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Professor Doctor Isabel Correia Neves

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STATEMENT OF INTEGRITY

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I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

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Fator de transcrição embrionário T-box Brachyury como biomarcador de resposta e alvo terapêutico em cancros da próstata e pulmão

Resumo

A Brachyury tem sido reconhecida pelo seu papel em várias neoplasias, tais como cancro da próstata (CPa) e pulmão (CP), entre outros, devido às suas propriedades oncogénicas como por exemplo, a indução da EMT, propriedades estaminais, metastização e mau prognóstico, assim como resistência à terapia em ambos os modelos. Todavia, os mecanismos moleculares através dos quais esta proteína atua no cancro não estão completamente elucidados.

Deste modo, os objetivos fundamentais deste trabalho consistiam em elucidar o papel biológico de Brachyury em CP, estudar o seu valor preditivo na resposta a terapias antiandrógenas e a inibidores de EGFR em CPa e CP, respetivamente, bem como explorar estratégias terapêuticas anti-Brachyury. Assim, modulámos a expressão de Brachyury em linhas celulares de CPa e LC, e avaliámos o seu efeito no comportamento biológico das células, na expressão de várias moléculas já associadas com Brachyury, e na modulação da resposta das células a terapias dirigidas a AR e EGFR. Por fim, avaliámos a especificidade dos fármacos afatinib e THZ1 na modulação da Brachyury, assim como desenvolvemos ainda *drug delivery systems* (DDS) baseados em estruturas zeolíticas e sílica mesoporosa com estes fármacos.

Em CPa foi possível observar alguns resultados consistentes com uma indução de EMT parcial e uma expressão aumentada de AR após a sobre-expressão da Brachyury, no entanto, isto não se refletiu na resposta a terapias antiandrógenas. Em relação ao CP, foi possível evidenciar o seu papel oncogénico, considerando o notório aumento de viabilidade celular, migração e formação de colónias, após a sua sobre-expressão. Foram ainda observadas alterações características de uma indução de EMT parcial e um aumento em marcadores de pluripotência. É ainda importante salientar que após a sobre-expressão da Brachyury foi verificada uma menor sensibilidade ao inibidor de EGFR AST1306, na linha celular H292, que poderá ser explicada por uma sobre-activação da via AKT. Por último, demonstrarmos que os fármacos afatinib e THZ1 inibem a expressão de Brachyury em ambos os modelos, e podem ser usados em DDS.

Desta forma, concluímos que a Brachyury poderá potencialmente ser uma biomarcadora preditiva em CP, mas não em CPa. Adicionalmente, sugerimos os fármacos afatinib e THZ1 como bons candidatos para reverter o fenótipo maligno induzido pela Brachyury em cancro.

Palavras-chave: Biomarcador, Brachyury, Cancro de pulmão, Cancro da próstata, Terapia de cancro.

Embryonic T-box transcription factor Brachyury as a predictive biomarker and therapeutic target in prostate and lung cancers

Abstract

Brachyury has been recognized for its role in several cancers, such as prostate cancer (PCa) and lung cancer (LC), among others, due to its oncogenic properties such as EMT induction, stem properties, metastasis and poor prognosis, as well as resistance to therapy in both models. However, the molecular mechanisms through which this protein acts in cancer are not completely elucidated.

In this work we intended to dissect the biological role of Brachyury in LC, study the predictive value of this transcription factor in antiandrogen therapies and EGFR-TKIs respectively in PCa and LC, and finally explore therapeutic strategies for Brachyury targeting, including the usage of drug delivery systems (DDS). To accomplish that, we modulated the expression of Brachyury in two PCa and LC cell lines and performed *in vitro* biological assays, assessed by western blot and qRT-PCR the expression of a number of molecules associated with Brachyury, and evaluated its impact on the modulation of cells response to antiandrogen therapies and EGFR-TKIs. Finally, we assessed the specificity of afatinib and THZ1 drugs at targeting Brachyury expression, and proceeded to develop zeolite and mesoporous silica-based DDS.

Overall, in our PCa models we observed some results consistent with an induction of an EMT partial state and upregulated levels of AR upon Brachyury overexpression (OE), however with no effects in antiandrogenic therapies response. Concerning LC, we evidenced the oncogenic role of Brachyury in this malignancy through the increased cell viability, migration and colonies formation. Then again, we were able to note changes characteristics of EMT partial states as well as an increase in stemness markers. Importantly, upon Brachyury OE we found that the H292 cell line became more insensitive to the EGFR inhibitor, AST1306 (allitinib), that might possibly be due to an overactivation of the AKT pathway. Finally, we proved that afatinib and THZ1 are able to inhibit Brachyury expression in PCa and LC, and successfully developed DDS using these drugs.

Thus, we conclude that Brachyury might have potential as a predictive biomarker to EGFR targeted therapies in LC, but not to antiandrogens therapies in PCa. Furthermore, our results suggest that afatinib and THZ1 can be used to target Brachyury and potentially revert the oncogenic Brachyury-associated phenotype.

Keywords: Brachyury, Cancer therapy, Lung cancer, Predictive biomarker, Prostate cancer

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List of abbreviations

5-FU: 5-fluorouracil

A

ADC: Adenocarcinoma

ADT: Androgen deprivation therapy

AKT: Protein kinase B

Al: Aluminum

ALK: Anaplastic lymphoma kinase

AMACR: Alpha-methylacyl-CoA racemase

AP-1: Activator protein 1

AR: Androgen receptor

AREs: Androgen response elements

ARv: AR splice variant

ATCC: American Type Culture Collection

ATM: Ataxia telangiectasia

ATP: Adenosine triphosphate

B

BPH: Benign prostate hyperplasia

BRAF: B-Raf Proto-Oncogene, Serine/Threonine Kinase

BRCA: Breast cancer gene

BTFs: Basal transcription factors

C

CAB: Combined androgen blockade

CCL2: C-C Motif Chemokine Ligand 2

CCL4: C-C Motif Chemokine Ligand 4

CD15: 3-fucosyl-N-acetyl-lactosamine

CDD: Computer-based differential display

CDK: Cyclin dependent kinase

CDKI: Cyclin dependent kinase inhibitor

cDNA: Complementary DNA

CHC: α-cyano-4-hydroxycinnamic acid

CHEK2: Checkpoint kinase 2

ChIP-seq: Chromatin immunoprecipitation sequencing

CK: Cytokeratin

CRPC: Castration resistant prostate cancer

CSCs: Cancer stem cells

CUL1: Cullin 1

CYP17A1: Cytochrome P450 Family 17 Subfamily A Member 1

D

DBD: DNA binding domain

DDS: Drug delivery systems

DFS: Disease free survival

DHT: Dihydrotestosterone

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic acid

DRE: Digital rectal exam

DSMZ: German Collection of Microorganisms and Cell Cultures GmbH

E

EC: Extracellular

E-cadherin: Epithelial-cadherin

ECM: Extracellular matrix

EGF(R): Epidermal growth factor (receptor)

EMA: European Medicines Agency

EMT: Epithelial-to-mesenchymal transition

ErbB: Epidermal growth factor receptor family

ERK: Extracellular Signal-Regulated Kinase

F

FAK: Focal adhesion kinase

FAU: Faujasite

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

FGF(R): Fibroblast growth factor (receptor)

G

G418: Geneticin

GIST: Gastrointestinal stromal tumor

GnRH: Gonadotropin-releasing hormone

GR: Glucocorticoid receptor

H

HCC: Hepatocellular carcinoma

HER2: Human Epidermal Receptor 2

HOXB13: Homeobox B13

Hsp: Heatshock protein

I

IC_{50}: Half maximal inhibitory concentration

IGFR1: Insulin growth factor receptor 1

IHC: Immunohistochemistry

IL: Interleukin

IUPAC: International Union of Pure and Applied Chemistry

J

JXM: Juxtamembrane

K

KRAS: Kirsten rat sarcoma viral oncogene homolog

L

LBD: Ligand binding domain

LC: Lung cancer

LCC: Large cell carcinoma

LDCT: Low-dose computed tomography

LH: Luteinizing hormone

LNM: Lymph node metastasis

LUTS: Lower urinary tract symptoms

M

MAPK: Mitogen-activated protein kinase

MCM-41: Mobil Composition of Matters no 41

mCRPC: Metastatic castration resistant prostate cancer

MED1: Mediator Complex Subunit 1

MEK: Mitogen-activated protein kinase kinase

Met: Hepatocyte growth factor receptor

MET: Mesenchymal-to-epithelial transition

MHC: Major histocompatibility complex

MLH1: mutL homolog 1

MMP: Metalloproteinase

MMR: Mismatch repair

mRNA: Messenger RNA

MSH2: mutS homolog 2

MSH6: mutS homolog 6

mTOR: Mechanistic target of rapamycin

N

N-cadherin: Neural-cadherin

NE: Neuroendocrine

NEtD: Neuroendocrine transdifferentiation

NF-κβ: Nuclear transcription factor kappa beta

NRAS: Neuroblastoma RAS viral oncogene homolog

NSCLC: Non-small cell lung cancer

NTD: N-terminal domain

NTRK1: Neurotrophic Receptor Tyrosine Kinase1

O

O: Oxygen

Oct4: Octamer-binding transcription factor 4

OE: Overexpression

ORR: Objective response rate

OS: Overall survival

P

p-AKT: Phosphorylated AKT

PARP: Poly (ADP-ribose) polymerase

PBS: Phosphate Buffered Saline

PCa: Prostate cancer

PD1: Programmed cell death protein 1

p-EGFR: Phosphorylated EGFR

p-ERK: Phosphorylated ERK

PFS: Progression-free survival

PI3K: Phosphoinositide 3-kinase

PI3KCA: Phosphatidylinositol 3-kinase oncogene

PIN: Prostate intraepithelial neoplasia

PLCγ: Phosphoinositide phospholipase C

pMEK: Phosphorylated MEK

PMS2: Postmeiotic segregation increased 2

PSA: Prostate-specific antigen

PTEN: Phosphatase and tensin homolog

Q

qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction

R

RB: Retinoblastoma

RET: Ret Proto-Oncogene

RNA: Ribonucleic acid

ROS1: ROS Proto-Oncogene 1

RPMI: Roswell Park Memorial Institute

RTKs: Receptor tyrosine kinases

RT-PCR: Reserve transcription polymerase chain reaction

S

SA: Salicylic acid

SBA-15: Mesoporous Silica

SCC: Squamous cell carcinoma

SCLC: Small cell lung cancer

siRNA: Small interfering RNA

SRB: Sulforhodamine B

STAT: Signal transducer and activator of transcription

T

T: Testosterone

TBS: Tris-Buffered Saline

TBXT: Brachyury

TCA: Trichloroacetic acid

TGF-β: Transforming growth factor beta

TK: Tyrosine kinase

TKI: Tyrosine kinase inhibitor

TM: Transmembrane

TME: Tumor microenvironment

TMPRSS2: Transmembrane protease serine 2

TNF: Tumor necrosis factor

TNM: Tumor-node-metastasis

TRUS: Transrectal ultrasonography

W

WB: Western blot

Y

YAP: Yes-associated protein

YY1: Yin Yang

Y: Zeolite Y

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CHAPTER 1:

General Introduction

1.1. Cancer

Cancer is a major health problem and is the second leading cause of death worldwide, expecting to rank as the leading cause of death and the most important barrier to increase life expectancy in the 21st century. Incidence and mortality rates of this disease are rapidly growing, with 18.1 million new cases and 9.6 million cancer deaths worldwide estimated in 2018[1]. Meanwhile, in Portugal were estimated 58199 new cases and 28960 cancer related deaths[2].

Cancer is a complex disease that involves dynamic changes in the genome, including mutations that activate the so-called oncogenes and inhibit tumor suppressor genes[3]. To simplify all the complex and intricate alterations that occur during the formation of these malignant neoplasms, Hanahan and Weinberg defined in 2000 the "Hallmarks of Cancer", in other words, functional capabilities that allow cancer cells to survive, proliferate, and disseminate[3]. In 2011, the same authors redefined and expanded these Hallmarks of Cancer (Figure 1), adding four more aspects that are characteristic of this disease[4].

Figure 1. Hallmarks of cancer defined by Hanahan and Weinberg in 2011[4].

1.2. Prostate cancer

Prostate cancer (PCa) ranks as the 2nd most common diagnosed malignancy in men and is the 5th leading cause of cancer related deaths across the world. Moreover, is the most frequently diagnosed malignancy among men in over half of the countries of the world, including Portugal[1,2].

Development of PCa has some well-established risk factors, such as ethnicity, age and family history of disease[5,6]. Age is by far the strongest risk factor for PCa incidence, which rises abruptly with age, being diagnosed in very few people under 50 years (<1 % of all patients) and the mean age at the time of

diagnosis is 72-74 years old[7-9]. Overall, when PCa is diagnosed at an early age it is generally associated with a family history of disease[6-8], which might be due to a genetic factor or even environmental factors within a family. Some genes and common gene variations have been correlated with an increased risk of PCa, such as mutations in breast cancer genes 1 and 2 (*BRCA1* and *BRCA2*, respectively), in homeobox B13 (*HOXB13*) and in some DNA mismatch repair genes (MMR) namely mutL homolog 1 (*MLH1*), mutS homologs 2 and 6 (*MSH2* and *MSH6*, respectively), postmeiotic segregation increased 2 (*PMS2*) and other DNA repair genes like, checkpoint kinase 2 (*CHEK2*), and ataxia telangiectasia mutated (*ATM*)[6,7,10,11]. Finally, regarding ethnicity, there is a great heterogeneity in clinical PCa incidence worldwide, and it is known that black men have a higher risk of PCa incidence and death compared to men from white or Asian backgrounds[7].

PCa presents a number of challenges for primary care clinicians, mainly because the majority of men with PCa are asymptomatic until the tumor has progressed. Advanced PCa causes symptoms such as lower urinary tract symptoms (LUTS), erectile dysfunction and haematuria[7], which also occur in other conditions like benign prostate hyperplasia (BPH) and prostatitis, making it very challenging to distinguish between them based only on symptomatology[7]. Currently, serum prostate-specific antigen (PSA), the most clinically validated biomarker in PCa, is very used for screening, early diagnosis and for evaluate disease progression as well[12,13]. PSA, encoded by an androgen-responsive gene, is a serine protease secreted by prostate epithelial cells[14], and was first detected in the serum of PCa patients in 1980[15]. In 1986 it was approved by the Food and Drug Administration (FDA) as a biomarker for PCa progression monitoring, and as such, in the 1990's this test became widely used as a screening method with early detection as a main goal, with the consequent lowering of the mortality rates[15]. Unfortunately, PSA is not completely specific, since conditions such as BPH and prostatitis increase PSA levels[7,13], and some men with PCa also have normal PSA levels[7,14], leading to false positive and negative results. These limitations result in overdiagnosis and overtreatment, which constitute the most important adverse effects of PCa screening, being these events vastly more common than in screening for breast, colorectal, or cervical cancer[12,16]. In spite of these disadvantages, PSA is still a useful biomarker of PCa, but new ones are in need for diagnosis and to predict PCa progression, as well as for distinguish between clinically significant from indolent tumors. In despite of the aforementioned limitations of PSA testing, when a patient has a suspicious high level of PSA it is recommended a digital rectal exam (DRE), and then a transrectal ultrasonography (TRUS) guided biopsy, which remains the standard tool for PCa diagnosis[17].

Depending on the cell of origin, which is still a controversial topic and an area of active investigation[18-20], prostatic tumors can be grossly classified as adenocarcinomas (ADC), squamous carcinomas and neuroendocrine (NE) tumors[19] (Figure 2), being the vast majority of prostate tumors ADC[21]. Despite the existence of this classification, prostate tumors are normally multifocal, having multiple independent histologic *foci* that are often genetically distinct[19]. Prognostication and treatment stratification at the time of diagnosis are based on serum PSA levels, clinical stage (TNM system), and Gleason score, which is a widely used grading system based on histological patterns of the tumor, originally defined by the pathologist Donald Gleason[17,22]. Depending on the stage and grade of the tumor, treatment for PCa may involve watchful waiting/active surveillance, surgery, cryosurgery, radiotherapy, brachytherapy, chemotherapy, hormonal therapy, or combinations. In the case of localized disease, the therapeutic approach consists in watchful waiting/active surveillance for low risk tumors[16,23,24], and surgery and radiotherapy for intermediate and high risk tumors[5,25-27]. However, for advanced PCa the first line of treatment is hormonal therapy[26,28].

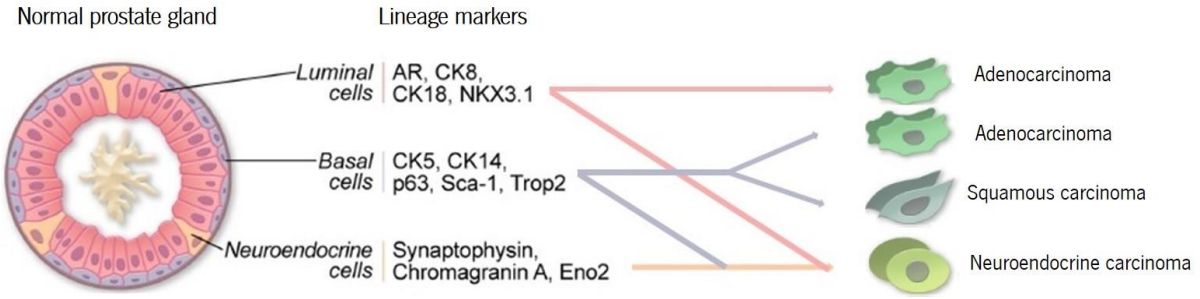

Figure 2. Prostate epithelium characterization and origin of the different PCa histological subtypes. Luminal, basal and NE cells, all of which express a specific panel of characteristic proteins, comprise the normal prostate epithelium: luminal cells are defined by expression of cytokeratin (CK) 8, CK18 and androgen receptor (AR); basal cells express high levels of CK5, p63 and very low levels of AR; NE cells, the smallest population, express NE markers such as synaptophysin and chromogranin A and do not express AR. Studies have demonstrated that both luminal cells and basal cells can serve as the cell of origin for PCa; both basal and luminal cells can give rise to ADC, but only basal cells give rise to squamous carcinoma. It remains unknown whether NE cells can be transformed to generate a malignant neoplasm. Adapted from[19].

