigma-Aldrich, MO, USA), at a concentration of 20 µg/mL. Stock solution of EGF (PeproTech, London, UK) was reconstituted in sterile phosphate-buffered saline (PBS) containing 0.1% BSA, at a concentration of 50 µg/mL.

2.2 Western blotting

To evaluate whether EMT occurred or not, the expression of some epithelial (β-catenin) and mesenchymal (N-cadherin and vimentin) markers was carried out by Western Blot (WB), after a 48-h treatment with TGF-β (10 ng/mL) and EGF (50 ng/mL), separately or in combination. As EMT is also thought to induce a phenotypic cell shift towards a stem-like phenotype, the expression of two stemness-related markers – ALDH2 and SOX2 – was analysed as well.

2.2.1 Cellular extracts

HT-1376 and UM-UC3 cells were plated in 6-well plates (Orange Scientific, Belgium) at a density of 200×10^3 cells/well and allowed to attach overnight. The growth factors mentioned above were then added to the culture medium and, after 48 h of incubation, total protein lysates were prepared.

For this purpose, the cell culture medium was discarded and cells were washed with PBS and scraped using 100 µL of lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl (Merck, NJ, USA), 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate (SDS; 161-0307, Bio-Rad, CA, USA) and 2 mM EDTA], containing a mixture of proteases and phosphatases inhibitors (Roche, Switzerland), 2 mM sodium orthovanadate, 1mM NaF and 1mM dithiotreitol (DTT; D0632, Sigma-Aldrich, MO, USA). After a 30-min incubation

at 4°C in lysis buffer, samples were sonicated, using an ultrasound device (Vibra cell Sonics and Materials Inc. Danbury, CT, USA), at 40 MHz, with 3-5 pulses for 5 seconds, while dipped in ice.

Protein concentration was determined using the bicinchoninic acid (BCA; B9643, Sigma-Aldrich, MO, USA) method with BSA (A2153, Sigma-Aldrich, MO, USA) as a standard, in a 96-well cell culture plate.

The protein samples were mixed (1:1) with an equal volume of 2× denaturing solution [0.25 M Tris (pH 6.8; T1378, Sigma-Aldrich, MO, USA), 200 mM DTT, 4% (w/v) SDS, 20% (v/v) glycerol (G2025, Sigma-Aldrich, MO, USA) and bromophenol blue] and heated at 95ºC during 5 min for protein denaturation. Samples were stored frozen at -20ºC until their usage.

2.2.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransference

Samples (40-50 µg) were loaded into SDS-polyacrylamide gels [8 or 12% acrylamide/bisacrylamide solution (GRiSP Research Solutions), 20% SDS, 10% ammonium persulfate (APS; 0486, AMRESCO, USA) and tetramethylethylenediamine (TEMED; T9281, Sigma-Aldrich, MO, USA)] and proteins were separated considering their molecular weight by electrophoresis for 10 min at 100 V followed by 1 h at 140 V in buffer solution [100 mM Tris-HCl containing 100 mM bicine (B3876, Sigma-Aldrich, MO, USA) and 0.1% (w/v) SDS]. A protein marker (5 µL; Precision Plus Protein All Blue Standards, 161-0373, Bio-Rad, CA, USA) was also used in electrophoresis for accurate target protein band identification.

After electrophoresis, proteins were transferred from the polyacrylamide gel to a methanol-activated hydrophobic polyvinylidene difluoride (PVDF) membrane. The electrotransference was performed in electrotransfer buffer solution [12.5 mM Tris-HCl (pH 8.0-8.5) containing 96 mM glycine (G8898, Sigma-Aldrich, MO, USA) and 20% (v/v) methanol (VWR International, PA, USA)] during 90 min at 110 V and 4ºC.

2.2.3 Immunoblotting and quantification

Promptly after transfer, PVDF membranes were blocked in 5% (w/v) non-fat dry milk in tris-buffered solution T [TBS-T; 20 mM Tris (pH 7.6) and 137 mM NaCl, containing 0.1% (v/v) Tween 20 (437082Q, VWR International, PA, USA)] for 1 h at room temperature (RT), with soft agitation. This step is essential for covering up all protein-free parts of the membrane (i.e., portions of the membrane that were not filled with protein) in order to prevent non-specific interactions between the membrane and the antibody used for detection, and to reduce the background. After blocking, membranes were incubated overnight at 4ºC with primary antibodies at appropriate dilutions in 1% (w/v) (β-catenin) or 5% (w/v) (N-cadherin, vimentin and ALDH2) non-fat dry milk in TBS-T, or 5% (w/v) BSA in TBS-T (SOX2). After the incubation period, membranes were washed 5 times for 5 min in TBS-T on an orbital shaker and then incubated for 1 h at RT with alkaline phosphatase-conjugated secondary antibodies. The dilution of primary and secondary antibodies is shown in Table 2.1. Lastly, membranes were washed again as previously described and revealed using an enhanced chemifluorescent substrate (ECF; RPN5785, ECF Western Blotting Reagent Packs, GE Healthcare Life Sciences, IL, USA). Membranes were then scanned in Typhoon FLA 9000 (GE Healthcare Life Sciences, IL, USA) to visualize reactive protein bands. Subsequent quantification of band density was performed using ImageJ (National Institutes of Health), a java-based imaging software.

Table 2.1 – Brief illustration of the parameters used for WB analysis.

Protein	Molecular weight (kDa)	% of acrylamide in electrophoresis gel	Dilution of primary antibody	Secondary antibody	Dilution of secondary antibody
β-catenin	92	8	1:250	Anti-Mouse	1:10000
N-cadherin	130	8	1:500	Anti-Rabbit	1:20000
Vimentin	57	12	1:1000	Anti-Mouse	1:10000
ALDH2	56	12	1:1000	Anti-Rabbit	1:20000
SOX2	35	12	1:1000	Anti-Rabbit	1:20000
β-actin	43	–	1:5000	Anti-Mouse	1:10000

The membranes were stripped using 0.2 M NaOH (28244.295, VWR International, PA, USA) for 5 min and then reprobed with anti–β-actin antibody as loading control, followed by incubation with secondary antibody and revelation as previously described. The band intensities of target proteins were normalized to their corresponding β-actin controls.

2.3 Cell proliferation studies

To evaluate whether exposure to TGF-β or EGF increases the proliferation rate of BC cell lines, cell proliferation was measured for 4 days, with 24 h time intervals, after incubation with the growth factors by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. This experiment is based on the reduction of the yellow tetrazolium salt into purple formazan crystals by the mitochondrial enzyme succinate dehydrogenase of metabolic active cells. The amount of formazan crystals formed can be measured spectrophotometrically, after dissolution with acidified isopropanol, using an ELISA microplate reader. The colour intensity resulting from the dissolution of the formazan crystals is directly proportional to the enzymatic activity and, consequently, to the number of viable cells.