1.2.1. Antiandrogen therapies

Hormonal therapy arose due to the unraveled role of androgens in PCa, conducted by Charles Huggins[29]. He demonstrated that the reduction of testosterone levels, by surgical castration, had therapeutic implications in patients with metastatic PCa, awarding him with the Nobel Prize of Medicine

in 1966. Androgens and their receptor, androgen receptor (AR), play a pivotal role in PCa development and progression, and the underlying rationale of hormonal therapy is the blockade of their action[30,31].

AR is a type I hormone receptor that belongs to the nuclear receptors superfamily[32,33], which besides being a key factor in PCa it also plays a role in the development and maintenance of the reproductive, musculoskeletal, cardiovascular, immune, neural, and hemopoietic systems[34], and is implicated in other cancer types such as breast, ovarian and pancreatic cancers[35-38]. Structurally, the AR, as the other nuclear receptors, is comprised by several domains, each one with a specific function: a N-terminal domain (NTD), with an activation function, being also responsible for receptor dimerization through interaction with the C-terminal ligand binding domain (LBD); and a hinge region that connects the DNA biding domain (DBD) and the LBD[32,33] (Figure 3A). In the basal unbound state, the AR is located in the cytoplasm and bound to heatshock proteins (Hsp90, Hsp70), and other chaperone proteins in a conformation that prevents DNA binding[32,34,39] (Figure 3B). The androgens testosterone and dihydrotestosterone (DHT) bind to the LBD of AR, displacing the Hsp and promoting an interaction between the NTD and LBD of the receptor. AR is further translocated into the nucleus, where it dimerizes and bind to androgen response elements (AREs) in the promoter regions of target genes, such as *PSA*, *transmembrane protease serine 2* (*TMPRSS2*), etc[32,40,41]. This results in the recruitment of various coregulators to enhance or repress transcription[42], activating the transcriptional program of AR and amplifying the signal initiated by hormone binding, that leads to biological responses like growth and survival[32,40] (Figure 3B). This constitutes the known genomic functions of AR, however a growing body of evidence suggests that this receptor also has non-genomic functions, that occur in a short time frame being incompatible with the activation of the AR transcriptional program[38,43,44]. Several studies using cell lines reported that upon ligand binding, AR is able to interact with cytoplasmic proteins, such as the non-receptor tyrosine-kinase Src and phosphoinositide 3-kinase (PI3K) leading to the activation of the mitogen-activated protein kinase (MAPK) and PI3K/Akt/mTOR signaling pathways[45,46], respectively (Figure 3B). These non-genomic functions demonstrate that AR plays a much more complex role than first thought.

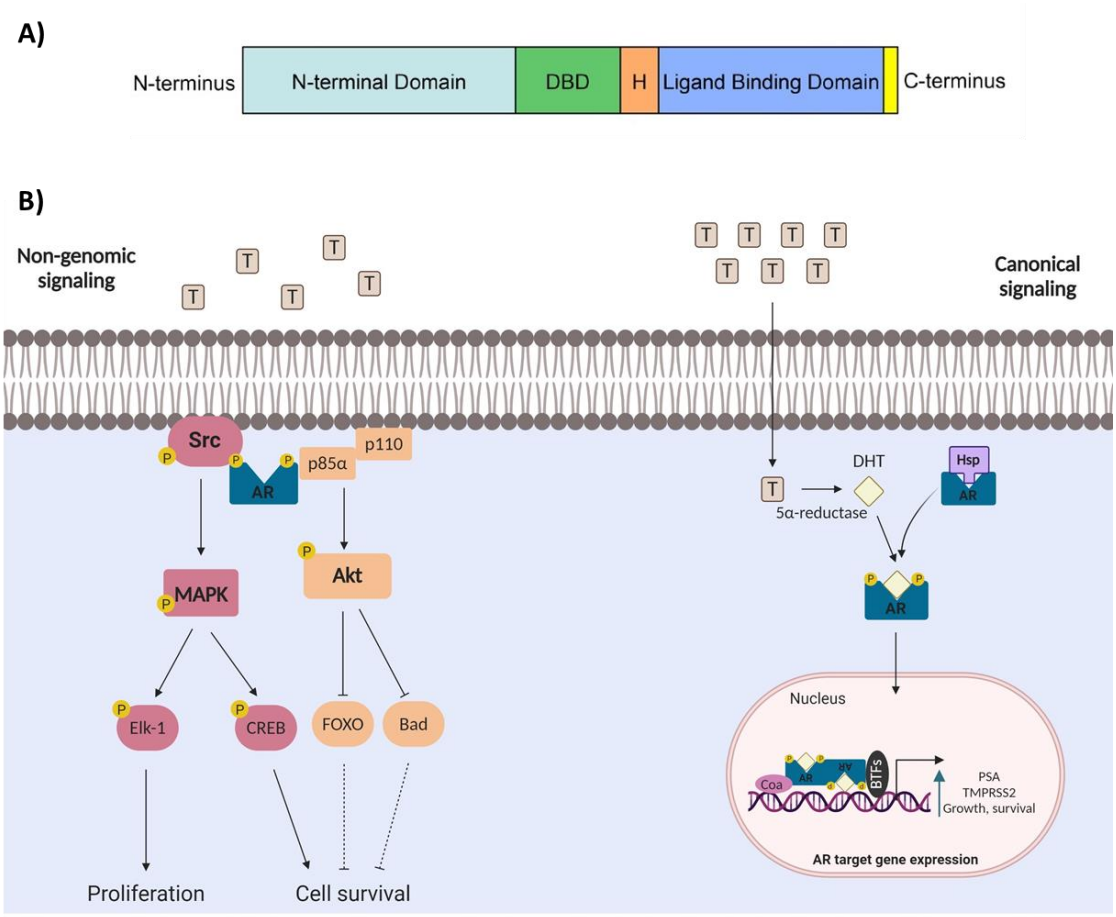

Figure 3. AR structure domains and respective signaling. A) AR is comprised by a N-terminal domain (NTD), a DNA binding domain (DBD), a C-terminal ligand binding domain (LBD) and a hinge region (H) that connects the DBD with the LBD. Retrived from [34]. B) AR has a non-genomic function and is able to interact with cytoplasmic proteins, such as the non-receptor tyrosine-kinase Src and the p85α regulatory subunit of PI3K leading to the activation of the MAPK/ERK and PI3K/Akt/mTOR signaling pathways, respectively, and culminating in increased proliferation and cell survival. Concerning AR genomic function, testosterone enters the cells, the majority being converted into dihydrotestosterone (DHT) by the 5α-reductase enzyme, DHT then binds to AR, displacing Hsp and entering the nucleus. At the nucleus AR dimerizes, interacts with the promoter regions of androgen responsive genes and recruits the basal transcription machinery (BTFs) and coactivators regulating the expression of target genes, such as *PSA*, *TMPRSS2*, etc. Adapted from [43].

Inhibition of androgen signaling can be achieved through two main tactics, the inhibition of testosterone production by the testis and adrenal gland, and the direct targeting of the AR. The decreased levels of circulating androgens with the following decline in AR activation and it's signaling is the rationale underlying androgen deprivation therapy (ADT), the gold standard treatment for PCa[47]. This can be achieved by castration either surgical (orchiectomy) or chemically, acting in two crucial molecules that control androgen synthesis, luteinizing hormone (LH) and gonadotropin-releasing hormone (GnRH), using for that GnRH (also known as LH releasing hormone (LHRH)) receptor agonists and antagonists[32]. GnRH agonists desensitize the GnRH receptor by interrupting its physiological intermittent stimulation, whereas

the GnRH antagonist blocks directly the stimulation of the receptor[32,48], resulting in a decrease of serum testosterone levels, being reported that more than 80-90% of patients show a positive response to androgen ablation[9,30,32].

The class of drugs that directly target AR, through binding to the LBD and competing with androgens in order to block receptor activation, are denominated antiandrogens or AR antagonists[49]. Antiandrogens are mostly often used in combination with ADT, being this therapeutic approach known as combined androgen blockade (CAB)[32]. Flutamide was the first nonsteroidal antiandrogen to be clinically approved by the FDA in 1989 for use in advanced PCa. Few years later, other derivatives of flutamide, namely nilutamide and bicalutamide were also approved, and altogether comprise what is called the 1st generation of antiandrogens[32,49]. These AR antagonists possess relatively weak affinity for the AR and are not potent enough to completely block AR signaling. The patients often develop resistant mutations, which normally converts them into AR agonists[49,50], or other resistance mechanisms, in a time frame of 14-20 months[28]. When patients no longer respond to ADT and/or CAB, cancer progresses even under castrate levels of testosterone, being this disease now classified as castration resistant prostate cancer (CRPC) with a median survival of 2.8 years[28,32,51]. With this in mind, a lot of efforts were made, and a 2nd generation of antiandrogens was developed. Enzalutamide was the first AR antagonist of this generation to be approved in 2012 and represented a breakthrough in CRPC treatment due to its properties and effectiveness[52,53]. Enzalutamide binds to AR with strong affinity, prevents AR nuclear translocation and DNA binding, and led to an improvement on progression of the cancer and in overall survival (OS)[52,53]. Other 2nd generation antiandrogens, namely apalutamide and darolutamide, were approved for non- and metastatic CRPC (mCRPC) treatment by the FDA in 2018 and 2019, respectively, being subsequently approved by the European Medicines Agency (EMA)[49,54-56]. Furthermore, the androgen synthesis inhibitor abiraterone was developed and approved just before enzalutamide[57,58]. This specific drug inhibits the CYP17A1 enzyme that is involved in androgen biosynthesis, preventing this way androgen production in the adrenal glands and in the tumor itself, being reported to result in a survival benefit in CRPC patients[57]. These two former drugs, abiraterone and enzalutamide, were at the time of their development major breakthroughs for the lethal CRPC, that until then was limited only to the chemotherapeutics docetaxel and cabazitaxel[26,27,32,59].

Unfortunately, development of resistance to these newer and improved antiandrogens eventually happens leaving CRPC patients with restricted therapeutic options[60]. More recently, some advances were made with Poly (ADP-ribose) polymerase (PARP) inhibitors, namely rucaparib and olaparib, and anti-Programmed cell death protein 1 (PD1) inhibitor, pembrolizumab, that were approved for a subset of

these patients[61,62]. Several mechanisms that confer resistance were uncovered and comprise two main types, AR- dependent and independent mechanisms. Initially it was thought that CRPC was completely independent of AR signaling, however it was demonstrated that in the majority of the cases, CRPC had restored AR signaling which was confirmed by efficacy of the 2nd generation antiandrogens for its treatment[63].

Mechanisms that restore AR signaling after ADT/CAB treatment are the most common, and comprise AR amplification/overexpression, intratumoral androgen synthesis, AR mutations, ligand independent AR activation, aberrant expression of AR coregulators, expression of AR splice variants (ARvs), and glucocorticoid receptor (GR) upregulation[30,32,40,51,64]. This plethora of events highlight the importance of AR in PCa carcinogenesis, aggressiveness and metastasis.

As mentioned before, CRPC can be totally independent of AR signaling, being the activation of receptor tyrosine kinases (RTKs) and common oncogenic pathways[65,66], the epithelial-mesenchymal transition (EMT), acquisition of stem cell properties and neuroendocrine transdifferentiation (NEtD) examples of this kind of mechanisms that overall demonstrate the high plasticity of PCa cells[64,67]. The concept of EMT was first introduced in 1968 by Elizabeth Hay, that later in 1995 described and defined EMT as a reversible developmental process during which epithelial cells are converted into invasive mesenchymal cells[68] (Figure 4), being also involved in processes like inflammation, wound healing and tissue regeneration[69,70]. More recently, EMT has been identified as a crucial event in cancer invasion and metastasis [4,70], being associated with therapy resistance and cancer stem cells (CSCs) that altogether contribute to a more aggressive state of disease[71,72]. During this complex process, epithelial tumor cells lose the expression of proteins involved in cell-to-cell adhesion, such as Epithelial-cadherin (E-cadherin) and integrins, and gain expression of proteins typically associated with mesenchymal cells, including Fibronectin, Neural-cadherin (N-cadherin), and Vimentin[69,70]. The phenotypic switch results in enhanced tumor cell motility and invasiveness and, as a consequence, tumor cells undergoing EMT are thought to be able to detach from the primary tumor and to initiate the cascade of events leading to the establishment of metastasis[4,69,70]. Regarding the control of this process, it is known that some transcription factors, usually referred as key masters of EMT, like Snail, Slug, Twist1, and Zeb1/2 are activated to promote the molecular changes that occur during the EMT program[69,70,73-75] (Figure 4). These transcription factors are in turn controlled by signaling pathways such as nuclear transcription factor κB (NF-κβ), Wnt, Notch, Hedgehog, Activator protein 1 (AP-1), and growth factor signaling, that converge at the level of the aforementioned transcription factors[69,70]. In the specific case of PCa, it was shown that ADT promotes EMT

in animal models as well as in tumor samples of patients after hormonal therapy[71,76]. Additionally, the previously mentioned ARvs that emerge as an adaptive response to therapy and are increased in CRPC cases, have a regulatory role in EMT as well[77]. Another interesting aspect of EMT is that it seems to promote the acquisition of stem cell properties by tumor cells[72,78]. This capacity of the EMT program to generate what some authors affirm to be CSCs[72] poses several threats, namely increased migration and invasion, the capacity to form new tumors and the intrinsic resistance to conventional therapies demonstrated by this kind of cells, which ultimately results in cancer metastasis and relapse[79,80].

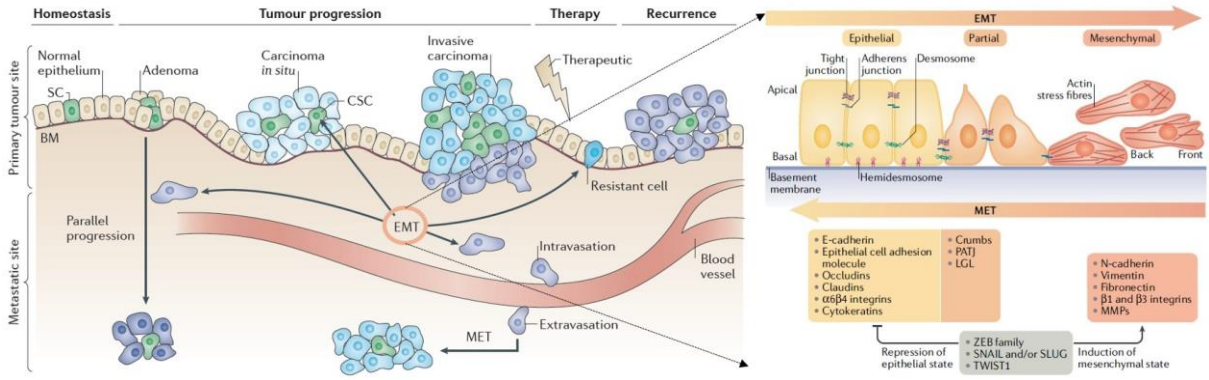

Figure 4. Schematic representation of EMT at a molecular level and its role in cancer progression. Induction of EMT leads to the expression of specific transcription factors, such as ZEB, Snail, Slug and Twist, which result in the decrease of epithelial markers and in the increase of mesenchymal ones, leading to the disassembly of epithelial cell–cell junctions and of apical–basal cell ultimately resulting in higher motile and invasion capabilities. Cancer cells undergoing EMT can disseminate from the primary tumor site, migrate into a new location in the body, revert to the epithelial state by undergoing mesenchymal-to-epithelial transition (MET) forming a metastatic lesion. EMT has also been associated with stemness features and therapy resistance, leading to recurrence and a poor prognosis. Adapted from [70,81].

Another mechanism associated with therapy resistance in PCa is the NEtD process[64,67,82], that occurs in about of 17-25% of patients with mCRPC[83,84]. Various studies demonstrated that high grade and high stage prostate tumors, specially CRPC, possess a NE phenotype that is associated with poor prognosis[64,82,84,85]. There is some doubt about the origin of these kind of tumors, but increasing evidence suggests that originate from ADC cells rather than being a *de novo* secondary tumor, meaning that cancer cells undergo NEtD, acquiring a similar phenotype of the normal NE prostate cells[82,85], which is supported by the fact that these NE tumor cells exhibit the same genetic profile as the non-NE cancer cells[82,85,86]. Tumor cells that had undergone NEtD become more elongated with a neuron-like phenotype, gain cytoplasmic secretory granules, undergo growth arrest, lose AR expression and express higher levels of NE markers such as synaptophysin, chromogranin A, neuron specific enolase (NSE), gastrin and neurotensin[82,84-87]. In the specific case of PCa it has been shown that patients who had received a longer

course of ADT have higher levels of NE PCa cells and increased serum levels of some NE markers as well, suggesting that ADT promotes NEtD[82,83,85,88]. This process has been observed in both *in vitro* and *in vivo* studies, after the removal of androgens [82,85,88], and it was also noticed that NEtD could be reverted upon reestablishment of androgens levels[82,89]. Regarding the underlying mechanisms of this phenotypic switch little is uncovered, but it was reported that TP53, RB1 and PTEN play key roles in this process[64,83,84,90].

With all of these in mind, it is clear that CRPC poses a serious threat for PCa patients reinforcing the current need for novel improved and specific biomarkers, for early diagnosis purposes as well as to predict therapy resistance, and therapeutic targets that altogether can improve the clinical progression of these patients.

1.3. Lung cancer

Lung cancer (LC) is a major concern in our society nowadays, being the leading cause of cancer incidence and mortality worldwide[1], with rates that are still rising[1,91]. Specifically in Portugal, despite LC being only the 4th most common cancer, is still the deadliest malignancy accounting for 16.1% of all cancer related deaths in 2018[2].