For this assay, cells were seeded in 96-well plates (Orange Scientific, Belgium) at a density of 3×10^3 cells/well and allowed to attach overnight. They were then incubated with TGB-β (10 ng/mL), EGF (50 ng/mL) or both during 24 h, 48 h, 72 h and 96 h.

At each time-point, 50 µL of 0.5 mg/mL MTT (M2128, Sigma-Aldrich, MO, USA) were added to each well and the plate was maintained inside the CO_2 incubator at 37ºC during 4 h for formation of formazan crystals. Thereafter, the supernatant was removed and the blue formazan was dissolved using 50 µL of a mixture (1:1) of dimethyl sulfoxide (DMSO, Sigma-Aldrich, MO, USA) and 0.4 M HCl in isopropanol in an orbital shaker for 20 min at 37ºC. The absorbance was read at a wavelength of 570 nm, using a 620 nm filter as reference, in an automatic ELISA microplate reader (Synergy HT, BioTek Instruments, Inc., VT, USA). The

amount of proliferative cells at each experimental condition was expressed as the percentage of cell proliferation calculated for the untreated control at the 24-h time point, that was set as 100%.

2.4 Drug cytotoxicity assays

To investigate whether the induction of EMT by TGF-β and EGF might alter the susceptibility of BC cells to conventional chemotherapy, the chemosensitivity of both HT-1376 and UM-UC3 BC cell lines to CIS was analysed, without and after EMT induction. CIS is currently the most commonly used chemotherapeutic drug in the treatment of BC patients with advanced disease.

Cells were seeded in 96-well plates (Orange Scientific, Belgium) at a density of 3.5×10^3 cells/well and allowed to attach overnight, followed by 24 h of incubation with the growth factors TGF-β (10 ng/mL) and EGF (50 ng/mL), separately or in combination, for EMT induction. After that, cells were incubated with increasing concentrations of CIS (Teva, Israel), ranging from 1 to 100 μM, for a period of 48 h. Stock solution of CIS (1 mg/mL) was diluted in PBS at appropriate working concentrations (0.1, 0.01 and 0.001 mg/mL), before being added to cells. Aliquots were stored at -20ºC.

Subsequent evaluation of the cytotoxic effects of CIS was carried out by using the MTT colorimetric assay as previously described in section 2.3, after exposure to CIS in the absence and in the presence of growth factors. Absorbance values were expressed as percentages relative to that of the untreated control, previously set as 100%.

2.5 Analysis of metabolic activity

The metabolic activity of BC cells was assessed based on the uptake of 2-deoxy-2-(^{18}F)fluoro-D-glucose (^{18}F-FDG), which is a radiopharmaceutical widely used in positron emission tomography (PET). Since ^{18}F-FDG is a glucose analogue, its uptake by tissues or cells is a marker for glucose uptake, which in turn is closely

correlated with cell metabolism. This radiopharmaceutical was provided by the Institute for Nuclear Sciences Applied to Health, ICNAS, University of Coimbra, Coimbra, Portugal. To perform this experiment, both BC cell lines were seeded in 12-well plates (Orange Scientific, Belgium) at a density of 100×10^3 cells/well and allowed to attach overnight. Then, TGF-β and EGF were added to the culture medium at a final concentration of 10 ng/mL and 50 ng/mL, respectively, individually or in combination, and cells were incubated for 48 h. After this period, cells were exposed to ^{18}F-FDG (1 MBq/mL) and left during 1 h within the incubator. The culture medium was subsequently collected to glass tubes and, after being scraped, cells were also collected to different glass tubes. All tubes were assayed for radioactivity in a Radioisotope Calibrator Well Counter (CRC-15W Capintec, USA) within the ^{18}F sensitivity energy window of 400-600 keV. The cellular uptake of ^{18}F-FDG was reported as the percentage of cell radioactivity in relation to the total radioactivity measured, normalized to control.

2.6 Wound-healing assay

To further analyse if TGF-β and EGF-induced EMT correlates with enhanced cancer cell migration, a wound-healing assay, a simple and inexpensive method to assess cell migration *in vitro*, was executed.

For that, BC cells were plated in 12-well plates (Orange Scientific, Belgium) at a density of 200×10^3 cells/well and allowed to attach overnight. On the following day, cells were exposed to TGF-β (10 ng/mL), EGF (50 ng/mL) or both during 48 h. After this incubation period, cells reached 100% confluence and a scratch was performed along the cell monolayer with a 200 μL pipette tip. The wells were washed once with culture medium to remove the debris and smooth the edge, and then refilled with 1 mL of fresh culture medium. Images were captured in an inverted microscope equipped with a digital camera (DMI3000 B, Leica Microsystems, Germany), immediately at the onset of scratch performance to record the initial area of the wound and then at regular time intervals during cell

migration to close the wound. The images acquired for experimental condition were subsequently analysed qualitatively.

2.7 Statistical analysis

Graphical artwork and statistical analysis were computed using GraphPad Prism version 6.0c (GraphPad Software, CA, USA). Values were considered to be significantly different if $P < 0.05$.

Chapter 3

Results

3.1 Effect of TGF-β and EGF exposure on the expression of EMT-related markers

As carefully described before, one of the main hallmarks characterizing a successful activation of an EMT program is a cellular phenotypic switching from an epithelial-like to a mesenchymal-like state. This mechanism drives several modifications by which cells that have undergone EMT pass through and is ultimately regulated by down- and upregulation of epithelial and mesenchymal markers, respectively. Based on this assumption, the expression levels of β-catenin (epithelial protein), and vimentin and N-cadherin (both mesenchymal proteins) were assessed through Western blotting. For that, both cell lines were incubated with 10 ng/mL TGF-β, 50 ng/mL EGF or both in combination for 48 h and then submitted to the protocol detailed in Section 2.2. Unfortunately, it was not possible to evaluate the effect of TGF-β in combination with EGF in N-cadherin expression, because the N-cadherin antibody c(H-63): sc-7937 that was initially used was discontinued, and the current-production one did not work properly in our samples, which made further experiments infeasible.

Data analysis showed a significant ($P < 0.05$) increase in vimentin expression exclusively for HT-1376 cells treated with TGF-β in combination with EGF when compared to control, although a slight increasing trend was observed in cells exposed to TGF-β or EGF separately. No significant differences were seen for the other two investigated proteins (Figure 3.1 A). Similarly, there were no significant changes in protein expression levels between control and treated groups in UM-UC3 cell line, although a slight increase in N-cadherin expression can be noted in cells exposed to TGF-β as compared to control (Figure 3.1 B). WB analysis of E-cadherin, an epithelial marker, did not show any detectable level of protein in any of the BC cell lines, neither for control groups nor in treated samples (data not shown).

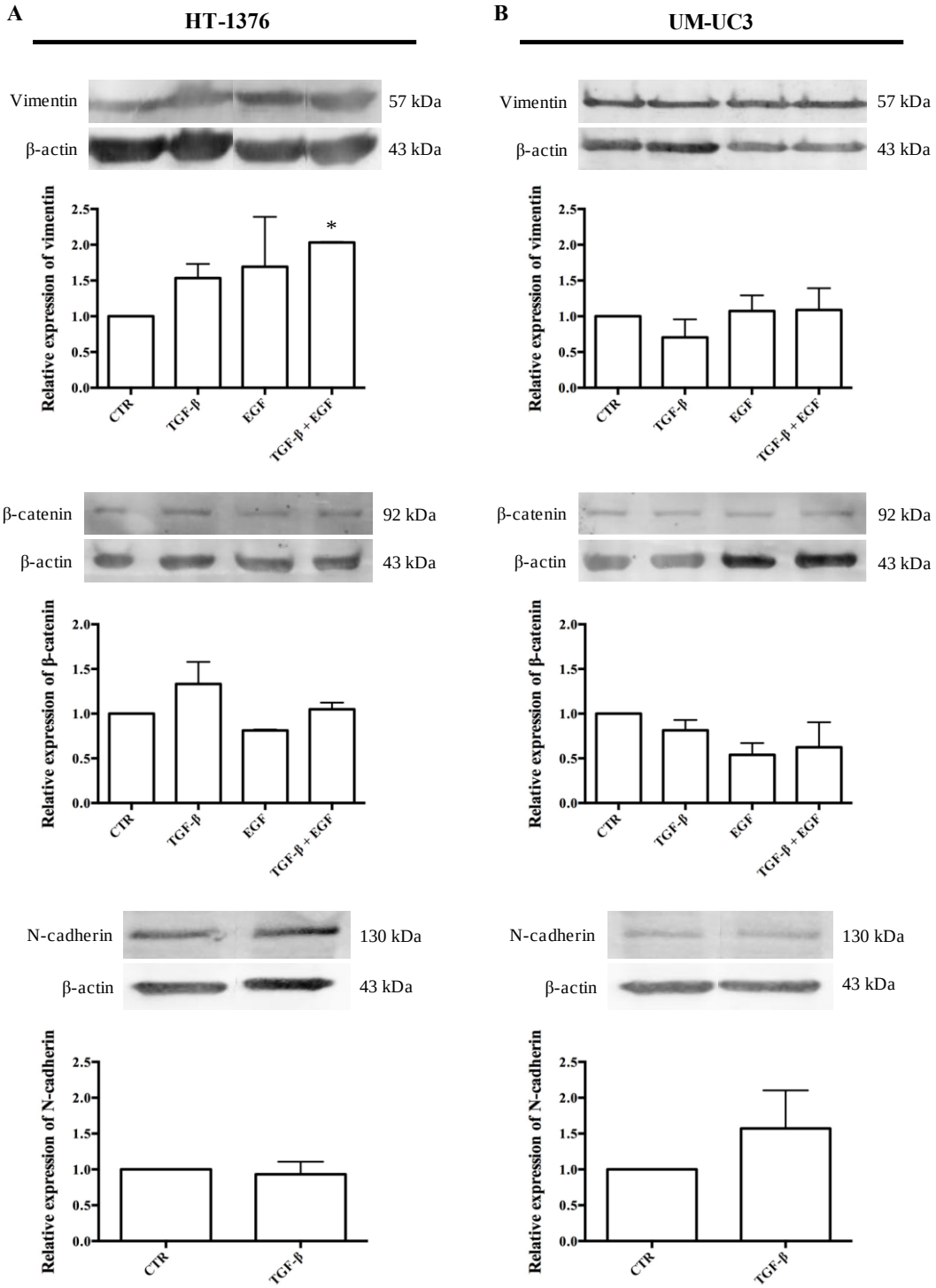

Figure 3.1 – Effect of TGF-β, EGF, and TGF-β + EGF exposure on vimentin, β-catenin, and N-cadherin expression levels in BC cells. Quantitative analysis of β-catenin (epithelial marker), and vimentin and N-cadherin (mesenchymal markers) expression after a 48-h incubation with the mentioned growth factors in **A |** HT-1376 and **B |** UM-UC3 cells. Above the bars, representative WB images of the analysed proteins are shown. All values were normalized to the constitutive protein β-actin and then to their respective untreated control. Results are presented as mean ± SEM, n=2-5, **P* < 0.05 when compared to the untreated control using one way ANOVA followed by Dunnet's multiple comparisons test.

3.2 Effect of TGF-β exposure on the expression of stemness-associated proteins

Previous studies performed within our research group revealed that putative bladder CSCs highly express the *ALDH* and *SOX2* genes, in addition to some other stem cell-related genes[300]. Hereupon, in order to evaluate whether eventually-occurred EMT (following a 48-h exposure to 10 ng/mL TGF-β) potentiates the acquisition of stem traits by BC cells, the expression of the ALDH2 and SOX2 proteins was also quantified by WB.

Analysis of Figure 3.2 A reveals approximately a two-fold increase in ALDH2 expression in HT-1376 cells after incubation with TGF-β, when compared to non-treated cells of the same cell line. This is however not true for the expression of SOX2, whose levels seem to remain unchanged after exposure to the aforementioned growth factor. Regarding the UM-UC3 cell line (Figure 3.2 B), TGF-β exposure seems to reduce the expression of ALDH2 to slightly less than half as compared to the levels found for the control group. On the other hand, SOX2 expression levels appear to suffer almost a two-fold increase in cells incubated with TGF-β when compared to non-treated cells. These results suggest that the effects of TGF-β on the expression of these two CSC-related markers are cell-type dependent.

3.3 Effect of TGF-β and EGF exposure on the chemo-sensitivity of BC cells to CIS

To investigate whether exposure to TGF-β and EGF promotes the acquisition of a chemoresistant profile by BC cells, both cell lines were assayed for sensitivity to CIS (a chemotherapeutic agent widely used in the fight against MIBC) after being treated with the growth factors, alone or in combination.