There are various risk factors for the development of LC such as tobacco smoking/exposure (secondhand smoking), radon, asbestos, biomass fumes, air pollution, diet, among others. Tobacco consumption is a well-known and the major risk factor for LC[92-94] and the patterns of tobacco consumption and cessation, as well as gender related patterns, are reflected in LC rate trends across the world[93,94].

LC is mostly often diagnosed in late stages since early stage tumors are normally asymptomatic, having this a tremendous impact in OS and in accordance, LC has the lowest 5-year survival rate, 19.4% in 2019, compared to other malignancies[92-94]. Unlike other types of cancer (breast, colorectal, cervical, etc.), LC doesn't have yet a strong implemented screening method that would be helpful to circumvent these late stage diagnoses and in consequence improve OS. Indeed, several efforts are being made in the research of screening methods and studies on using low-dose computed tomography (LDCT) as a screening method in high-risk population showed an increased benefit in LC mortality[94,95], being already applied in the US and implemented in the UK[96-99].

In resemblance to other types of cancer, including PCa, biopsy and histopathological evaluation by a pathologist is still the mainstay diagnostic approach. LC is a complex and heterogenous disease at a histological and molecular levels being these tumors divided into two major types, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC)[100]. NSCLC comprises about 85% of all lung tumors and

are further classified as adenocarcinoma (ADC), squamous cell carcinoma (SCC) and large cell carcinoma (LCC)[100,101]. ADC is the most common subtype of NSCLC comprising about 38.5% of all lung cancers, followed by SCC (20-30% of all LCs) and LCC (3-9%)[100,102]. As mentioned above, tobacco is the major risk factor, however is more associated with SCLC and SCC rather than ADC which besides being the most common histological subtype is also more prevalent in non-smokers[101].

Treatment of LC is chosen based on stage, subtype and genetic profiling and comprise surgery, radiotherapy, chemotherapy, targeted therapy and immunotherapies[101,103]. For early stage, localized LC, surgery and/or adjuvant therapy (chemo and radiotherapies) are the gold standard treatment, and for advanced stages (the majority of LC cases) platinum-based chemotherapy used to be the 1st line of therapy[101,103]. Due to major advances in research it was perceived that a great deal of lung tumors is molecularly driven and are referred as "oncogene addicted". "Oncogene addiction" refers to the phenomenon where tumors are dependent on a single oncogenic protein or pathway to maintain their malignant properties[104,105]. This can be exploited for treatment purposes, since a great clinical response is attained when the oncogene or its downstream signaling pathway is effectively blocked by specific small molecule inhibitors, and as a matter of fact LC is one of the best examples for this.

In the past decades, several molecular alterations and oncogenic drivers were identified in NSCLC and the corresponding molecular targeted therapies were developed or are currently under development and evaluation (summarized in Figure 5), and have revolutionized LC treatment and improved survival[101,105-110]. Most of these genetic alterations involve RTKs, the oxidative response and cell cycle regulation and in light of these discoveries it is now clinically routine for NSCLC patients, especially those with ADC, to be profiled for molecular aberrations in *EGFR*, *KRAS*, *ALK*, *ROS1* and *BRAF*[95,101,105,110] in order to choose the most appropriate therapeutic regimen. About 69% of NSCLC harbor driver oncogenes[107,111], being the most common KRAS, EGFR, and ALK, which are almost exclusive to ADC being rare in SCC[101,110,112].

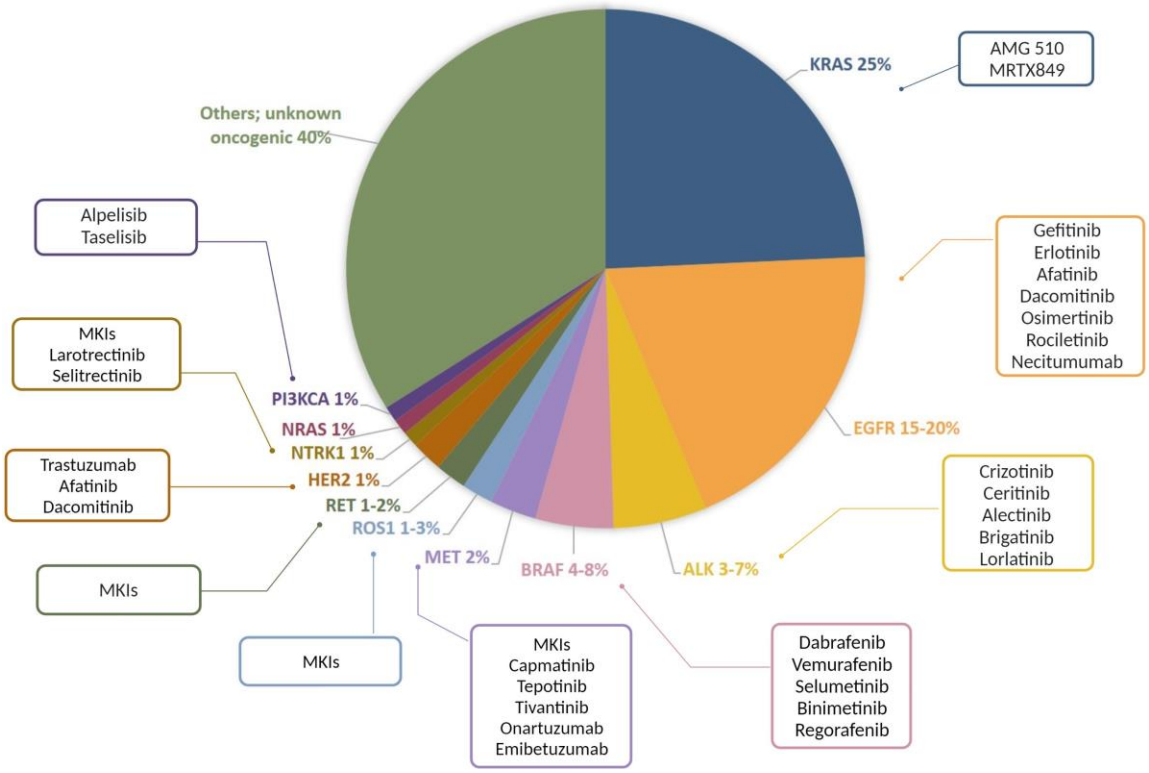

Figure 5. Driver oncogenes in NSCLC patients and respective targeted therapies. It is worth to mention that these are median percentages that vary between populations of patients. Mutations in any of these genes sensitize the affected patients to therapies targeting the respective oncogene being considered predictive biomarkers of response. In this figure are summarized clinical approved therapies and some that are currently under development or clinical evaluation. For some of these oncogenic drivers specific targeted therapies were not yet developed, nonetheless, some multikinase inhibitors (MKIs) can be used to target various targets with kinase activity. For example, due to similarity between proteins, the therapies for ALK-targeting presented here can also be used to target MET, RET and ROS1. Information retrieved from [101,105,107-109,113,114].

1.3.1. Molecular targeted therapies: anti-EGFR therapies

Followed by KRAS, the 2nd most common oncogenic driver in NSCLC is EGFR, that is mutated in about 10-20% of Caucasian patients and in 30-50% of Asian patients, being more prevalent in women and never smokers[100,107,115]. The *EGFR* gene is located on the short arm of chromosome 7 at position 12[100,116] and encodes a type I RTK that belongs to the epidermal growth factor receptor (ErbB) family consisting of 4 different proteins, namely ErbB/HER1-4[117]. In structural terms, EGFR possesses an intracellular region (C-terminus) with a short juxtamembrane segment, a protein kinase domain, and a long carboxyterminal tail; an extracellular region (N-terminus) where the binding of the ligands occurs and is divided in four parts (domains I-IV); and a hydrophobic transmembrane region[117,118] (Figure 6). EGFR possesses seven ligands, being the most known the epidermal growth factor (EGF), that when bound at

the extracellular domain induces dimerization with another EGFR molecule (homodimerization) or even with other ErbB family members like HER2 (heterodimerization)[117,118]; this in turn causes the activation of the tyrosine kinase domain resulting in the autophosphorylation of tyrosine residues in the intracellular domain[117], ultimately leading to the activation of several signaling pathways such as RAS-RAF-MEK-ERK, PI3K-AKT-mTOR, STAT, PLCγ and SRC that regulate cell proliferation, differentiation and apoptosis[117,119] (Figure 6).

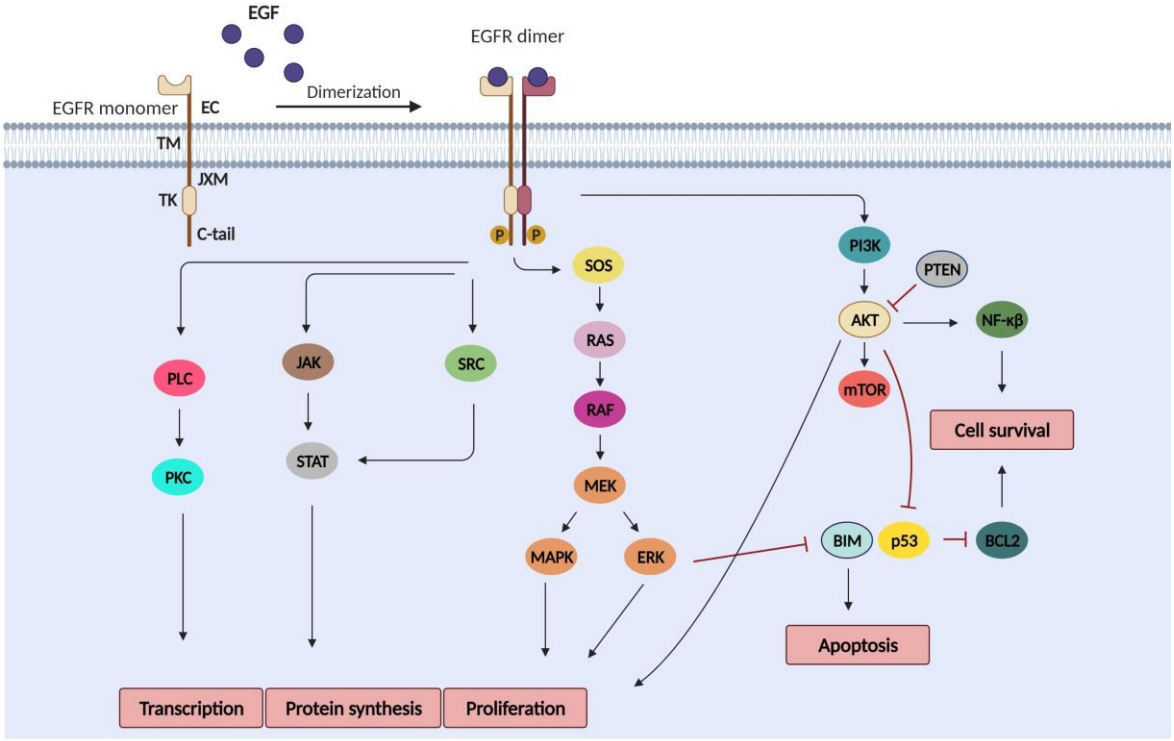

Figure 6. EGFR structure and signaling. As other RTKs, EGFR is structurally comprised by an extracellular domain (EC) where the ligand binds, a hydrophobic transmembrane region (TM), a juxtamembrane segment (JXM) and a tyrosine kinase domain (TK) with a carboxyterminal tail (C-tail). Upon binding of a ligand, normally EGF, the receptor dimerizes with other EGFR monomer or other ErbB monomer, activating the TK domain, which results in autophosphorylation of tyrosine residues in the intracellular region and activation of several signaling pathways, ultimately resulting in responses such as transcription and protein synthesis, proliferation, apoptosis, differentiation, etc. Adapted from [119].

In the specific case of NSCLC, especially in ADC, several *EGFR* mutations were already identified occurring almost exclusively in the tyrosine kinase domain: the most common are deletions in exon 19 (del19) and a point mutation in exon 21 L858R (substitution of leucine for arginine in codon 858) that comprise about 80-85% of all *EGFR* mutations in lung ADC[101,120,121]; less common mutations (<15%) comprise exon 20 and 19 insertions, point mutations in exons 18 (G719X) and 21 (L861X)[101,120,122]. Those mutations lead to hyperactivation of downstream oncogenic signaling pathways and consequently

increase of tumor cell proliferation and survival[105,123,124], but fortunately, the abovementioned del19 and L858R *EGFR* mutations for example, sensitize most patients to EGFR tyrosine kinase inhibitor (TKI) treatment, being denominated "activating" or "sensitizing" mutations. This was the driving force that led to the development of small molecule inhibitors that specifically target EGFR.

Currently, in the clinic there are three generations of EGFR TKIs approved by FDA and EMA, for 1st and 2nd lines of NSCLC treatment (Table 1). Gefitinib and erlotinib are the 1st generation of reversible EGFR inhibitors and represented major breakthroughs in *EGFR* mutant NSCLC treatment, since were demonstrated to be superior to classical chemotherapy (platinum-based drugs) with improved safety profiles, objective response rate (ORR) and progression-free survival (PFS)[125,126], however, both erlotinib and gefitinib did not improved OS compared to platinum-based chemotherapies[127]. Second generation EGFR TKIs, namely afatinib and dacomitinib, are pan-ErbB inhibitors and inhibit EGFR irreversibly[101]. In clinical trials both afatinib and dacomitinib demonstrated an improvement in PFS and ORR compared to platinum-based chemotherapy and gefitinib, granting their approval by the FDA and EMA as 1st line treatment and in the case of afatinib also as 2nd line, for NSCLC patients with sensitizing *EGFR* mutations[128-130].

Despite the tremendous impact these drugs had on *EGFR* mutant NSCLC patients, virtually all patients develop resistance in approximately 10-14 months[101,116]. Currently, several mechanisms behind this resistance were described, being the most common the development of the secondary T790M (substitution of threonine for methionine at codon 790) mutation in exon 20 of EGFR, that occurs in 50-60% of patients treated with the 1st and 2nd generation EGFR TKIs[100,101,107,121,123,131]. In this sense a 3rd generation of EGFR TKIs was developed to specifically target this mutation and overcome resistance, and among them is osimertinib that demonstrated striking properties to irreversibly and effectively target the resistance T790M mutation while sparing wild-type (WT) *EGFR*[32]. Studies demonstrated that osimertinib in a 2nd line therapy setting had a response rate of 62.6% and PFS of 12 months (in 52% of patients)[133] and it is also superior to platinum-based chemotherapy for the subset of patients who progressed after EGFR TKIs treatment and developed the T790M mutation[134]. After the FLAURA clinical trial in 2018 and given its superiority to gefitinib and erlotinib, osimertinib was also approved as 1st line treatment for NSCLC patients with *EGFR* activating mutations[135] (Table 1).

Table 1. FDA and EMA approved EGFR inhibitors for LC.[118,136,137]

Gen	Name (trade name)	Therapeutic setting	Approval
1st	**Gefitinib** (Iressa)	2nd line for unselected NSCLC patients	2003 (FDA)
		1st line for patients with *EGFR* mutant L858R or del19	2015 (FDA)/2009 (EMA)
	Erlotinib (Tarceva)	2nd line for unselected NSCLC patients	2004 (FDA)
		1st line for patients with *EGFR* mutant L858R or del19	2011 (FDA)/2005 (EMA)
2nd	**Afatinib** (Giotrif)	1st line for patients with *EGFR* mutant L858R or del19	2011 (FDA)/2013 (EMA)
		2nd line for patients with SCC who progressed after CT	2016 (FDA)
	Dacomitinib (Vizimpro)	1st line for patients with *EGFR* mutant L858R or del19	2018 (FDA)/2019 (EMA)
3rd	**Osimertinib** (Tagrisso)	2nd line for patients with T790M *EGFR* mutation	2015 (FDA)/2016 (EMA)
		1st line for patients with *EGFR* mutant L858R or del19	2018 (FDA and EMA)

Gen: generation; CT: Chemotherapy

In addition, some tumors may undertake different resistance mechanisms that do not involve EGFR, the so-called "off target" resistance mechanisms, such as bypass mechanisms, mutations in downstream effectors of important signaling pathways and histological transformation. Bypass mechanisms are essentially a way for tumors to compensate the inhibitory effect of EGFR TKIs in the downstream signaling pathways to continue to drive tumor development, surpassing this way the initial oncogene dependence[136,138], and some examples comprise MET amplification[139-142], *ALK* fusions such as EML4-ALK fusion[143]; *BRAF* and *KRAS* mutations and fusions[109,141,144-146]. Histologic transformation, namely EMT, squamous and SCLC transformation, has been reported in NSCLC patients who have progressed after EGFR TKIs treatment[67,136,138,142,147-150]. Similarly to PCa, these mechanisms are less common, but represent a more clinic aggressive behavior, difficult to target, being responsible for a significant amount of the unavoidable patient relapses[67], and are also reported alongside with some bypass signaling mechanisms, such as overexpression of AXL, IGFR1 and FGFR[151].

Even though the therapies discussed here represented major advances and revolutionized the concept of personalized medicine in the clinic, the abovementioned resistance mechanisms are an obstacle to achieve curative results. With this being stated, novel predictive biomarkers and therapeutic targets are an imperative unmet need, being an area of active research.

1.4. Embryonic T-box transcription factor Brachyury

Transcription factors are defined as proteins that bind to DNA in a sequence-specific manner and are capable of regulate gene transcription. Given to this function, transcription factors are key elements in controlling biological processes, determining how cells function and respond to environmental cues, and their dysregulation is implicated in several diseases including cancer[152].

The ancient T-box family of transcription factors shares a highly conserved DNA binding domain, known as T-box domain, and the members of this family play a key role in embryonic development which is highlighted by the fact that mutations in these genes are associated with anatomical abnormalities and drastic embryonic phenotypes[153]. *Brachyury* (or *TBXT*) is the founding member of this ancient family and in 1927[154] it was found that mutations in this gene in mice resulted in dead *in utero* in null homozygotes and in a short tail phenotype in heterozygotes[155]. Only later, after cloning and sequencing it was classified as a DNA-binding protein[156-158], the T-domain, was unraveled[157] and since these discoveries several Brachyury orthologues in other species were identified, including in humans[159-162].

The human *TBXT* gene is located on chromosome 6q27 and consists of eight exons that spans 10 kb, and its open reading frame encodes a protein of 435 amino acids that shares 91% identity with the mouse ortholog[162]. Brachyury has a pivotal conserved role in embryonic development of vertebrates, specifically in cell movements during gastrulation, mesoderm formation and differentiation, notochord development in chordates and even plays a role in extraembryonic tissues (Figure 7), like the allantois that ultimately gives rise to the umbilical cord[162-167], explaining this way why homozygous mutants die *in utero*. At an early embryonic phase Brachyury is expressed in mesoderm precursors in the primitive streak during gastrulation and later becomes restricted to the notochord, that ultimately develops into the vertebral column in vertebrates[163,164,168]. These important functions in the proper embryo development were discovered mainly through studies with mutant organisms and loss- and gain-of-function experiments and in accordance with the mutant phenotype firstly described in mice, similar phenotypes were observed in other vertebrates as well, demonstrating in fact the conserved role in embryonic development of Brachyury[169-172].

Later it was found that genetic aberrations in human *Brachyury* are associated with spinal cord defects such as sacral agenesis[173], congenital scoliosis[174], neural tube defects and spina bifida[175], and also chordoma[176-180], which is a rare bone tumor that is thought to arise from notochord remnants[176,180].

1.4.1. Brachyury and cancer

As mentioned above, genetic alterations in the *Brachyury* gene, such as single nucleotide polymorphisms (SNPs) and gene duplications, are associated with an increase susceptibility to develop chordoma and are often detected in familial and sporadic tumors of this kind. Moreover, Brachyury seems to be expressed in virtually all chordoma patients, having an oncogenic role in this notochord-derived tumor and is considered part of the molecular identity of chordomas, being used as a specific biomarker for diagnostic purposes[176,181-183].

The association between chordoma and Brachyury was the first link suggesting and supporting an implication of this molecule in cancer, drawing attention of scientists to further study Brachyury in contexts other than embryonic development. One of the first studies that reported an association between Brachyury and cancer was conducted by Palena and collaborators, that using a computer-based differential display (CDD) analysis tool demonstrated that Brachyury is expressed in several carcinomas and is undetectable in most human normal tissues, which was then confirmed at an mRNA level[184]. In fact, since then a multitude of studies reported this tumor associated pattern of Brachyury, at mRNA and protein levels, being undetectable in most normal tissues, except for testis, thyroid and a few small cell populations detected in some studies[185-189], and overexpressed in several malignancies that affect the lung, esophagus, stomach, small intestine, kidney, bladder, uterus, testis, breast, and prostate[185-197], and cancer cell lines[184-186,189,190,197-199]. Through several studies, Brachyury was strongly associated with oncogenic properties, for instance viability, proliferation, migration and invasion *in vitro* and metastasis formation *in vivo*[187,189,190,195,196,200-205]. Furthermore, Brachyury was correlated with various clinical aspects namely, tumor grade, stage of disease, recurrence and distant metastasis[185-187,189,190,193-196,206-208], and shown to be a predictor of poor prognosis in a number of neoplasms such as hepatocellular carcinoma (HCC), gastrointestinal stromal tumors (GISTs), high-risk testicular germ cell tumors, oral squamous cell carcinoma, colorectal, breast, lung and prostate cancers[185-187,189-194,207,209,210]. Moreover, Brachyury expression was not only detected in primary tumors but also in metastatic tissue[200,209], and a limited number of studies reported that Brachyury expression is even higher in metastasis than in the primary tumor[186,190,210], which points Brachyury as a key player in metastization.

These reports gave insight on Brachyury's role in cancer and prompted a greater interest of research in this molecule in the oncology field. One of these first studies, conducted by Fernando et. al[195], demonstrated in lung and pancreatic cancer cell lines that Brachyury is an EMT inducer given that its overexpression increased the expression of mesenchymal markers (Snail, Slug, Vimentin, N-cadherin, etc.), decreased the expression of epithelial ones (E-cadherin, Plakoglobin, ZO1, etc.) and in accordance the migratory and metastasis formation capacities were also increased. These interesting events were further corroborated by other studies in different models, namely HCC, adenoid cystic carcinoma, colorectal, breast, lung and prostate cancers[186,187,189,190,196,197,204,207,208,211] *in vitro* and more importantly, in a clinical context through tumor tissue analysis[189,190,193,205,210,211]. Another interest aspect of Brachyury in cancer is its association with stemness: Brachyury overexpression results in an increased self-renewal capacity, measured by tummorsphere formation *in vitro* and the extreme limiting dilution assay *in vivo*, and in the expression of stem cell and pluripotency markers Nanog, Oct4, Sox2, Nestin and other CSCs

markers[186,200,211,212]. Both EMT and stemness are intimately related to therapy resistance, and accordingly Brachyury correlates with therapy resistance to a number of drugs, namely cisplatin, docetaxel, cabazitaxel, tamoxifen, vinorelbine, and radiation as well in various cancer models[186,187,198,203-205,213,214].

In order to understand how Brachyury functions, several researchers investigated the downstream targets of this transcription factor in mesoderm formation[215-217], however how and through which downstream targets Brachyury mediates its effects in cancer is less understood (Figure 7).

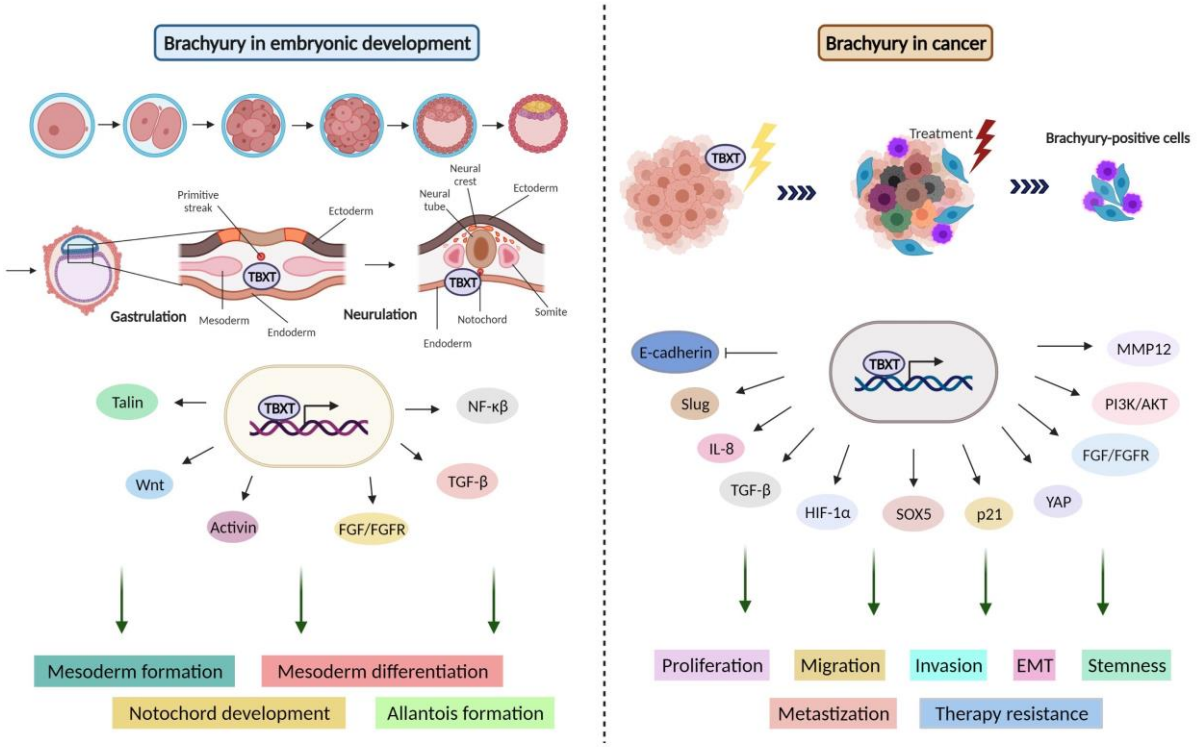

Figure 7. Role of Brachyury in embryonic development and cancer contexts. Brachyury was first described by its vital role in embryonic development, more specifically in cell movements during gastrulation, mesoderm formation and differentiation, notochord development and it also plays a role in extraembryonic tissue formation. These are all typical EMT processes since they involve the massive conversion of epithelial cells into migratory mesenchymal cells. Brachyury mediates its functions through several pathways such as Wnt, TGF-β, NF-κβ, FGFR, etc. Cancer cells hijack several pathways normally involved in embryonic development to sustain their characteristic malignant behavior, and this was demonstrated to be the case of Brachyury as well. This transcription factor has been identified in a number of malignancies, with a transcriptional program that involves a number of known oncogenic pathways and it has been strongly correlated with oncogenic properties, EMT, stemness, and therapy resistance that eventually leads to metastasis and patient relapses.

Some insights regarding this subject were uncovered mainly by transcriptomic approaches performed both *in silico* and *in vitro* using cancer cell lines of different models, for example chordoma, colorectal and breast cancers, and demonstrated that Brachyury is associated with several cancer pathways involved in cell cycle regulation, production of extracellular matrix (ECM) proteins, adhesion proteins and

cytokines, calcium signaling pathway, steroid biosynthesis, tumor necrosis factor (TNF) signaling pathway and its gene regulatory network was also associated with the PI3K/AKT signaling[200-202,206,218]. Some specific molecular players of the Brachyury-mediated effects were unraveled, and it was reported that the E-cadherin promoter possesses a Brachyury binding site, and that this transcription factor functions as a repressor of E-cadherin[195,219], being its effects partially mediated by Slug transcription factor[195], and moreover some authors reported a positive feedback loop between Brachyury and interleukin-8 (IL-8)/IL-8 receptor axis in lung cancer[193,220], TGF-β in prostate and lung cancers[214], FGF/FGFR in chordoma[221], and also a link between Brachyury and YAP axis in chordoma, glioblastoma and lung cancer[200], HIF-1α in breast cancer[196], MMP12[222] and p21 in lung cancer[195,204], SOX5 in breast cancer[208] and chordoma[201], EGF in chordoma[201], which all have a well-known role in cancer and may explain Brachyury's oncogenic properties (Figure 7). Even though these studies represent an advance attained in the research of the molecular players underlying Brachyury's actions, much more remains to be elucidated.

Despite of the already stated evidence of Brachyury as a molecule with an oncogenic function in several models, some contradictory studies reported this transcription factor as a tumor suppressor in gliomas and lung cancer[223,224], which suggest that the role of Brachyury might be context-dependent, which needs further addressing.

1.4.1.1. Brachyury in prostate cancer

The study conducted by Larocca et al that reported the connection between Brachyury and TGF-β, using a prostate cancer cell line, was the first study to implicate Brachyury in PCa aggressiveness[214]. Given that a characterization of Brachyury in prostate tumorigenesis was missing, our group evaluated the role of Brachyury in this malignancy[190,213]. In agreement with the previously tumor associated pattern of Brachyury, it was verified in human samples that Brachyury is aberrantly overexpressed in prostate intraepithelial neoplasia (PIN) lesions, primary and metastatic PCa when compared with normal tissues[190]. Through *in vitro* studies, Brachyury showed to have some oncogenic properties: was associated with increased proliferation, viability, migration and invasion; was associated *in vitro* and in *silico* with EMT-related molecular changes, such as a decrease of E-cadherin and concomitant increased expression of mesenchymal genes, N-cadherin, fibronectin, and Snail, and with upregulation of metalloprotease MMP14 which is implicated in ECM matrix degradation and invasion[190]. Thus, the group confirmed for the first time an association between Brachyury and PCa aggressiveness, as well as showed to be a predictor of poor prognosis in this malignancy[190].

In a subsequent study, the group demonstrated that Brachyury regulates several biological mechanisms associated with PCa therapy resistance, such as EMT, stem cell properties, NEtD and AR regulation[213]. Brachyury shown to be a direct regulator of the strongly PCa associated molecules, AR and Alpha-methylacyl-CoA racemase (AMACR), suggesting the possibility to use this transcription factor for clinical diagnosis of PCa[213]. Furthermore, it was confirmed the role of Brachyury in EMT program, as pointed in the previous study[190], being a direct regulator of the mesenchymal markers Snail and Fibronectin, as well as its role in stemness, since it increases prostate-spheres formation capacity and expression of the stem cell markers CD44 and CD15[213]. Moreover, through *in silico* analysis Brachyury was associated with the NE markers, chromogranin A and synaptophysin, suggesting the involvement of this transcription factor in the NEtD process, that as previously mentioned gives rise to more aggressive tumors.

The T-box transcription Brachyury has been already linked to therapy resistance to chemotherapy in other models and likewise, the group demonstrated that this transcription factor promotes resistance of PCa to 1st line chemotherapeutic drugs used in the treatment of CRPC, docetaxel and cabazitaxel[213]. Thus, it seems that Brachyury might play a central role in therapy resistance in PCa as well as in the development of CRPC, since it correlates with EMT, stemness and NEtD, and it is a direct regulator of AR.

1.4.1.2. Brachyury in lung cancer

Following chordoma, LC is probably one of the cancer models in which Brachyury has been more extensively studied. Several researchers evaluated the mRNA and protein expression of this transcription factor in LC samples (summarized in Table 2) and demonstrated a significant correlation between Brachyury and tumor stage and grade, vascular invasion, lymphatic permeation[193,195,205,210], being also a biomarker of poor prognosis[193,205,210].

The first report in the literature regarding Brachyury in LC[223] examined Brachyury expression and promoter methylation in ADC and normal lung samples and cell lines, and the data suggested a possible tumor suppressor role for this transcription factor, however several posterior studies demonstrated quite the opposite. Fernando and his coworkers[195] were the first group to point out the oncogenic properties of Brachyury in this model, demonstrating that high mRNA expression of this transcription factor in LC (vs normal lung tissue) is correlated to late stage tumors; moreover, through gain- and loss-of-function approaches and *in vitro* and *in vivo* assays, these researchers showed Brachyury as a driver of EMT as well as its association with migration, invasion and metastasis formation capacities. Subsequently,

numerous studies evaluated not only Brachyury mRNA but also protein expression in tumor samples and established various significant clinical correlations as already mentioned[185,205] (Table 2), and reinforced the involvement of this transcription factor in EMT and stemness[193,204,205], which altogether suggests that Brachyury plays a key role in LC aggressiveness.

As previously mentioned, Brachyury is associated with cell cycle regulation, and in fact some studies performed in LC supported this connection[195,204,218]. Firstly, the abovementioned study carried out by Fernando et al showed that knockdown of this transcription factor in the H460 cell line increased proliferation *in vitro*, downregulating *CUL1* and *p21* and upregulating cyclin D1 levels, all involved in cell cycle, proposing that Brachyury impairs cell cycle, probably at the G1-S transition[195]. Concordantly, Huang and collaborators also demonstrated that Brachyury negatively regulates the cell cycle, since low levels of this transcription factor resulted in increased proliferation *in vitro* and tumor growth *in vivo*. The underlying molecular players of this effect were also studied, however in contrary to the results reported by Fernando et al, the data showed an inverse correlation between Brachyury and cyclin D1, pRb and p21, which was later found to be a direct target of Brachyury[204]. More recently, Xu et al[218] reported dissimilar results to Fernando et al and Huang et al[204], more specifically Brachyury knockdown resulted in a decrease of cellular viability and further analyses suggested a positive effect of Brachyury on cell cycle progression and in apoptosis inhibition, which highlights the need to fully elucidate the role of this transcription factor.

Another interesting study, performed by Chen et al, proposed a new role for Brachyury in LC, given that it was shown through *in vitro* experiments that Brachyury suppresses macrophage infiltration, mediated by CCL2 and CCL4 chemokines, an association that was confirmed in lung tumor specimens[199]. This study suggests a role of Brachyury as a modulator of cancer tumor microenvironment (TME) that until now it was not reported in any cancer model and needs to be further addressed.

The T-box transcription factor Brachyury was already associated with therapy resistance in a number of cancer models and the similarly was reported in the specific case of LC. Roselli et al showed for the first time that Brachyury overexpression is associated with resistance to EGFR targeted therapy, namely the AG1478 inhibitor, whereas the knockdown resulted in increased susceptibility to treatment with this drug in NSCLC cell lines[185]; in the study of Huang and collaborators, the researchers observed that overexpression of this transcription factor conferred a survival advantage *in vitro* to radiation treatment and to the chemotherapeutic drugs docetaxel, cisplatin, vinorelbine and the cisplatin plus vinorelbine combination, and the opposite after Brachyury knockdown was reported[204]; these results

regarding Brachyury-mediated cisplatin resistance was corroborated by Xu et al[205]. Additionally, Huang et al also reported an interesting finding regarding therapy resistance, in which the NSCLC A549 cell line was treated with the abovementioned drugs in a chemotherapy regimen and it was demonstrated that the survival cells possessed higher Brachyury protein levels *in vitro* and *in vivo*, which strongly points for a role of this transcription factor in recurrence of disease[204].

Table 2. Review of the literature regarding Brachyury expression and its clinical impact in LC patients.