With that purpose, a dose-response study was performed. HT-1376 and UM-UC3 cells were plated, and control and treated groups were incubated for 48 h with

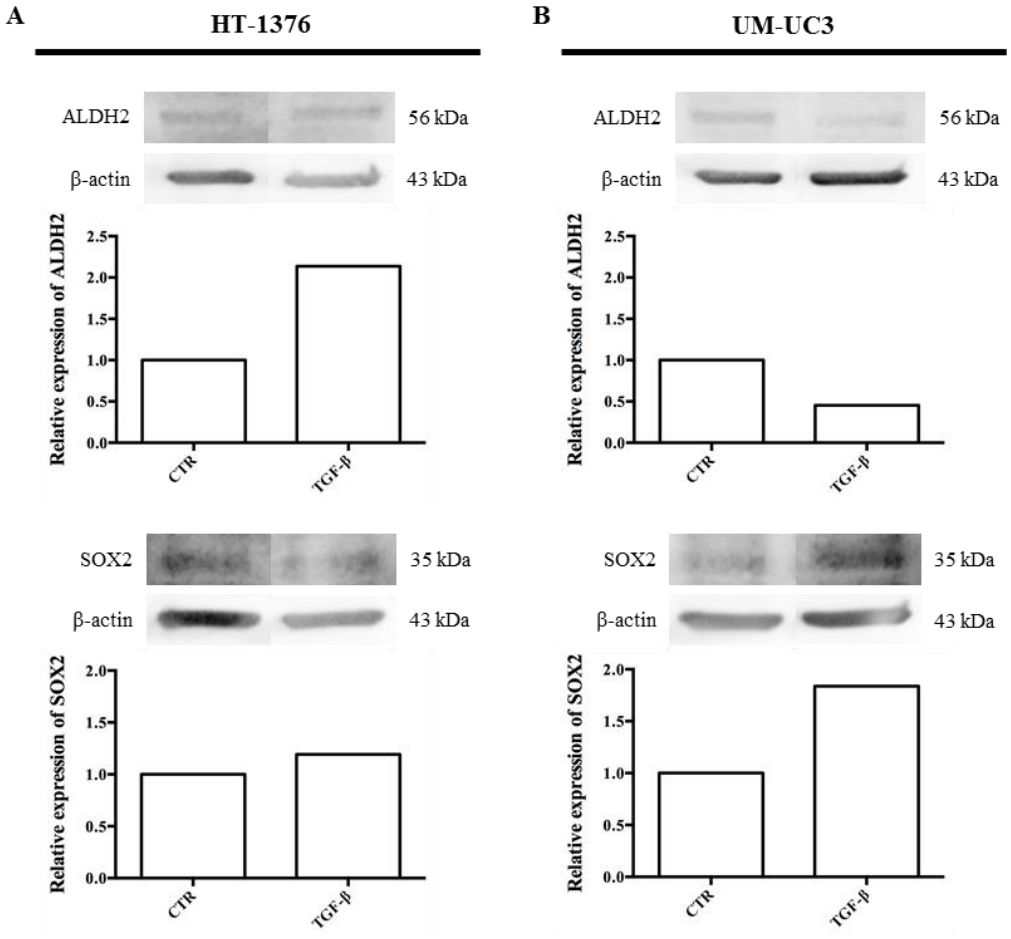

Figure 3.2 – Effect of TGF-β, EGF, and TGF-β + EGF exposure on ALDH2, and SOX2 expression levels. Quantitative analysis of ALDH2, and SOX2 (stemness markers) expression after a 48-h incubation with the mentioned growth factors in **A |** HT-1376 and **B |** UM-UC3 cells. Above the bars, representative WB images of the analysed proteins are shown. All values were normalized to the constitutive protein β-actin and then to their respective untreated control, n=1.

increasing concentrations of CIS (ranging from 1 to 100 μM), without and after being treated with the growth factors. The evaluation of cell viability was accomplished using the MTT colorimetric assay.

Incubation with CIS resulted in reduced cell viability in both cell lines, in a concentration-dependent manner. However, treatment with TGF-β and EGF had practically no considerable effects on the chemosensitivity of BC cells to CIS, with just a few exceptions (Figure 3.3). For the HT-1376 cell line, exposure to EGF, either alone or in combination with TGF-β, provided protection against CIS-induced cytotoxicity at 10 μM, significantly increasing cell viability from 36.0 % ±

2.7 % to 50.5 % ± 3.2 % ($P < 0.01$) and from 36.0 % ± 2.7 % to 46.5 % ± 3.8 % ($P < 0.05$), respectively. However, no significant changes in cell viability were observed for the other concentrations of CIS nor amongst cells previously treated with TGF-β (Figure 3.3 A). On the other hand, for the UM-UC3 cell line, exposure to TGF-β significantly reduced cell resistance to CIS at the highest concentrations (50 and 100 μM), decreasing cell viability from 18.6 % ± 3.2 % to 9.8 % ± 1.9 % ($P < 0.01$) and 6.0 % ± 0.9 % to 3.8 % ± 0.5 % ($P < 0.01$), respectively. However, as in the HT-1376 cell line, no significant changes were observed for the additional concentrations of CIS, as well as amongst cells previously submitted to EGF or TGF-β + EGF exposure (Figure 3.3 B).