	Positive Brachyury Expression (%)				Prognostic Value	Clinical Correlations	Molecule Analyzed (Technique)
	ADC	SCC	Other/Unspecified	Nontumour			
Park J *et al.*, 2008[223]	Low expression: 80% (12/15)	-	-	100% (10/10)	-	-	mRNA (RT-PCR)
Fernando R *et al.*, 2010[195]	-	-	Stage I: 37.5 % (12/32) Stages II, III, IV: 62.5 % (30/48)	12.5% (2/16)	-	Stage	mRNA (qPCR)
Roselli M *et al.*, 2012[185]	48% (10/21)	25% (3/12)	Undifferentiated carcinoma: 50% (2/4) Bronchioloalveolar carcinoma: 100% (1/1) SCLC: 0 % (0/1)	43.8% (7/16)	-	-	Protein (IHC[1])
	-	-	52.5% (42/80)	12.5% (1/8)[2]	-	-	mRNA (qPCR)
Haro A *et al.*, 2013[193]	-	-	Low expression: 25% (26/104) Medium expression: 50% (52/104) High expression: 25% (26/104)		Yes (5-year DFS and OS rates)	Vascular invasion; Lymphatic permeation; Histological grade; TNM stage	mRNA (qPCR)
	-	-	Yes*	No expression			Protein (IHC)[3]
Xu K *et al.*, 2015[205]	55.56% (25/45)	31.43% (22/70)	-	0% (0/115)	Yes (OS)	TNM stage; LNM	Protein (IHC)[4]
Shimamatsu S *et al.*, 2016[210]	Low expression: 58.54% (48/82) High expression: 41.46% (34/82)	Low expression: 45.45% (10/22) High expression: 54.55% (12/22)	Primary tumor: low 63.64 % (7/11), high 36.36 % (4/11) LNM: low 48.7 % (56/115), high 51.3 % (59/115)	-	Primary tumor: Yes (OS after surgery) LNM: Yes (OS)	Histological grade	Protein (IHC)[3]
Chen S *et al.*, 2015[199]	-	-	Yes*	-	-	-	Protein (IHC)*
Wan Z *et al.*, 2016[222]	-	-	Primary tumor: low 33.33 % (10/30), medium 23.33 % (7/30), high 13,33% (4/30) Metastatic tissues: low 22.73% (5/22), medium 27.27 % (6/22), high 18.18% (4/22)	-	-	-	Protein (IHC)[5]
Hu Y *et al.*, 2016[225]	-	-	45 % in different lung tumors	-	-	-	Protein (IHC)[6]

ADC: adenocarcinoma; SCC: Squamous Cell Carcinoma; SCLC: Small Cell Lung Carcinoma; LN: Lymph Node; LNM: Lymph Node Metastasis; IHC: Immunohistochemistry; WB: Western Blot; TMA: Tissue Microarray; RT-PCR: semi-quantitative PCR: qPCR: Real Time PCR; DFS: disease free survival; OS: overall survival. † Comparison of expression between surrounding healthy tissue and tumour tissue out of the 16 Brachyury-positive primary tumours was performed. [1] anti-Brachyury mAb (ab57480; Abcam) (Dilution: 1:100). [2] Using a commercial panel of cDNAs obtained from 40 lung tumour tissues and 8 histologically normal lung tissues obtained from lung cancer patients. [3] anti-Brachyury rabbit polyclonal antibody (Abcam, ab2068) (Dilution: 1:100). [4] goat anti-human polyclonal anti-Brachyury (sc-17745; Santa Cruz Biotechnology) (Dilution: 1:100). [5] rabbit anti-human polyclonal anti-Brachyury (sc-20109; Santa Cruz Biotechnology) (Dilution: 1:400). [6] anti-Brachyury (Santa Cruz Biotechnology) (Dilution: 1:400). * Values not discriminated on the paper

1.4.2. Brachyury as a therapeutic target

Due to the pivotal role of Brachyury in therapy resistance and progression in several types of malignancies as well as its tumor associated pattern, therapies targeting this transcription factor are extremely attractive strategies for cancer treatment. In agreement, Robinson et al, recently highlighted the importance of targeting Brachyury in cancer and reviewed the different types of approaches to do so[226] (Figure 8).

A group of investigators focused on a T-cell mediated approach to target the EMT driver Brachyury. By using an MHC-peptide-binding prediction algorithm, a nonameric Brachyury epitope was selected to generate Brachyury specific T cytotoxic cells from the blood of both normal donors and cancer patients, which was later modified to improve its binding and stimulating properties[227]. These specific T cells were able to lyse, *in vitro* and *in vivo*, tumor cells expressing Brachyury including lung, breast, and colorectal carcinoma cells[184,185,187,195,227,228]. After these preliminary studies, vaccines expressing the full length human Brachyury, yeast-brachyury (GI-6301) and MVA-brachyury-TRICOM[187,228,229], entered clinical trials and were the first vaccines targeting an EMT driver to successfully do that. GI-6301 and MVA-brachyury-TRICOM vaccines showed in Phase I clinical trials to be safe and capable of generate Brachyury-specific T cell immune responses in cancer patients demonstrating evidence of little clinical activity in some of the enrolled patients[230,231], being at the moment under evaluation in phase II trials combined with other therapies[232-234]. Currently, other approaches combining Brachyury vaccines with other therapies are now recruiting patients to initiate clinical trials[235,236].

Similar to other transcription factors, Brachyury is not easy to directly target, and no small-molecule inhibitor has been developed yet, however, some small molecule inhibitors have been reported to indirectly target this transcription factor. Recently, Magnaghi et. al[237] showed that afatinib is the only EGFR inhibitor active in several chordoma cell lines (EGFR signaling driven). As previously mentioned, Brachyury is overexpressed in virtually all chordomas and silencing of this transcription factor in chordoma cell lines was shown to decrease tumor growth both *in vitro* and *in vivo*. Since afatinib also downregulates Brachyury protein, this is thought to be the reason underpinning the great activity of this drug against chordoma[237]. Furthermore, Sharifnia et. al demonstrated that the transcriptional cyclin-dependent kinase (CDK) inhibitor targeting CDK7/12/13, THZ1, also targets Brachyury, suppressing this way chordoma cell proliferation, both *in vitro* and *in vivo*[183]. Both studies are an incredible advance for chordoma treatment, given the lack of effective treatment options available in the clinic, and might also be applied in other cancers that overexpress the EMT driver Brachyury, like PCa and LC.

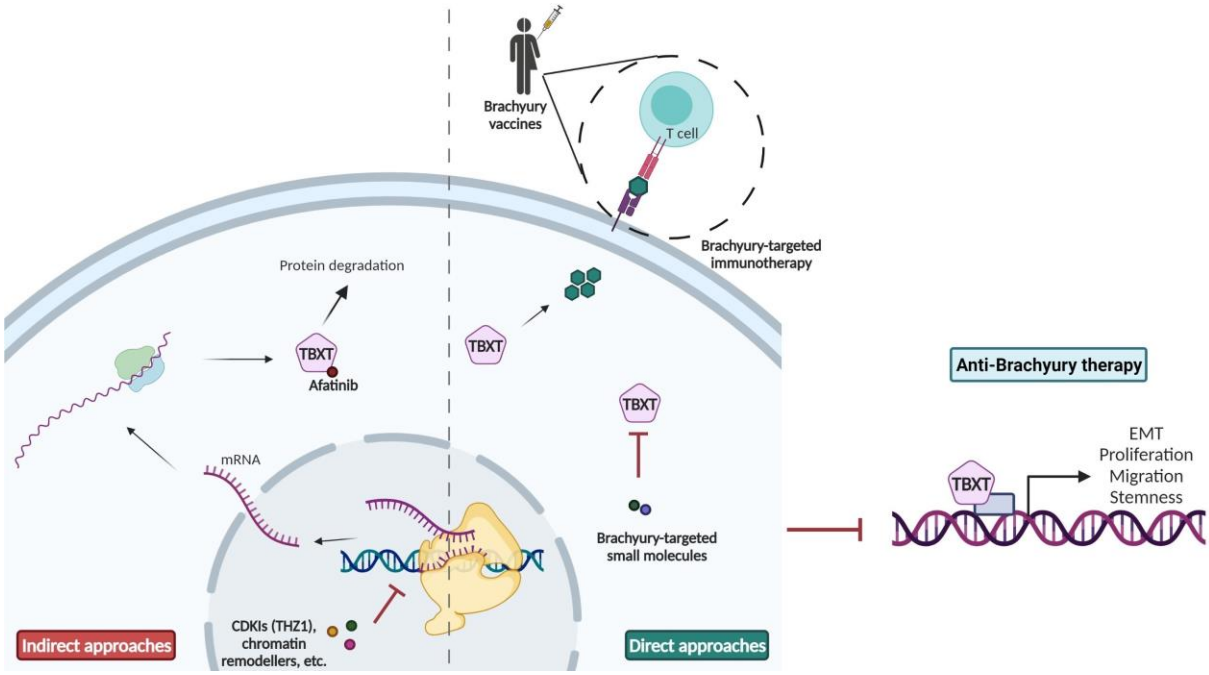

Figure 8. Different strategies for Brachyury targeting. The main goal of targeting the embryonic transcription factor Brachyury in cancer is to inhibit its transcriptional program to ultimately eradicate its oncogenic functions. Strategies to target Brachyury can be direct or indirect approaches. Currently, the only available direct strategy is a T-cell mediated approach, being these anti-Brachyury vaccines under evaluation in clinical trials. Small molecules specific to directly target this transcription approach weren't developed yet but yield tremendous potential. Indirect approaches identified so far comprise the recently identified afatinib[237] and THZ1[183] drugs. Adapted from [226].

1.5. Drug delivery systems

Major advances are being accomplished regarding anticancer therapy, such as the discovery of novel therapeutic targets, predictive biomarkers of response and so on, however this is not sufficient to greatly improve cancer treatment and overcome the underlying problems of conventional systemic therapies, namely poor specificity, high toxicity and the development of drug resistance[238]. This mirrors the current need for improved therapeutic approaches and in that sense, drug delivery is an area of active and attractive research with the aim of develop more efficient and less toxic strategies. Drug delivery systems (DDS) can be defined as technologies that are designed to improve the specificity of therapeutics by stabilizing them *in vivo*, controlling their release, and localizing their effect[238-240]. These systems alter pharmacokinetics and biodistribution of the associated drugs, and are able to reduce side effects, improve bioavailability and reduce degradation in the human body, protecting them from harsh environments[238-240]. Due to the promising results obtained with this kind of approaches, several DDS were already approved by FDA and EMA for clinical use, and a lot more are currently being evaluated in clinical trials[241]. In the biomedical field, it is worth to mention that these DDS besides having therapeutic applications can also be used in diagnostic settings[241,242]. Currently, there is a plethora of materials that can be used for drug

delivery purposes, among them metal nanoparticles, organometallic and porous compounds, polymers, liposomes, etc[240,242,243] (Figure 9).

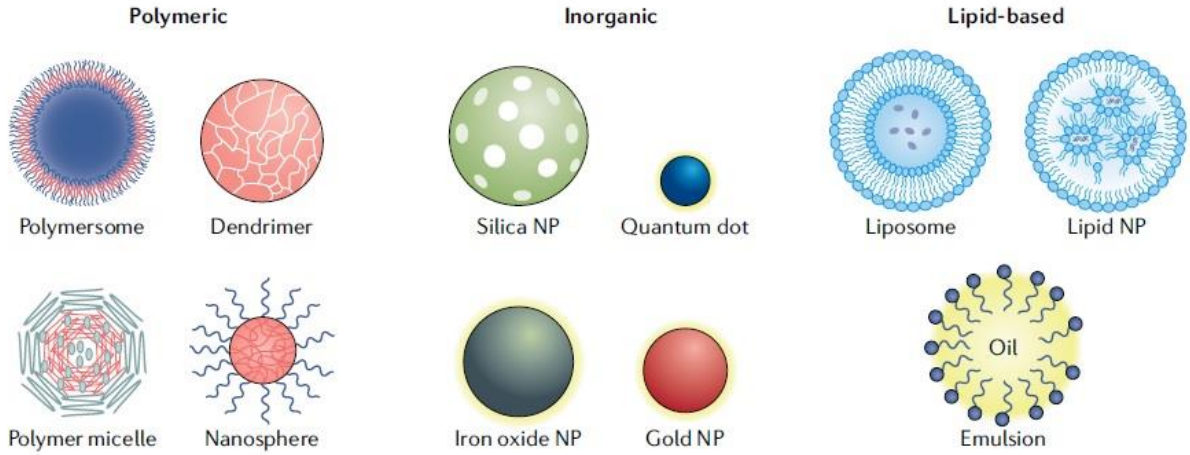

Figure 9. Different classes of nanomaterials used for drug delivery purposes. Adapted from [242].

One class of inorganic materials that have received a lot of attention, and that in fact comprise the matrix of one of the formulations that was approved for clinical trials[244,245], are porous materials. International Union of Pure and Applied Chemistry (IUPAC) classified porous materials, considering the pore size, as microporous (<2 nm), mesoporous (2-50 nm) and macroporous (>50 nm)[246,247]. Zeolites and mesoporous silicas are examples of this kind of materials and accordingly with the mentioned classification are considered microporous and mesoporous, respectively[247]. They present many attractive properties, such as thermal stability, chemical inertia, non-toxicity, biocompatibility, high inner surface area, are inexpensive and easy to synthesize, allow the adsorption of many molecules and possess a surface susceptible to functionalization, that altogether make them suitable for a vast number of applications[238,239,246,248,249].

1.5.1. Zeolites and mesoporous silicas

Zeolites were first discovered in 1756 and belong to the family of aluminosilicates, being solid inorganic crystalline materials, composed of silicon (Si), aluminum (Al) and oxygen (O)[249,250]. These materials possess a $[Si/Al]O_4$ tetrahedral framework arranged in a three-dimensional structure forming a network of channels and pores with regular dimensions on a nano- and subnanometer scale[250,251]. Currently, over 150 different zeolite frameworks have had their structure elucidated with pores and channels ranging from ~0.6 to 1.4 nm, that are potentially large enough to accommodate a wide variety of different molecules[251]. Zeolite structures have a wide range of applications in industry, agricultural, environmental, and biomedical fields[251-254]. Focusing in oncologic applications, zeolites can be used for

diagnostic purposes such as magnetic resonance imaging[255-257] and also as DDS for anticancer therapy. Several authors reported the successful hosting of different anticancer drugs, for example paclitaxel[258], temozolomide[259], cyclophosphamide[260], cisplatin[261], doxorubicin[262], among others. One of the most known and widely used zeolites is the synthetic zeolite Y, with a faujasite (FAU) framework structure, composed of eight sodalite cages connected by O bridges forming a large central cavity[263-267] (Figure 10). In fact, our research group already explored the potentiality of this specific zeolite as a carrier for anticancer drugs, namely α-cyano-4-hydroxycinnamic acid (CHC)[264,265], 5-fluorouracil (5-FU)[266] and salicylic acid (SA)[267], and successfully demonstrated the efficacy of these systems *in vitro*.

Even though zeolites are very interesting materials that can host a wide range of molecules, their micropores restrict the size of biomolecules that can be used. In this sense, new materials that can surpass this restriction were in need, and in the 1990s mesoporous silica's were developed being gained attention for drug delivery purposes since then. These materials are composed by hundreds of pores arranged in a honeycomb-like structure with tunable sizes of 50-300 nm, large surface area and pore volume, high loading capacity, controllable pore diameters ranging from 2 to 50 nm which allows them to accommodate a broader range of molecules compared to zeolites[238,268,269]. Similar to zeolitic structures, mesoporous silica's can be used for several medical applications such as drug delivery, in therapeutic devices[270], cell imaging[271], enzyme immobilization[272], and in fact these materials were already reported as nanocarriers for anticancer drugs, like paclitaxel, doxorubicin, methotrexate[273], tamoxifen[274], cisplatin and transplatin[275], etoposide[276] and lapatinib[277]. Mobil Composition of Matters (MCM) and Santa Barbara Amorphous (SBA), synthetized in 1992 and 1998 respectively, comprise two of the most well-known families of mesoporous silica[278,279] (Figure 10), being MCM-41 and SBA-15 the most widely used for drug delivery purposes[280]. These both materials share some properties, besides the ones already stated above that are common to zeolite structures as well, such as a ordered hexagonal architecture of pores[278-280] (Figure 10). However, SBA-15 possesses larger pores (pore diameter of 4.6-30 nm) and thicker walls that make it more thermal stable than MCM-41 (pore diameter of 2.5-6 nm) and other mesoporous silica materials[280,281].

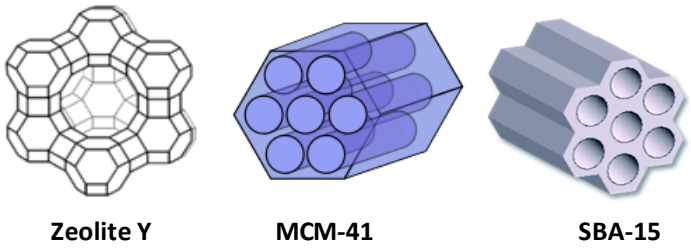

Figure 10. Schematic representation of zeolite Y and mesoporous silica's MCM-41 and SBA-15 materials. Adapted from[249,264].

CHAPTER 2:

Research Objectives

The T-box transcription factor Brachyury was first described and extensively studied in embryonic development context, but more recently has been implicated in cancer as well. Brachyury was described as an EMT driver, an important therapy resistance mechanism that is also associated with metastization, and has been correlated with radio- and chemotherapy resistance in several malignant neoplasms. In the specific case of PCa, our group provided some evidence that this transcription factor might be a regulator of AR and regarding LC, it was suggested that Brachyury could be associated with resistance to EGFR targeted therapy. Furthermore, Brachyury has been associated with cancer aggressiveness *in vitro* and *in vivo* and also correlated with poor prognosis in several malignancies, including PCa and LC. With this in mind, the major aim of this work was first to dissect whether Brachyury can in fact be a predictor of response to antiandrogens and EGFR targeted therapies in PCa and LC, respectively, and secondly determine the potentiality of this transcription factor to be a therapeutic target in these two models. Specifically, it is aimed to:

- ✓ Dissect the biological role of this transcription factor in LC, similarly to what was already done in PCa by our group;
- ✓ Study the predictive value of Brachyury to the most commonly used therapies in PCa and LC and elucidate the mechanisms behind therapy response modulation;
- ✓ Explore therapeutic strategies for Brachyury targeting, including the usage of DDS.