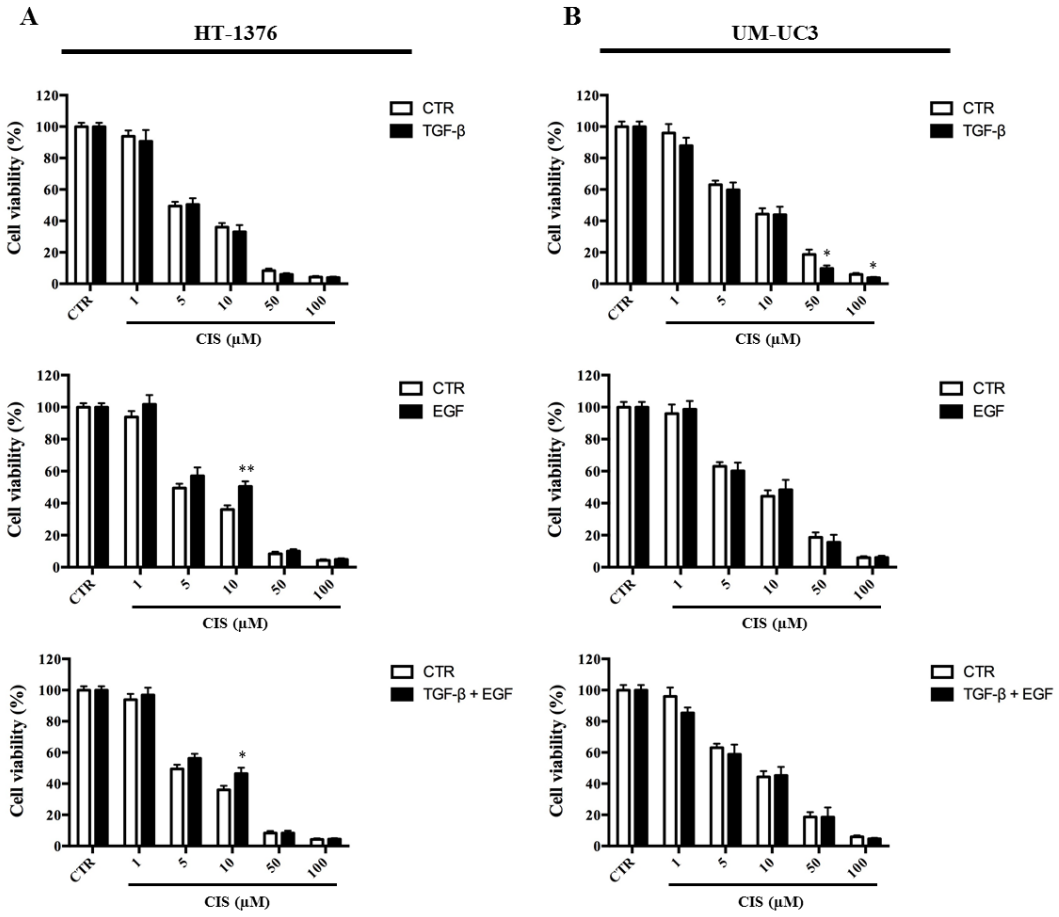

Figure 3.3 – Cytotoxic effects of CIS in the viability of A | HT-1376 and B | UM-UC3 cells. Percentage of viable cells after treatment with 10 ng/mL TGF-β, 50 ng/mL EGF or both in combination and subsequent incubation with increasing concentrations (1-100 μM) of CIS, determined through the MTT assay. All values were normalized to their respective untreated control. Results are presented as mean ± SEM, n=5-7, $*P < 0.05$ and $**P < 0.01$ when compared to respective CIS controls using unpaired t test with Welch's correction.

3.4 Effect of TGF-β and EGF exposure on HT-1376 and UM-UC3 cell proliferation

In order to analyse the effect of TGF-β and EGF on the ability of BC cells to proliferate, HT-1376 and UM-UC3 cells were plated and incubated with the appropriate concentrations of the growth factors. Subsequently, they were subjected to an MTT assay at regular time intervals of 24 h for quantification of the amount of viable proliferative cells. Results are shown in Figure 3.4 and Table 3.1, and expressed as percentage of the absorbance values measured for the control group at 24 h (which was used as the reference measure, i.e., set as 100%).

For cells belonging to the HT-1376 cell line, a significant increase in cell proliferation was seen for the EGF-treated group when compared to the control group, but exclusively after 72 h of incubation (256.2 % ± 13.3 % *vs.* 216.5 % ± 6.4 %; $P < 0.05$). Additionally, a significant reduction in cell proliferation was observed after 24 h of incubation for the group who undergone treatment with TGF-β in combination with EGF as compared to control (78.3 % ± 7.1 % *vs.* 100.0 % ± 3.7 %; $P < 0.01$), although this difference was not found to be significant for the following time points (Figure 3.4 A; Table 3.1 A). With respect to the UM-UC3 cell line, no significant variations were detected (Figure 3.4 B; Table 3.1 B). These results suggest that none of the growth factors used, individually or in combination, caused significant alterations on BC cell proliferation rates.

3.5 Effect of TGF-β and EGF exposure on the metabolic activity of BC cells

To fulfil the huge metabolic demands of tumours, a set of modifications occur across their metabolic landscape. In fact, the Warburg effect[301] is a widely-recognized feature of the metabolic profile of tumour cells, which implies a shift of ATP synthesis from oxidative phosphorylation to glycolysis, even in oxygen-rich situations. As highly proliferative cells, tumour cells need rapid ATP generation – to maintain energy status, increased biosynthesis of macromolecules and strict

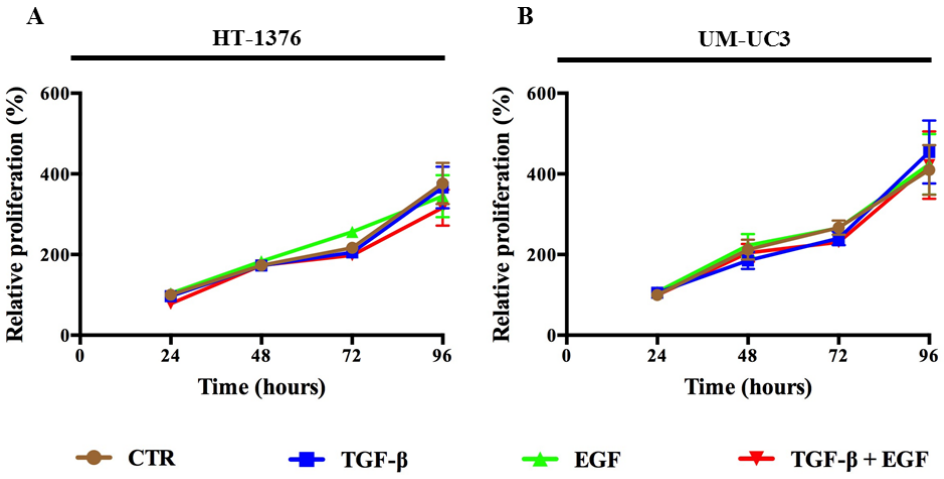

Figure 3.4 – Comparison of proliferation rates between control and treated cells from A | HT-1376 and B | UM-UC3 cell lines. BC cells were plated and incubated with 10 ng/mL TGF-β, 50 ng/mL EGF or both growth factors in combination. After regular time intervals of 24 h, proliferation-related cell viability was assessed by an MTT assay. All values were normalized to the 24-h untreated control. Results are presented as mean ± SEM, n=8.

Table 3.1 – Comparison of proliferation rates between control and treated cells from A | HT-1376 and B | UM-UC3 cell lines.

A

HT-1376				
	24h	**48h**	**72h**	**96h**
CTR	100.0 ± 3.7	173.1 ± 11.8	216.5 ± 6.4	376.1 ± 51.0
TGF-β	96.9 ± 3.0	173.0 ± 12.6	205.0 ± 12.4	366.5 ± 51.6
EGF	104.0 ± 4.3	183.3 ± 12.6	256.2 ± 13.3*	344.8 ± 51.9
TGF-β + EGF	78.3 ± 7.1**	173.1 ± 14.7	198.2 ± 3.7	316.0 ± 44.9

B

UM-UC3				
	24h	**48h**	**72h**	**96h**
CTR	100.0 ± 3.1	212.2 ± 24.7	266.7 ± 17.7	410.0 ± 61.6
TGF-β	105.4 ± 9.7	185.2 ± 21.2	240.1 ± 16.9	454.1 ± 78.1
EGF	107.1 ± 4.7	223.0 ± 28.0	265.7 ± 12.3	423.9 ± 74.9
TGF-β + EGF	99.4 ± 4.5	204.4 ± 22.3	230.6 ± 8.2	421.8 ± 83.5

BC cells were plated and incubated with 10 ng/mL TGF-β, 50 ng/mL EGF or both growth factors in combination. After regular time intervals of 24 h, proliferation-related cell viability was assessed by an MTT assay. All values were normalized to the 24-h respective control. Results are presented as mean ± SEM, n=8, $*P < 0.05$ and $**P < 0.01$ when compared to the respective untreated control using one way ANOVA followed by Dunnet's multiple comparisons test.

controlled cellular redox conditions – and the aerobic glycolysis is more efficient in providing such demands[302]. Since EMT represents a particularly complex and many-sided mechanism, it probably entails changes at the energetic axis as well[303].