To answer our main questions, the present work was divided into the following tasks:

- Characterization of Brachyury expression, EMT, stemness and signaling pathways in PCa and more deeply in LC cell lines;
- Assess the Brachyury impact in LC aggressiveness *in vitro*, through viability, migration and colony formation assays;
- Evaluate the effects of Brachyury in the modulation of prostate and lung cancer cells response to AR and EGFR inhibitors, respectively.
- Validate the efficacy and specificity of drugs described as targeting Brachyury, afatinib and THZ1;
- Develop DDS using porous silica materials, NaY, MCM-41 and SBA-15 as hosts to entrap the abovementioned small molecule inhibitors.

CHAPTER 3:

Materials and methods

3.1. Cell lines and cell culture

Four human prostate and seven human lung cancer cell lines (Table 3) were used in this work and were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) or from German Collection of Microorganisms and Cell Cultures GmbH (DSMZ; Braunschweig, Germany). These cell lines were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Invitrogen) or in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, Invitrogen) supplemented with 10% of Fetal Bovine Serum (FBS, GIBCO, Invitrogen) and 1% penicillin/streptomycin (GIBCO, Invitrogen) at 37°C and 5% CO_2.

Table 3. Panel of human cancer cell lines used in this work.

Cancer model	Cell line	Histological type	Molecular Characteristics
Prostate	LNCaP	ADC	Hormone sensitive; *AR* mutant (T877A)
	22RV1	ADC	Hormone resistant; AR and ARv expression
	DU145	ADC	Hormone resistant; no AR expression
	PC3	NE	Hormone resistant; no AR expression
Lung	H292	Mucoepidermoid carcinoma	*EGFR* and *KRAS* WT
	HCC827	ADC	*EGFR* mutant (exon19del)
	A549	ADC	*KRAS* mutant
	PC9	ADC	*EGFR* mutant (exon19del)
	H322	ADC	*EGFR* and *KRAS* WT
	H1975	ADC	*EGFR* mutant (L858R; T790M)
	HCC4006	ADC	*EGFR* mutant (exon19del; A750P)

ADC: adenocarcinoma; NE: neuroendocrine; WT: wild type; del: deletion

3.2. Drugs and porous materials

Afatinib, AST1306, Osimertinib and THZ1 were obtained from Selleck-Chemicals (Houston, USA) and Abiraterone, Enzalutamide, Flutamide and Bicalutamide were obtained from MedChemExpress (USA). These drugs were prepared as stock solutions in Dimethyl Sulfoxide (DMSO) and stored at -20°C, and in all experimental conditions the drugs were diluted in 0.5% FBS culture medium. The vehicle control (DMSO) was also used in all experiments.

NaY (CBV100) zeolite was obtained from Zeolyst International (USA), and the mesoporous MCM-41 and SBA-15 structures were previously synthetized as described[267].

3.3. *In vitro* Brachyury overexpression

An *in vitro* overexpression (OE) of Brachyury (TBXT) was performed in LNCaP, 22RV1, H292 and HCC827 cell lines, using a pCMV6-AC-GFP vector (Origene) containing the TBXT full cDNA. This overexpression is based on a transfection with a vector that contains a multiple cloning site, where the

full cDNA of TBXT is inserted together with Geneticin (G418) resistance gene, that further is exploited for selection of successfully transfected cells.

LNCaP, 22RV1, H292 and HCC827 cells were plated on 6-well plates at a density of $5x10^5$ cells per well in RPMI/DMEM 10% FBS and allowed to adhere overnight. In the next day, transfection was done using the FUGENE HD reagent (Roche) according to the manufacturer's protocols, with 2 µg of the plasmid at a ratio of 6:2 (reagents:plasmid), in serum free Opti-MEM media. After 48 hours, stable LNCaP, 22RV1, H292 and HCC827 cell pools with TBXT expression were maintained with 500-800 µg/mL G418 treatment. The Empty vector was used as control and as such in this study the resultant cell clones from transfection with this vector will be further designated as Empty and the ones resulting from transfection with the vector containing the TBXT full cDNA will be called TBXT.

3.4. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) analysis

The cells were plated on a 6-well plate at a density of $1x10^6$ cells per well and allowed to adhere overnight. In the next day, the cells were subjected to more 24 hours in 10% FBS culture media.

The total RNA was isolated using TRIZOL reagent (GRiSP Research Solutions) according to the manufacturer's instructions, through a chloroform-based phase separation and isopropanol RNA precipitation. One microgram of RNA was reverse transcribed into cDNA with the Xpert cDNA Synthesis Mastermix (GRiSP Research Solutions). The expression levels of several genes were then assessed by qRT-PCR and the primers used are presented in Table 4. Real-time PCR was conducted by using 200 ng of cDNA as template and the reagent SsoFast™ EvaGreen® Supermix (Bio-Rad), using the Thermal cycler CFX96 (BioRad). The thermocycler program used was as follows: 95°C for 10 minutes for enzyme activation, and 40 cycles at 94°C for 15 seconds for denaturation, 58°C for 30 seconds for annealing and 72°C also for 30 seconds for extension. PCR mixture without the cDNA template was used as negative control and β-actin was used as an internal control to normalize gene expression. Data was analyzed using the formula: $Ratio=2^{\Delta Ct}$.

Table 4. Primers sequences used for qRT-PCR.

Transcript target	Forward primer	Reverse primer
Snail	5'-CTCTAGGCCCTGGCTGCTAC-3'	5'-TGACATCTGAGTGGGTCTGG-3'
Slug	5'-CTTTTTCTTGCCCTCACTGC-3'	5'-ACAGCAGCCAGATTCCTCAT-3'
E-cadherin	5'-TGCCCAGAAAATGAAAAAGG-3'	5'-GTGTATGTGGCAATGCGTTC-3'
β-catenin	5'-GAAACGGCTTTCAGTTGAGC-3'	5'-CTGGCCATATCCACCAGAGT-3'
Vimentin	5'-GGGACCTCTACGAGGAGGAG-3'	5'-AAGATTGCAGGGTGTTTTCG-3'

Nanog	5'-ATACCTCAGCCTCCAGCAGA-3'	5'-CTGGGGTAGGTAGGTGCTGA-3'
Oct4	5'-GCTCCTGAAGCAGAAGAGGA-3'	5'-CTCCAGGTTGCCTCTCACTC-3'
Sox2	5'-GAGAACATGCTCTTGGCACA-3'	5'-GCACATCTCTGCCAGTTGAA-3'
β-actin	5'-GGACTTCGAGCAAGAGATGG-3'	5'-AGCACTGTGTTGGCGTACAG-3'

3.5. Western Blot analysis

The cells were plated on a 6-well plate at a density of $1x10^6$ cells per well and allowed to adhere overnight. In the next day, the cells were serum starved for two hours, and when necessary, two hours followed by a 24-hour treatment with drugs. In some experiences when necessary, PCa cells were also stimulated with 10 nM of DHT for 24 hours and LC cells were stimulated with 10 ng/ml of EGF for 15 minutes in 0.5% FBS medium.

To obtain the protein extracts the cells were washed with Phosphate-Buffered Saline (PBS) and then scrapped in lysis buffer containing 50 mM of Tris (pH 7.6–8), 150 mM of NaCl, 5 mM of EDTA, 1 mM of Na_3VO_4, 10 mM of NaF, 10 mM of sodium pyrophosphate, 1% of NP-40 and 1/7 of protease inhibitors (Roche, Amadora, Portugal). After a centrifugation of 13000 rpm for 15 minutes, total protein was quantified using the Bradford method (Sigma-Aldrich). Aliquots of 40 μg of total protein from each sample were separated on 8/10% polyacrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis (100V) and transferred onto a nitrocellulose membrane (Amersham Biosciences) in 25mM Tris-base/glycine buffer using the Trans-Blot Turbo Transfer System (25V, 1A for 30 minutes). The membranes were blocked with milk 5% Tris-Buffered Saline/0.1% Tween (TBS-Tween) for 1 hour at room temperature (RT) and incubated overnight with the primary antibodies at 4°C (Table 5). Next, after washing in TBS-Tween, the membranes were incubated with the respective secondary antibody coupled to horseradish peroxidase (1:2500, Cell Signaling). Tubulin was used as loading control. Blots detection was done by chemiluminescence (Supersignal West Femto kit, Pierce, Thermo Scientific) using the Sapphire Biomolecular Imager (Azure Biosystems).

Table 5. Primary antibodies used for Western Blot.

Protein target	Reference	Dilution (Secondary antibodies)
Brachyury	D2Z3J (CS)	1:500 (Rabbit)
Snail	C15D3 (CS)	1:1000 (Rabbit)
E-cadherin	24E10 (CS)	1:1000 (Rabbit)
N-cadherin	D4R1H (CS)	1:1000 (Rabbit)
Vimentin	D21H3 (CS)	1:1000 (Rabbit)
β-catenin	D10A8 (CS)	1:1000 (Rabbit)

FAK	3285T (CS)	1:1000 (Rabbit)
Paxilin	D9G12 (CS)	1:1000 (Rabbit)
Talin	C45F1 (CS)	1:1000 (Rabbit)
Vinculin	4650T (CS)	1:1000 (Rabbit)
YY1	SC-7341	1:1000 (Mouse)
AR	ab108341	1:1000 (Rabbit)
p-EGFR (Tyr1068)	D7A5 (CS)	1:1000 (Rabbit)
EGFR	D38B1 (CS)	1:1000 (Rabbit)
p-AKT (Ser473)	D9E (CS)	1:1000 (Rabbit)
AKT	C67E7 (CS)	1:1000 (Rabbit)
p-MEK1/2 (Ser217/221)	41G9 (CS)	1:1000 (Rabbit)
MEK1/2	L38C12 (CS)	1:1000 (Mouse)
p-ERK1/2 (Trh202/tyr204)	D13.14.4E (CS)	1:2000 (Rabbit)
ERK1/2	137F5 (CS)	1:2000 (Rabbit)
p21	2947 (CS)	1:1000 (Rabbit)
α-Tubulin	SC-73242	1:2500 (Mouse)

CS: Cell Signaling Technology; SC: Santa Cruz Biotechnology; ab: Abcam

3.6. Immunofluorescence analysis

The cells were seeded on glass cover slips placed on 12-well plates until ~60% of confluence and were allowed to adhere overnight. The cells were then fixed and permeabilized in cold methanol for 5 minutes. After blocking with Ultra V Block solution (Thermo Scientific) for 10 minutes, the cells were incubated overnight at 4°C with the primary antibody for Brachyury (1:200, #81694, Cell Signaling). After washing in PBS, the TRITC Alexa Fluor-conjugated secondary antibody (Molecular Probes, Invitrogen) was used at a dilution of 1:500 for 1 hour at RT protected from light. Finally, after washing in PBS, cells were mounted in Vectashield Mounting Media with 4',6-diamino-2-phenylindone (Sigma) and images were obtained with a fluorescence microscope (Olympus BX61), using Cell P software.

3.7. Cellular viability assay

To assess cellular viability overtime, H292 cells were plated on 48-well plates in triplicate at a density of $3x10^4$ cells per well and allowed to adhere overnight in DMEM 10% FBS. In the following day, the cells were submitted to DMEM 0.5% FBS and let incubate for 24, 48 and 72 hours. The total biomass was quantified in time zero after the cells were fixed with cold 10% trichloroacetic acid (TCA) for at least 1 hour at 4°C and stained with Sulforhodamine B (Sigma-Aldrich) for 30 minutes. To remove the excess of dye, cells were repeatedly washed with 1% acetic acid and protein-bound dye was dissolved in 10mM of Tris-Base solution (pH=10.5) for absorbance measurement at 490nm using the Thermo-Scientific Varioskan Flash SkanIt software (Thermo-Scientific). The same was done to quantify the remaining time

points. The results were calibrated to the starting value (time 0 hours, considered as 100% of proliferation) and expressed as the mean ± SD. The assay was done in triplicate at least three times.

To perform all cytotoxicity assays, cells were plated on 96-well plates at a density of 5-8x10^3cells per well, depending on the cell line, and allowed to adhere overnight in DMEM/RPMI 10% FBS. On the next day, the cells were treated with increasing concentrations of the drugs, zeolite/mesoporous silica and DDS or with DMSO alone (in the case of the drugs), both diluted in 0.5% FBS culture medium. After 72 hours, cell viability was quantified using the Cell Titer96 Aqueous cell proliferation assay (Promega). The results were expressed as the mean percentage ± SD of viable cells relative to the DMSO or medium alone (considered as 100% viability). The IC_{50} was calculated by nonlinear regression analysis using GraphPad Prims software version 8.

3.8. Wound Healing migration assay

H292 cells were seeded on 6-well plates and cultured to at least 95% confluence. Monolayer cells were scrapped with a plastic 1000µl pipette tip, washed with PBS and then incubated with fresh DMEM 0.5% FBS medium. The "wounded" areas were photographed after 12, 24, 48 and 72 hours by phase contrast microscopy using the Olympus IX53 microscope. The migration distance was measured using the beWound software (version 1.7, BeSurg) and the relative migration distance was calculated by the following formula: $W\ (\%) = \frac{W_0 - Wt}{W_0} \times 100$, where W_0 is the width of the cell wounds before incubation, and Wt is the width of cell wounds after incubation. The results are expressed as the mean ± SD. The assay was done in triplicate at least three times.

3.9. Clonogenicity assay

H292 cells (750 cells/well) were seeded on 12-well plates and incubated overnight to adhere. Medium was replaced for fresh DMEM 0.5% FBS and was let incubate for 8-12 days, with medium renewal after 3 days. The colonies were fixed with cold methanol for at least 10 minutes at -20°C and stained with 5% Crystal violet for 30 minutes and manually counted. Results were expressed as the mean colonies ± SD. The assay was done in triplicate.

3.10. Drug Delivery Systems preparation

For the DDS development three porous materials, NaY, MCM-41 and SBA-15 were used, and only two were selected to encapsulate each drug (afatinib or THZ1) taking into consideration their molecular structure (Figure 11). The preparation of DDS based in porous materials was carried out based on a previously established method[259,265,266], that is simply a encapsulation method. Firstly, NaY, MCM-41 and

SBA-15 materials were dehydrated at 120°C for 2 hours in order to remove water from the pores, which is important given the poor water solubility of afatinib and THZ1. So, in order to prepare afatinib DDS, 200 mg of NaY and SBA-15 were each one added to a solution of 10 mg (20.58 μmol) of afatinib in 10 mL of ethanol; THZ1 DDS, 200 mg of MCM-41 and SBA-15 materials were added to a solution of 5 mg of THZ1 (7.825 μmol) in 10 mL of acetone. The resulting suspensions were stirred (100 rpm) for 48 hours at RT. In this case the suspensions were not filtered nor heated in order to minimize drug loss, instead they were allowed to sediment and the major part of the solvent was evaporated at RT during 48-120 hours. After this all the DDS were dried in an oven at 60 °C for 48 hours in order to fully evaporate the solvent, and further stored in a desiccator.

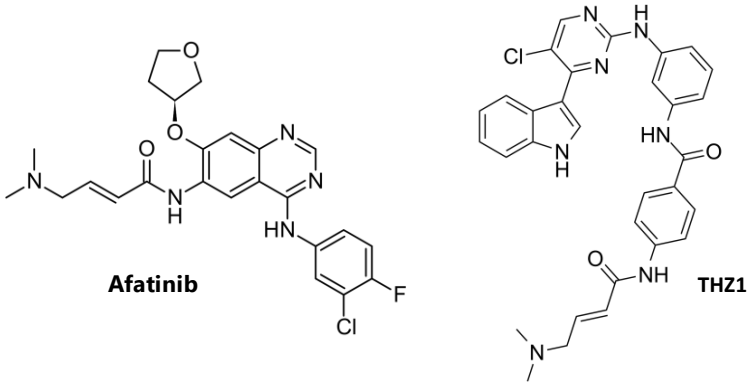

Figure 11. Molecular structure of Afatinib and THZ1.

3.11. Statistical analysis

Statistical analysis was done using GraphPad Prism 8 version. The level of significance in all statistical analysis was set at $p<0.05$. Student's t-test was used to do single comparisons between two different conditions.

CHAPTER 4:

Results

4.1. Biological and predictive role of Brachyury in Prostate Cancer

4.1.1. Role of Brachyury in modulation of EMT proteins in prostate cancer cell lines

The first main aim of this work was to study the impact of Brachyury expression in antiandrogen therapy response in PCa. For that we chose two androgen-dependent cell lines, namely LNCaP and 22RV1, which were described by our group[190] and others[195] as positive and negative, respectively, for Brachyury expression. Thus, we genetically modulated Brachyury, in order to achieve an OE of our molecule of interest, using an expression vector that was different from the one used in the first report of our group[190]. By immunofluorescence analysis (Figure 12), it was possible to observe a low cytoplasmic expression of Brachyury in LNCaP cells transfected with the empty vector (Empty cells), which is undetectable by western blot (Figure 13). The cells transfected to overexpress Brachyury (TBXT cells), presented high levels of this protein in the nucleus, which was indicative of a successful and functional transfection, and of a specific antibody, since Brachyury OE in the two transfected cell lines, was also confirmed by western blot (Figure 13A and 13B). Furthermore, it is important to notice a heterogeneous population of TBXT cells, in an expression level context, which is due to the fact that these cells were stably transfected but selected with antibiotic treatment instead of single cell cloning or cell sorting.