Considering these evidence, the ^{18}F-FDG (a glucose analogue radiotracer) uptake by BC cells was measured without and after being exposed to TGF-β and EGF, with the primary intent of investigating whether EMT plays a role in reinforcing cellular metabolism or not. The cellular uptake was measured 1 h after incubation with ^{18}F-FDG. As depicted in Figure 3.5, no significant differences in the cellular uptake of ^{18}F-FDG were observed between control and treated groups, neither for the HT-1376 (Figure 3.5 A) nor for the UM-UC3 (Figure 3.5 B) cell line. These results suggest that incubation with the growth factors does not affect the metabolic activity of BC cells, at least in the concentrations and time exposure used in this study.

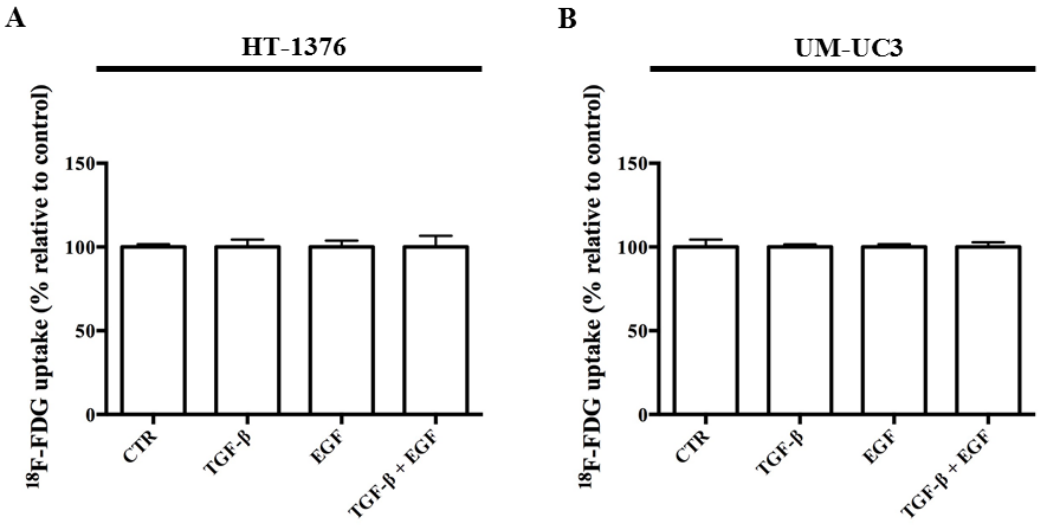

Figure 3.5 – Effect of TGF-β, EGF, and TGF-β + EGF exposure on BC cell metabolism. ^{18}F-FDG uptake by cells of **A |** HT-1376 and **B |** UM-UC3 cell lines after treatment with 10 ng/mL TGF-β, 50 ng/mL EGF or both for 48 h. Results are presented as a percentage ratio of the levels calculated for the respective untreated control. Values are shown as mean ± SEM, n=3.

3.6 Effect of TGF-β and EGF exposure on BC cell motility

As previously depicted, EMT greatly interferes with cell migration, making cancer cells able to escape from primary tumours and further migrate to distant body