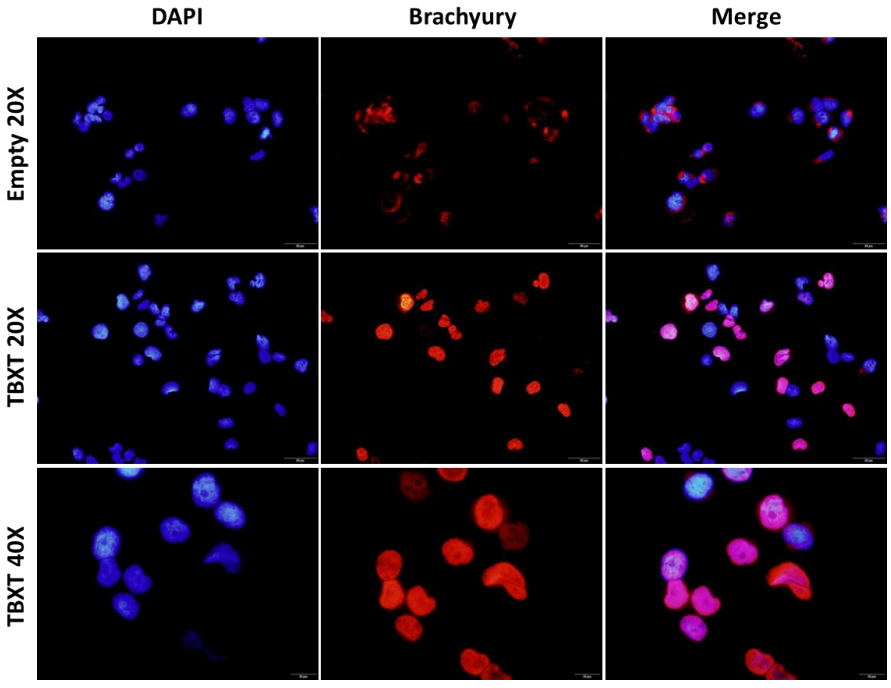

Figure 12. Immunofluorescence analysis of Brachyury expression in transfected LNCaP cells. Brachyury expression was analyzed in the control (Empty cells) and Brachyury OE (TBXT) cells by immunofluorescence analysis (20X and 40X magnification), in which the red and blue signals represent Brachyury and DAPI stained nucleus, respectively.

Further, as in this work we used either a different expression vector and antibody for Brachyury detection, we intended to validate the success of our transfection by recapitulating some of the previous results obtained by the group in the 22RV1 cell line (Figure 13A), for which was found a direct correlation between Brachyury mRNA expression and EMT related genes[190,213]. Herein we exposed the cells to different culture conditions, 0.5% FBS and 10% FBS, as well as treated them with 10 nM of DHT to stimulate AR, and determined the expression of both Brachyury and EMT related proteins (Figure 13).

Firstly, it is interesting to note that Brachyury expression increased over the stimulating conditions in 22RV1 cell line, but not in LNCaP cells (Figure 13A and 13B). Secondly, as it can be observed in Figure 13A, the 22RV1 epithelial cell line does not express several EMT related proteins and for the ones that they do express, E-cadherin and β-catenin (epithelial markers), a slight decrease was found between Empty and TBXT cells (Figure 13A). Regarding LNCaP cell line, for which there is no data concerning Brachyury's effects in EMT genes, we analyzed both protein (Figure 13B) and mRNA expression levels (Figure 13C). In this cell line, Brachyury OE resulted in upregulation of YY1 and β-catenin proteins, in this last case at mRNA level as well (Figure 13C). Vimentin protein level was also increased, but only in the control (CTR) condition (0.5% FBS), not being confirmed at mRNA level. In contrast, E-cadherin was found slightly downregulated at mRNA level (Figure 13C), but slightly overexpressed at protein level (Figure 13B). Regarding the two transcription factors that regulate EMT, Snail and Slug, LNCaP cells do not express them at a protein level, but they can be detected at mRNA level, however no differences were noted between Empty and TBXT cells (Figure 13C).

Complementary, we also evaluated some focal adhesion (FA) associated proteins, namely FAK, paxillin, talin and α-actinin and a significant downregulation of talin protein was found upon Brachyury upregulation, indicating a potential loss of adhesion properties.

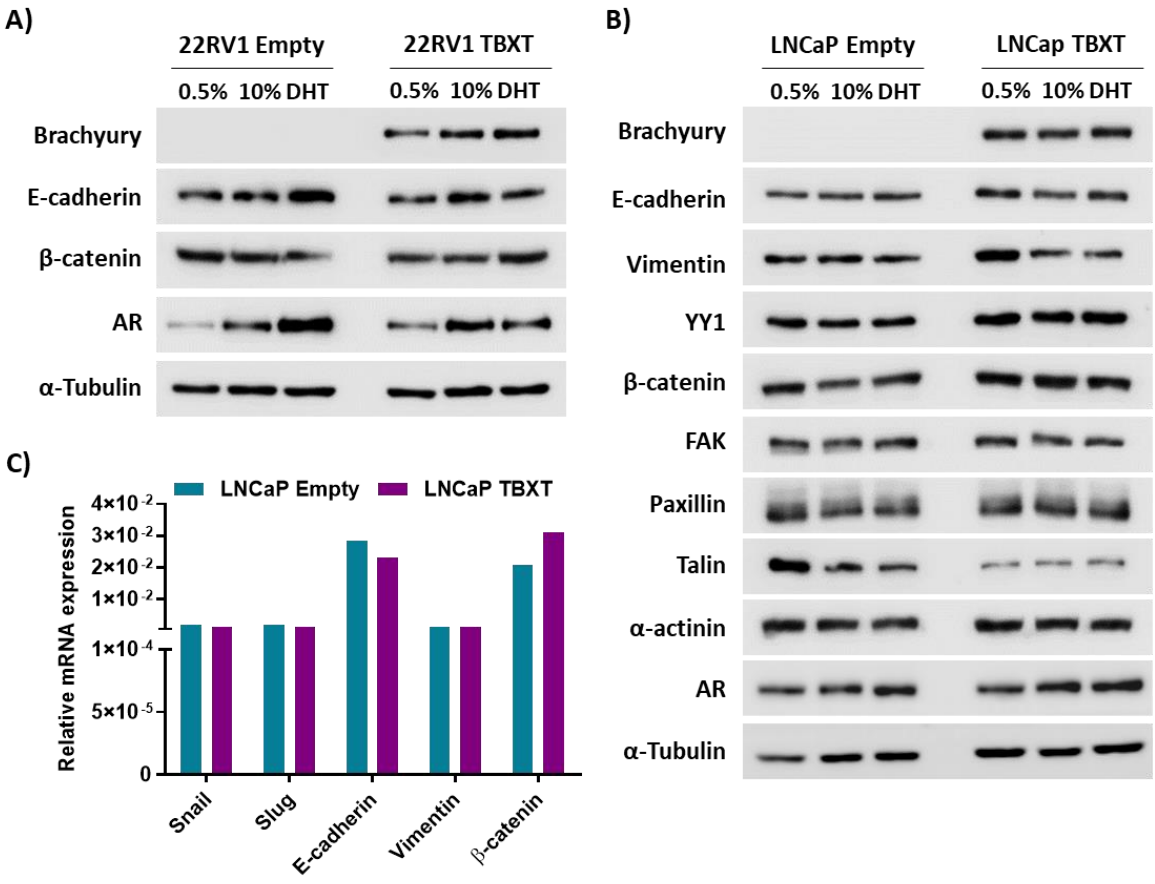

Figure 13. Characterization of Brachyury, AR, FA-, and EMT-related molecules in 22RV1 and LNCaP transfected cell lines. A) Western blot analysis of AR and of the epithelial markers E-cadherin and β-catenin in transfected 22RV1 cells. B) Analysis of AR, epithelial (E-cadherin) and mesenchymal (vimentin) markers, YY1 transcription factor and FA associated proteins (FAK, paxillin, talin and α-actinin) in our genetically modulated LNCaP model. All these analyses were conducted in 0.5% FBS, 10% FBS RPMI media and in DHT (10 nM, 24 hours) stimulating conditions. Tubulin was used as a loading control. The western blots presented here are representative assays of two independent experiments. C) Real time analysis (N=1) of EMT related genes in Empty and TBXT LNCaP cells (10% FBS growth condition). The experiment was done in triplicate and presented as relative expression in relation to β-actin.

Finally, given the reported positive association and identification of AR as a target of Brachyury in PCa[213], we also analyzed the AR expression levels in our models in basal and stimulating conditions (Figure 13A and 13B). Taking a closer look to the results and in agreement to the previously reported, Brachyury OE upregulated AR (full length) in LNCaP cells for all tested conditions, especially under stimulating conditions (10% FBS and DHT). However, the same was not observed in the 22RV1 cell line, in which TBXT cells had an increased full length AR expression in the conditions 0.5% and 10% FBS, but not in the stimulated one (DHT), where AR is slightly downexpressed in comparison to Empty cells.

Altogether, even though some apparent contradictory results were found in comparison to the group's published results, we herein show that Brachyury OE can induce EMT and AR expression changes

in the chosen cell lines, however, further analysis are in need, mainly regarding the biological behavior of TBXT cells that we established here (as invasion and migration assays). Importantly, by all the expression assays we present, and many other validation tests we conducted (data not shown), we have complete confidence in the overexpressing cell lines we generated as well as in the new commercial Brachyury antibody we chose, which allowed us to move to the next aim of the work with certitude.

4.1.2. Brachyury's effect in the modulation of prostate cancer cells response to AR inhibitors

As mentioned in the beginning and given the evidence of AR as a target of this transcription factor and the positive association between these two molecules in PCa[213], which we confirmed mainly in the more androgen-dependent cell line, LNCaP (Figure 13B), we hypothesized that Brachyury could be a potential predictive biomarker to antiandrogen therapy response in PCa.

To do so we performed cytotoxic assays with LNCaP and 22RV1 cells using several drugs widely used in the clinic, namely the 1st generation antiandrogens flutamide and bicalutamide, the 2nd generation antiandrogen enzalutamide and the CYP17A1 inhibitor abiraterone (Figure 14A). Analyzing Figure 14A, the sensitivity of these cell lines to the selected drugs is quite dissimilar, especially for bicalutamide, enzalutamide and abiraterone. Given that 22RV1 cell line, besides expressing the full length AR, also expresses a AR truncated version (ARv)[282] (Figure 14B) which is constitutively active and generally associated with resistance to antiandrogens[32,51,64], explaining in this way the sensitivity differences among the two cell lines. Unexpectedly, 22RV1 cell line was more sensitive to abiraterone than LNCaP cells and both were unresponsive to flutamide (Figure 14A).

Through these assays we were able to determine the IC_{50} concentrations of each drug, when possible, being these values summarized in Table 6. Looking at these results it is extremely clear that Brachyury OE did not impact the response to these androgen targeted therapies, since no statistically significant differences on IC_{50} values between Empty and TBXT cells were found (Figure 14A and Table 6).

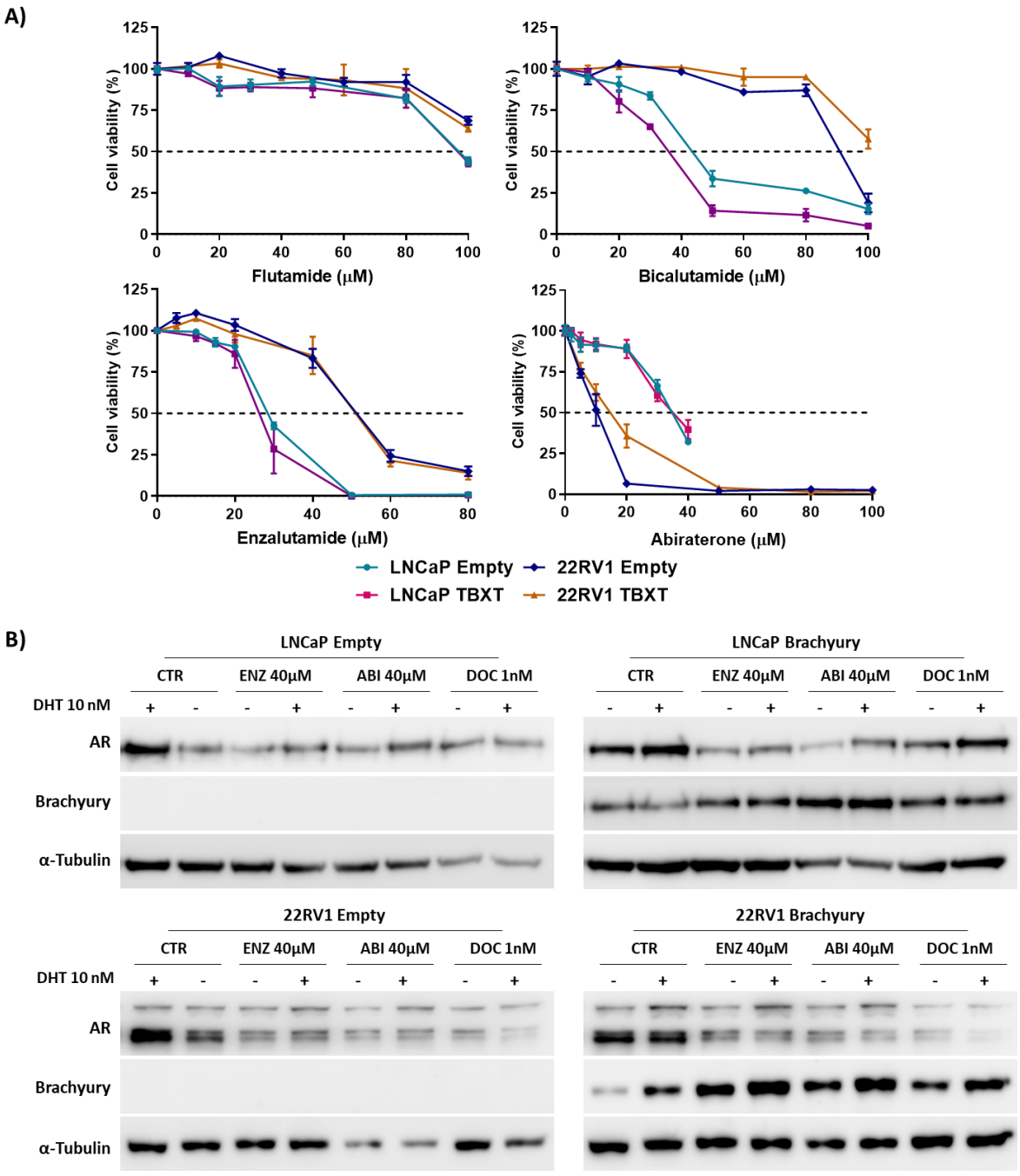

Figure 14. Evaluation of the response of our genetically modulated LNCaP and 22RV1 models to antiandrogen therapies. A) Cytotoxic assays in which Empty and TBXT cells were treated with increasing concentrations of flutamide, bicalutamide, enzalutamide and abiraterone for 72 hours, and the cell viability was measured by MTS assay. The graphs are represented as the mean ± SD (relative to DMSO alone) and are representative assays of at least two independent assays done in triplicate. B) Western blot analysis for both AR and Brachyury expression in LNCaP and 22RV1 transfected cells, upon a 24-hour treatment with the AR targeted drugs, enzalutamide (ENZ) and abiraterone (ABI), and the chemotherapeutic agent docetaxel (DOC). This analysis was done in duplicate under stimulating (+DHT, 10nM, 24 hours) and unstimulating conditions (-DHT). The western blots presented here are representative assays of two independent experiments, and tubulin was used as loading control.

Table 6. Mean IC_{50} values of antiandrogens in transfected PCa cell lines. IC_{50} values (μM) were expressed as the mean ± SD of, at least, two independent assays performed in triplicate.

Mean IC_{50} (μM)	LNCaP		22RV1	
	Empty	TBXT	Empty	TBXT
Flutamide	97.22±6.576	96.84±6.123	-	-
Bicalutamide	46.48±3.857	33.11±2.436	-	-
Enzalutamide	25.13±4.323	24.50±2.367	51.278±4.858	55.020±3.410
Abiraterone	34.36±2.027	34.99±2.265	10.474±1.720	11.129±2.829

In a way to further validate the previous findings and completely exclude the possibility of Brachyury being a predictive factor for antiandrogen therapies, we performed an western blot analysis (Figure 14B), in which the cells were treated with fixed doses of enzalutamide and abiraterone, that showed to be more potent than 1st generation antiandrogens (Figure 14A and Table 6), and analyzed both AR and Brachyury expression. As reflected in Figure 14B, it was confirmed that, at basal conditions (minus DHT), Brachyury increased both full-length AR in LNCaP cells and mainly ARv expression in 22RV1 cells, a variant that was not assessed before (Figure 13A). By this assay we were able to see that all drugs inhibited mainly the DHT-induced AR expression in both cell lines, being less efficient in TBXT cells, which retained more AR expression upon inhibition. Additionally, and as an experiment control, we also treated the cells with 1 nM of docetaxel, a chemotherapeutic drug to which our group already identified Brachyury as a resistance biomarker[213]. In that sense it was expected to see a significant reduction of AR expression upon docetaxel treatment, as we confirmed, and a more reluctance to AR downregulation in TBXT cells, which was evident in LNCaP but not in 22RV1 cells (Figure 14B). All the results were analyzed considering the tubulin expression differences we had.

Thus, with these assays we were able to demonstrate that even TBXT cells being more resistant to AR expression inhibition upon treatment, this seems to be insufficient to alter the cytotoxic effect of these drugs in AR-dependent cell lines, at least *in vitro*.

4.2. Biological and predictive role of Brachyury in Lung Cancer

4.2.1. Brachyury impact in lung cancer aggressiveness *in vitro*

In the first main aim of this project, it was also aimed to dissect the biological role of Brachyury in LC, since it is a tumor model of interest in the group and for which the existent literature is not concordant (Table 2)[185,193,204,205,210,218,222,223,283]. For that we used a panel of LC cell lines with different genetic backgrounds, as described in the Materials and Methods section (Table 3), and characterized them for

Brachyury expression (Figure 15). Similar to what was done in PCa, we herein also aimed to dissect how the most important oncogenic driver and therapeutic target in LC, EGFR, varies with Brachyury expression. In this first analysis (Figure 15A) it was verified that all cell lines expressed low basal levels of Brachyury protein, except H1975. All of them are positive for EGFR expression, having *EGFR* mutant cell lines activation of the receptor (p-EGFR), as expected.