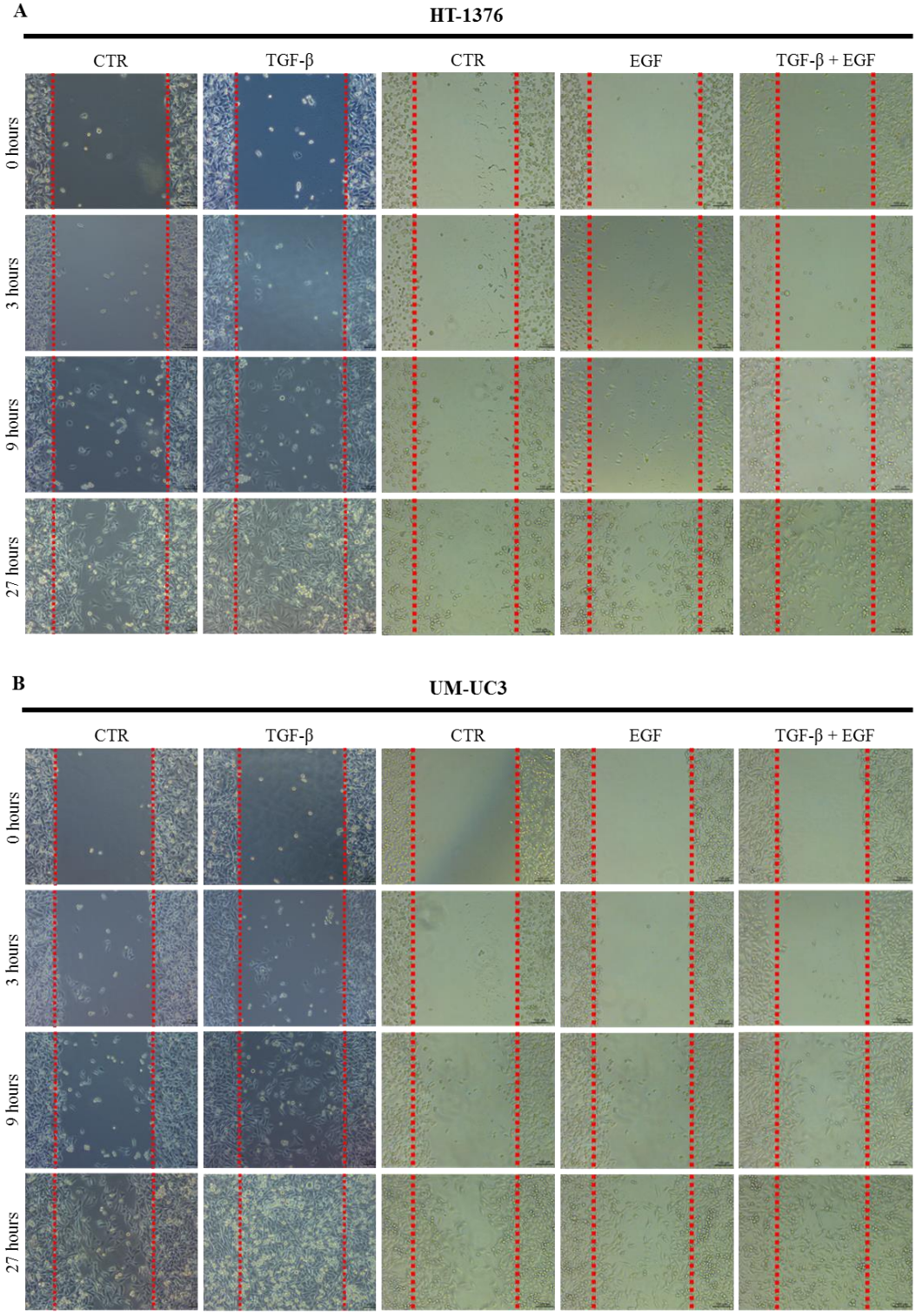

Figure 3.6 – Wound healing assay. A | HT-1376 and **B |** UM-UC3 cells were plated at high density and incubated with TGF-β (10 ng/mL), EGF (50 ng/mL) or both over 48 h. Migration of BC cells into the wound was assessed at 0, 3, 9, and 27 h after the scratch was made. The dotted red line indicates the boundaries of the scratch.

sites, where they settle and subsequently give rise to metastasis formation.

Therefore, a scratch wound-healing assay was performed to assess whether exposure of BC cells to TGF-β and EGF would increase cell migration. BC cells were exposed to both growth factors, alone or in combination, during 48 h prior to performing the scratch wound, with the untreated cells being used as a control. The wound closure was monitored for 27 h. Indeed, incubation with TGF-β alone or in combination with EGF facilitated the wound closure of both HT-1376 (Figure 3.6 A) and UM-UC3 (Figure 3.6 B) cells, as indicated by the high number of cells filling the denuded wound gap as compared to the untreated control cells. In fact, cells treated with TGF-β alone or in combination with EGF nearly filled the wound gap over 27 h, contrarily to the untreated control cells which only partially filled the gap. Exposure to EGF *per se* had no significant effects in the migration ability of both BC cell lines, relative to the control condition.

Chapter 4

Discussion

BC is part of the most widely diagnosed carcinogenic malignancies over the world, mainly in Western countries. It can be classified as non-muscle-invasive or muscle-invasive and, although the vast majority of the new BC cases are catalogued as superficial papillary tumours at initial diagnosis, they have a high propensity to relapse and progress to muscle-invasive forms or metastatic disease, with an overall poor prognosis and undesirable outcomes, which represents a serious concern. To date, despite the association between some risk factors and disease manifestation and progression, no substantial prognostic indicators are reliably established. Therefore, the identification of predictive tools to accurately foretell the evolution of the disease and contribute for the development of novel effective therapeutic approaches constitutes a major challenge in the management of BC patients.

The EMT, in turn, has revealed itself as a process that appears to be intimately related to cancer progression and metastization. Conversion of cells with an epithelial phenotype into cells with mesenchymal traits, referred to as EMT, leads to loss of cell-cell adhesion and cell apical-basal polarity. This simultaneously promotes the acquisition of enhanced migratory and invasive properties, fruit of rearrangements in the expression of some EMT-associated markers, more precisely an upregulation and downregulation of mesenchymal and epithelial markers, respectively. Such events favour the dissemination of tumour cells undergoing EMT to outlying parts of the body as well as the subsequent formation of metastases. These cells also develop survival strategies against apoptosis and antitumor drugs, and act as CSCs.

Hereupon, this work aimed at studying the effects of the activation of the above-mentioned mechanism by exposure to TGF-β in combination with EGF on BC progression and aggressiveness, in order to better understand how these growth factors, two potential EMT promoters, act and how they affect some tumour attributes, relatively to this specific illness.

Before the experiments, activation of EMT programs was induced in the two BC cell lines used, HT-1376 and UM-UC3, both established from tumour samples of patients diagnosed with high-grade muscle-invasive BCs, by exposing them to 10 ng/mL TGF-β along with 50 ng/mL EGF for 48 h. After that, the

expression of some EMT-related markers, namely the proteins vimentin, β-catenin and N-cadherin, was firstly analysed in those BC cells, without and after EMT induction. A significant increase in vimentin expression was observed in HT-1376 cells after treatment with TGF-β in combination with EGF as compared to control. This increment is an expected result, since vimentin is an ensign strongly associated with the mesenchymal phenotype. For the UM-UC3 cells, no significant changes in vimentin expression were seen. In addition, no significant alterations in β-catenin expression were detected in neither of the cell lines. This lack of significant results might be explained by the use of total protein extracts. As described before, during EMT, β-catenin degradation is prevented, enabling its translocation to the nucleus, where it plays a role in regulating gene expression. Therefore, for better and more reliable results, nuclear protein lysates should have been used instead of total ones. Similarly, for N-cadherin expression levels, no significant changes were observed between control and treated groups, neither for the HT-1376 nor for the UM-UC3 cell line, although a tendency for an increase was observed in the UM-UC3 cell line. Moreover, despite the attempt to evaluate possible variations in the expression levels of E-cadherin (an epithelial marker) after EMT induction, this protein was not detected in any of the samples, neither in those of untreated cells nor after TGF-β and EGF exposure. This can be partially explained by the fact that the primary antibody used for protein detection might not have been the most appropriated one for these cell lines.

The recent concept that the EMT program might be a critical regulator of the CSC phenotype prompted the analysis of the expression of two stemness-related markers overexpressed in bladder CSCs, ALDH2 and SOX2, according to previous work performed by our group[300]. Our results seem to demonstrate a cell-type dependency on the expression of these two proteins. In fact, while the expression of the ALDH2 protein seems to increase in HT-1376 cells after treatment with TGF-β, the same does not apply for the UM-UC3 cell line, whose ALDH2 expression levels seem to be either identic or even decreased after exposure to TGF-β, as compared to control. Conversely, although the expression levels of SOX2 do not seem to vary for the HT-1376 cell line after incubation with TGF-β, they appear to

be greatly increased in UM-UC3 cells treated with TGF-β, comparing to control. These differences amongst the two BC cell lines could eventually be explained by the fact these cells differ in the constitutive levels of these proteins, but such hypothesis does not apply, since the values calculated for both cell lines are very similar (0.212 a.u. and 0.279 a.u. for ALDH2, and 0.389 a.u. and 0.385 a.u. for SOX2, in HT-1376 and UM-UC3 cells, respectively). Nevertheless, WB analysis for these proteins' expression was performed only once, which is by far not sufficient to take trustworthy conclusions. Additionally, aside from looking over variations in the expression levels of EMT-related proteins, it would also be very interesting to inspect the expression of some TFs implicated in EMT, as they likewise represent reliable identifiers of this phenotype. From these results, we can premise that, although full induction of EMT could not be attained with this protocol, partial activation of this transition was achieved.

Knowing that CSCs are highly resistant to conventional therapies and considered to be the responsible for tumour relapse and metastasis formation, further investigation focused on whether treatment with TFG-β and EGF alters the chemosensitivity of BC cells to CIS, which is the main chemotherapeutic drug used in the treatment of MIBC. For that, BC cells were exposed to different concentrations of CIS, without and after a 48-h incubation with the growth factors for EMT induction. Overall, no meaningful alterations were observed in the chemosensitivity of HT-1376 or UM-UC3 cells to CIS after being exposed to TGF-β and EGF, when compared to non-treated cells, suggesting that the partial activation of EMT mediated by these growth factors does not induce chemoresistance, at least in these cell lines. These results are in disagreement with other previous studies reporting enhanced drug resistance in cells that have undergone EMT in several solid tumours. Indeed, Ma *et al.* verified that hepatocellular carcinoma cells with increased oxaliplatin resistance displayed an EMT phenotype[304]. Similarly, exposure to 2 ng/mL TGF-β or 50 ng/mL IL-6 for 72 h induced EMT activation in biliary tract cancer cells and caused cells to be resistant to gemcitabine treatment[305]. Likewise, suppression of EGF-induced (50 ng/mL) EMT has been demonstrated to promote an increase in chemosensitivity of cervical cancer cells to CIS[306].

A decrease in cell proliferation due to attenuated cell cycle progression, conditioned by the activation of some EMT-associated transcription factors, has been recognized as a hallmark of cells undergoing EMT[307,308]. Actually, TGF-β was found to induce EMT in epithelial cells with concomitant inhibition of apoptosis, but without altering this growth retardation effect[309–311]. Still, our results suggest that incubation with TGF-β alone or in combination with EGF has no considerable effects on BC cell proliferation.

Since the activation of EMT programs has been linked to reduced proliferation rates, one could postulate that cells going through an EMT process have lower metabolic needs, but that is not necessarily true. As a matter of fact, Li *et al.* believe that the uncontrolled metabolic requirements together with a non-favourable microenvironment are the primary driving forces which sustain the development of EMT. Additionally, they also defend that reprogramming of the metabolic phenotype of tumour cells, by regulation of their preferred metabolic pathways, is likewise necessary for enabling EMT[303]. In the present study, the cellular uptake of ^{18}F-FDG, a glucose analogue radiotracer used in PET imaging studies, was measured. Tumour cells with high energy demands exhibit enhanced ^{18}F-FDG uptake, however, no significant differences in ^{18}F-FDG uptake were observed amongst the treated and control groups for any of the cell lines, further suggesting there is no metabolic changes occurring during exposure to TGF-β and EGF for EMT induction. Nevertheless, it is important to mention that this kind of experiment does not give information about the rearrangements within the cell metabolic axis, but merely about the levels of the cellular metabolic demands.

Lastly, BC cell migration capability without and after TGF-β and EGF exposure was evaluated. This is the most evident and unquestionable attribute of cells subjected to EMT, along with enhanced invasion. Accordingly, incubation with TGF-β alone or in combination with EGF promoted the acquisition of motile traits by both HT-1376 and UM-UC3 BC cell lines, as denoted by a wound healing assay. These results are in line with previous studies that reported increased motility of tumour cells after EMT induction, not only but also through stimulation with these specific growth factors. For example, Yu *et al.* concluded that paracrine TGF-

β signalling, promoted by cancer-associated stromal fibroblasts, contributed to the development of aggressive breast cancer phenotypes, concomitant with enhanced tumour cell migratory and invasive properties, through the induction of EMT programs[312]. Likewise, Lin *et al.* reported an increase of cell migration and invasion in tongue squamous cell carcinoma cell lines after EMT activation by exposing them to 5 ng/mL TGF-β for 48 h[313]. There are also several studies which attest the EGF as a major EMT promoter. Furthermore, and with particular interest for this work, it was recently demonstrated that treatment with 10 ng/mL TGF-β in combination with 50 ng/mL EGF leads to, and is required for, EMT-associated changes in cell migration of lung adenocarcinoma cells, which further suggests that EGF potentiates TGF-β–induced EMT[314].

Taking our results as a whole, one can generically conclude that one out of two things might have happened. Considering our peculiar observations, we can either claim that: i) the TGF-β and EGF signalling pathways do not represent main routs within those responsible for triggering and regulating EMT in BC cells, which clearly does not correlate with the huge number of studies testifying EMT induction by interfering with these specific upstream elements or with downstream components of their corresponding cascades; or ii) that it might be of a great difficulty to detect differences eventually caused by EMT events in these particular BC cell lines mainly due to their high-graded tumour origin, which ultimately means that these cells might have already undergone EMTs and are just a few steps away of leaving the primary tumour site, evading to distant parts of the body and metastizing. In fact, this theory is supported by the evidence that notorious disparities were only observed between non-treated cells and cells in which EMT had been supposedly activated during the assessment of BC cells' motility, which represents a feature matching the last stages of tumour progression and dissemination, apart from the fact that it may provide an explanation for the lack of detection of the protein E-cadherin in our samples. Moreover, there are some authors that actually classify these cell lines, at least the UM-UC3 cell line, as mesenchymal instead of epithelial[286].

Hereupon, although the concentrations of TGF-β and EGF and the

incubation time used are widely found in literature, it might be of considerable importance to test other concentrations and distinct times of exposure in order to clarify whether it is possible or not to induce EMT in HT-1376 and UM-UC3 cell lines using these growth factors. It would also be relevant to further optimize the protocol by inspecting the influence of other factors related to the experimental design used. For instance, several studies have used cells that had been serum-deprived for a certain period of time before the onset of the experiments. Besides, aside from all these considerations, and since these cells derive from high-grade BCs, it would be very interesting to perform the same kind of study in the future, but using BC cell lines derived from low-grade tumours and even cell lines of normal, healthy urothelium for comparison purposes.

Undoubtedly, EMT plays a crucial role in tumour progression and metastization, contributing to an overall poor prognosis amongst patients diagnosed with cancer. For that reason, identifying and understanding the signalling mechanisms that are responsible for triggering EMT, as well as recognizing the physiologic context and dynamic nature of this process, is pivotal for the development of novel therapeutic strategies to impede this cellular transformation to occur, resulting in better outcomes for the patients.

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