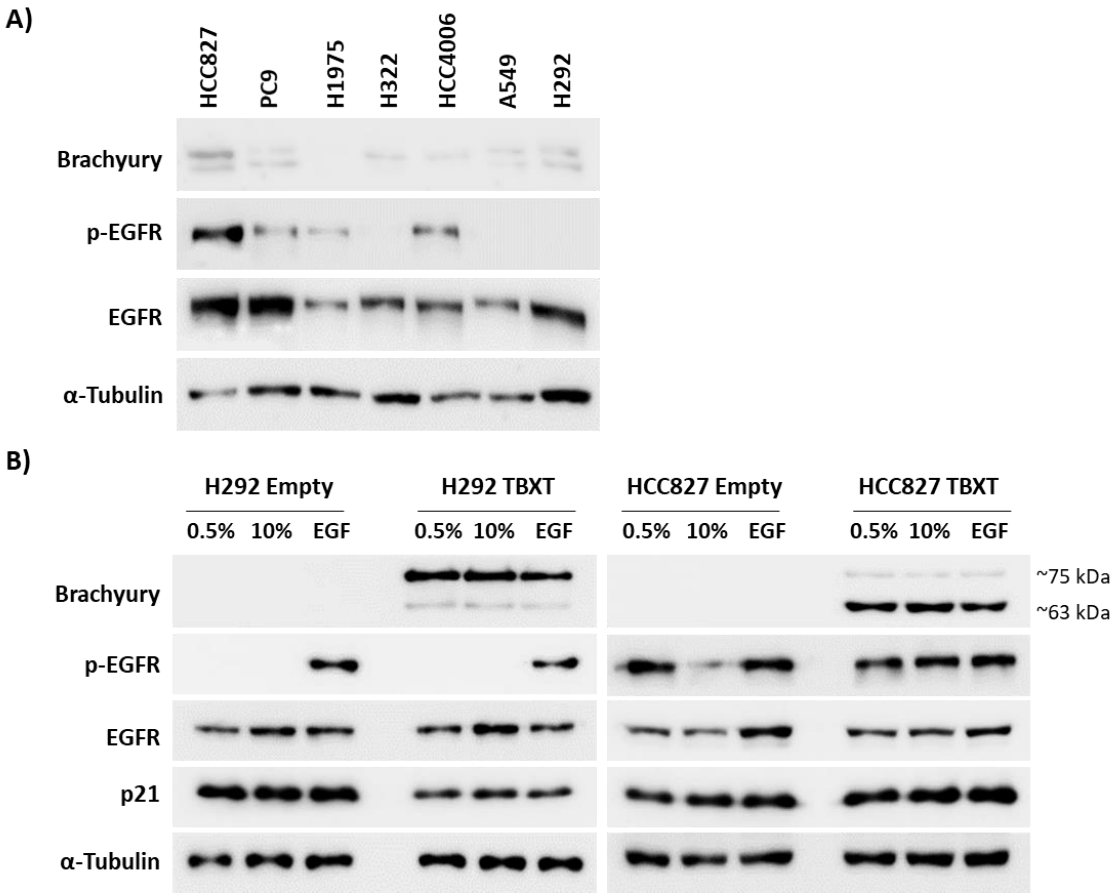

Figure 15. Western blot analysis of Brachyury and EGFR expression in LC cell lines. A) Assessment of Brachyury, EGFR and p-EGFR expression in our panel of LC cell lines at basal conditions. B) Validation of transfection in H292 and HCC827 cells and evaluation of its impact on EGFR levels and its activation (p-EGFR) as well as in the Brachyury downstream target p21. This analysis was carried out in three different conditions, 0.5% FBS, 10% FBS media and in EGF (10 ng/ml) stimulating conditions (15 minutes). Representative assays of at least two independent experiments. Tubulin was used as loading control.

Next, we chose two cell lines, H292 (*EGFR* WT) and HCC827 (*EGFR* mutant), to overexpress Brachyury and to move forward to the *in vitro* biological assays. To confirm the success of the transfection, we first analyzed Brachyury expression in the three different culture conditions cited above, and as it can be noted in Figure 15B, the cells were successfully transfected. Furthermore, given that p21 was described as a direct target of Brachyury in lung cancer[204], we also analyzed its expression as an additional confirmation of transfection assurance. In agreement with what was described by one group of

investigators[204,283], our results demonstrated a negative association between Brachyury and p21 expression in H292 cells, but for HCC827 no differences were found.

It is interesting to note that, in contrast to our PCa models (Figure 13A and 13B), LC cell lines expressed more than one Brachyury isoform (Figure 15B), even though being transfected with full-length Brachyury cDNA: H292 TBXT cells expressed higher levels of the long Brachyury isoform (~75kDa) whilst HCC827 TBXT expressed higher levels of the short isoform (~63kDa). Additionally, we can infer that the culture conditions tested did not affect Brachyury expression levels in both cell lines. Concerning EGFR expression, in H292 cells, the receptor was only activated upon EGF stimulation, while in the HCC827 *EGFR* mutant cell line was activated in all conditions, as expected. Comparing EGFR and p-EGFR expression levels between clones, no differences were observed between TBXT and Empty cells.

In addition, to further dissect the biological role of Brachyury in LC, we proceeded to assess the impact of its OE in LC aggressiveness *in vitro*, through viability, migration and colony formation assays (Figure 16). As illustrated by our results, Brachyury OE in H292 cell line conferred a significant advantage in cell viability (Figure 16A) and migration (Figure 16B) overtime, as well as in colonies formation (Figure 16C). Unfortunately, due to time constrains and being a harder cell line to work with, we were unable to validate these results with HCC827 cell line.

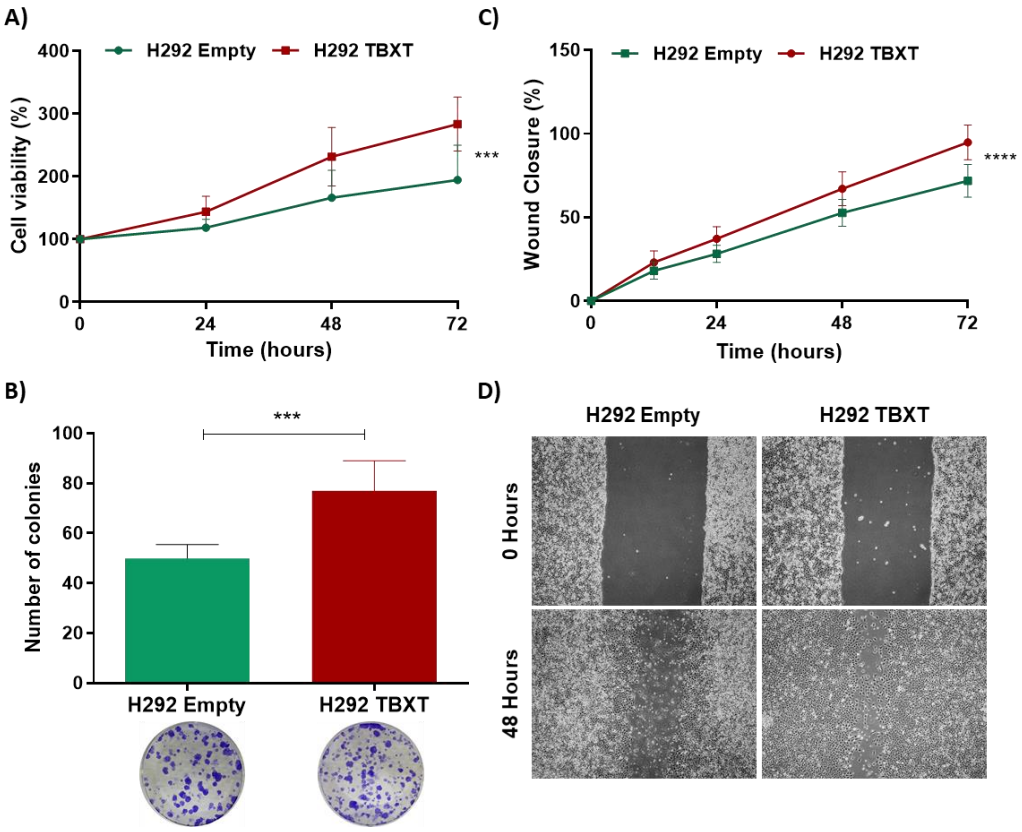

Figure 16. *In vitro* assessment of the Brachyury-associated phenotype in H292 transfected cell line. A) Cellular viability was measured by MTS assay (N=4) overtime (24, 48 and 72 hours timepoints). B) Wound healing migration assay (N=4) and the respective wound closure was evaluated by phase contrast microscopy overtime (12, 24, 48 and 72 hours timepoints) and determined through the calculated relative migration distance. C) Colony formation ability was assessed by clonogenicity assay (N=1) in which the multicellular colonies were photographed after 10 days. D) Representative photographs of wound healing migration assays at the 0- and 48-hours timepoints. All assays were performed in 0.5% FBS DMEM media condition.

Given the strong association between Brachyury and EMT described in the literature[195,204,205,210], we also evaluated the impact of Brachyury OE in EMT-associated molecules in H292 and HCC827 cells in the above cited culture conditions, through RT-qPCR and Western Blot (Figure 17). Analyzing Figure 17A, H292 cell line expresse both epithelial (ZO1 and β-catenin) and mesenchymal (N-cadherin and vimentin) proteins and Brachyury OE barely had an impact in these proteins, excepting for vimentin for which a slight upregulation was noted at 10% FBS condition. Regarding HCC827 cell line, it also expresses epithelial (E-cadherin and β-catenin) proteins but only the mesenchymal marker Snail, not expressing N-cadherin neither vimentin, representing in this way a more epithelial-like model. Concerning the effect of Brachyury OE in these proteins the only difference that can be observed between Empty and TBXT cells was the sharp increase of Snail levels, which strongly points for a potential EMT induction (Figure 17B). Finally, as for PCa, we also studied some FA associated proteins (FAK, vinculin and paxilin), but no differences were found (Figure 17A and 17B).

At mRNA level, in H292 cell line (Figure 17C), the picture was quite different: Snail and Slug were detected and increased after Brachyury upregulation, which is in agreement to the role of EMT inducer of this transcription factor; in agreement with the Western Blot results (Figure 17A), the N-cadherin levels remain unchanged in TBXT cells; however, upon Brachyury OE a tremendous increase in the epithelial marker E-cadherin was detected, which besides disagreeing to the information provided by the literature it also did not translate to the protein level, suggesting a post transcriptional mechanism that prevents mRNA to be translated (Figure 17C); finally it can also be noted a slight decrease in vimentin mRNA in TBXT cells, however given the results obtained at the protein level, this result is certainly not relevant as the qPCR was done only once. Moreover, since Brachyury has been associated with stemness and CSCs[200,211,212] we also assessed the expression of some stemness related genes (Nanog, Sox2 and Oct4) in H292 Empty and TBXT cells (Figure 17C). Looking closer at these results and in accordance to what it has been described, the levels of these genes were upregulated in TBXT cells.

Altogether, the results from this part of the work, strongly point to an oncogenic role of Brachyury in LC, in which EMT regulation and stemness induction could be some of the mechanisms behind the aggressive phenotype of Brachyury-overexpressing LC cells.

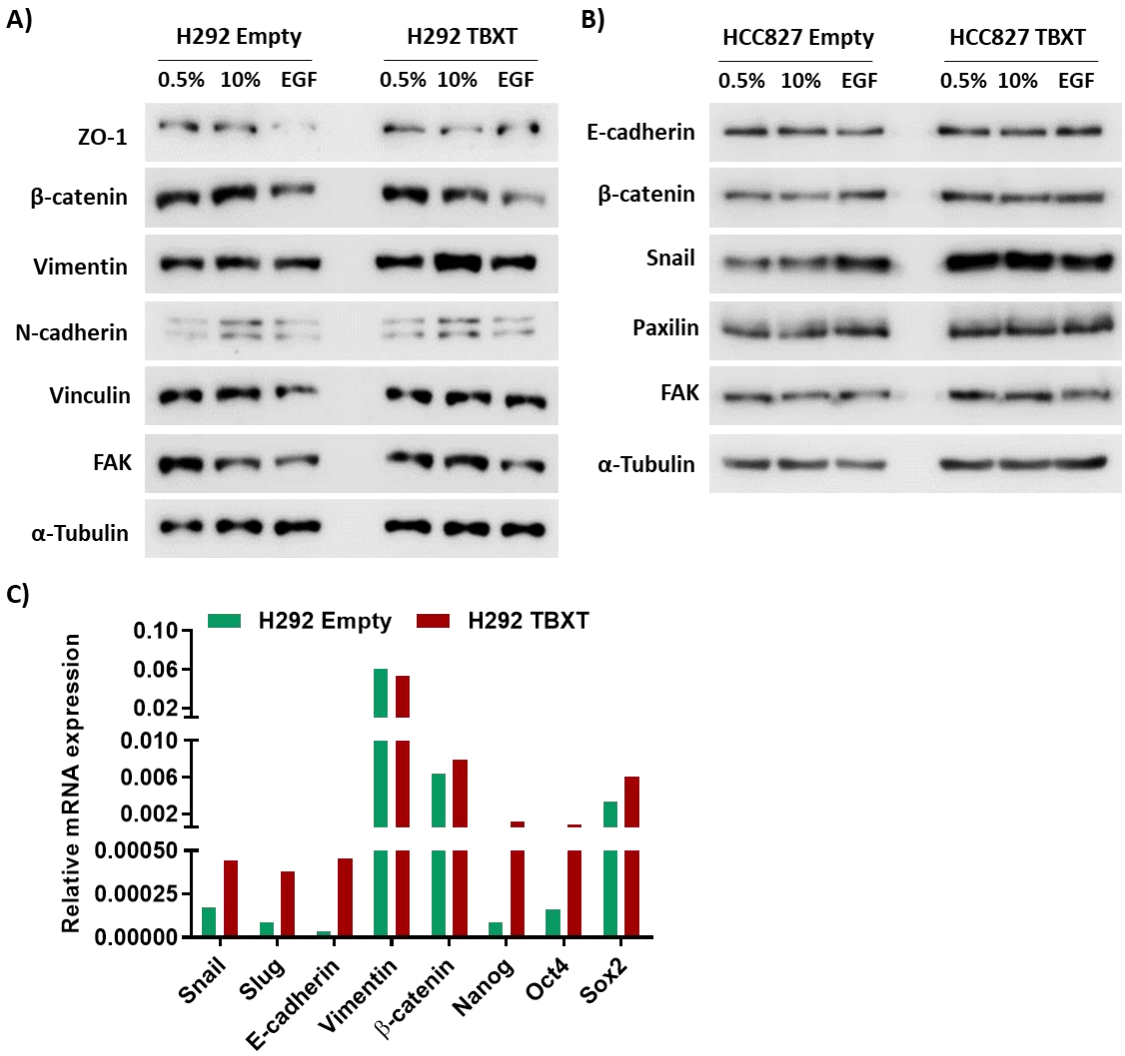

Figure 17. Characterization of FA-, EMT- and stemness-associated molecules in transfected LC cells. A, B) Western blot analysis of several EMT related proteins such as epithelial (ZO1, β-catenin and E-cadherin) and mesenchymal markers (N-cadherin, Vimentin) and one of the key players in EMT regulation (Snail) and also FA associated proteins (FAK, vinculin, paxillin) in H292 (A) and HCC827 (B) transfected cells, at basal and stimulating conditions (10% FBS and EGF 10ng/ml). Tubulin was used as loading control. C) Real time analysis (N=1) of EMT- and stemness-related genes in H292 Empty and TBXT cells (10% FBS growth condition). The experiment was done in triplicate and presented as relative expression in relation to β-actin.

4.2.2. Brachyury's effect in the modulation of lung cancer cells response to EGFR inhibitors

To conclude this section of the work concerning the LC model, we aimed to dissect whether Brachyury can modulate LC cells response to EGFR targeted therapies. For that we chose to use some clinically relevant EGFR inhibitors, such as 2nd and 3rd generation EGFR inhibitors, namely afatinib, AST1306 (allitinib) and osimertinib, respectively. Cytotoxic assays were performed using those drugs in

Empty and TBXT clones of both H292 and HCC827 cell lines (Figure 18). As evidenced in Figure 18, the two cell lines are differentially sensitive to EGFR inhibitors, which is in accordance to their *EGFR* mutational status, with HCC827 cell line being more responsive. Concerning the drugs actions, AST1306 was the most potent drug to H292 cell line, with a lower IC_{50} value (Table 7), while for HCC827, afatinib was the one that demonstrated to be the most potent, not excluding the possibility of osimertinib being as potent as the latter mentioned 2nd generation EGFR inhibitor since it was not possible to determine the osimertinib IC_{50} value (Figure 18 and Table 7). Comparing the response between Empty and TBXT cells, no differences were detected, except in the case of AST1306 inhibitor, for which H292 TBXT cells showed a significantly higher IC_{50} value than Empty cells (Figure 18B and Table 7). It should be noted that only one assay was performed with the HCC827 cell line and that the dose scale for afatinib and osimertinib drugs are not fully optimized, but even so these preliminary data (Figure 18A) showed that Brachyury had no effect on HCC827 cells response to EGFR targeted inhibitors.

Table 7. Mean IC_{50} values for afatinib, AST1306 and osimertinib in transfected LC cell lines. For H292 cells, IC_{50} values (μM) are presented as the mean ± SD of, at least, three independent assays, whilst the IC_{50} values (nM) for HCC827 cells were determined from only one assay. All assays were performed in triplicate.

Mean IC_{50}	**H292 (μM)**		**HCC827 (nM)**	
	Empty	TBXT	Empty	TBXT
Afatinib	2.599±0.219	2.606±0.558	10.59	10.36
AST1306	1.422±0.222	2.324±0.272	64.95	37.72
Osimertinib	2.840±0.403	2.436±0.276	<100	<100

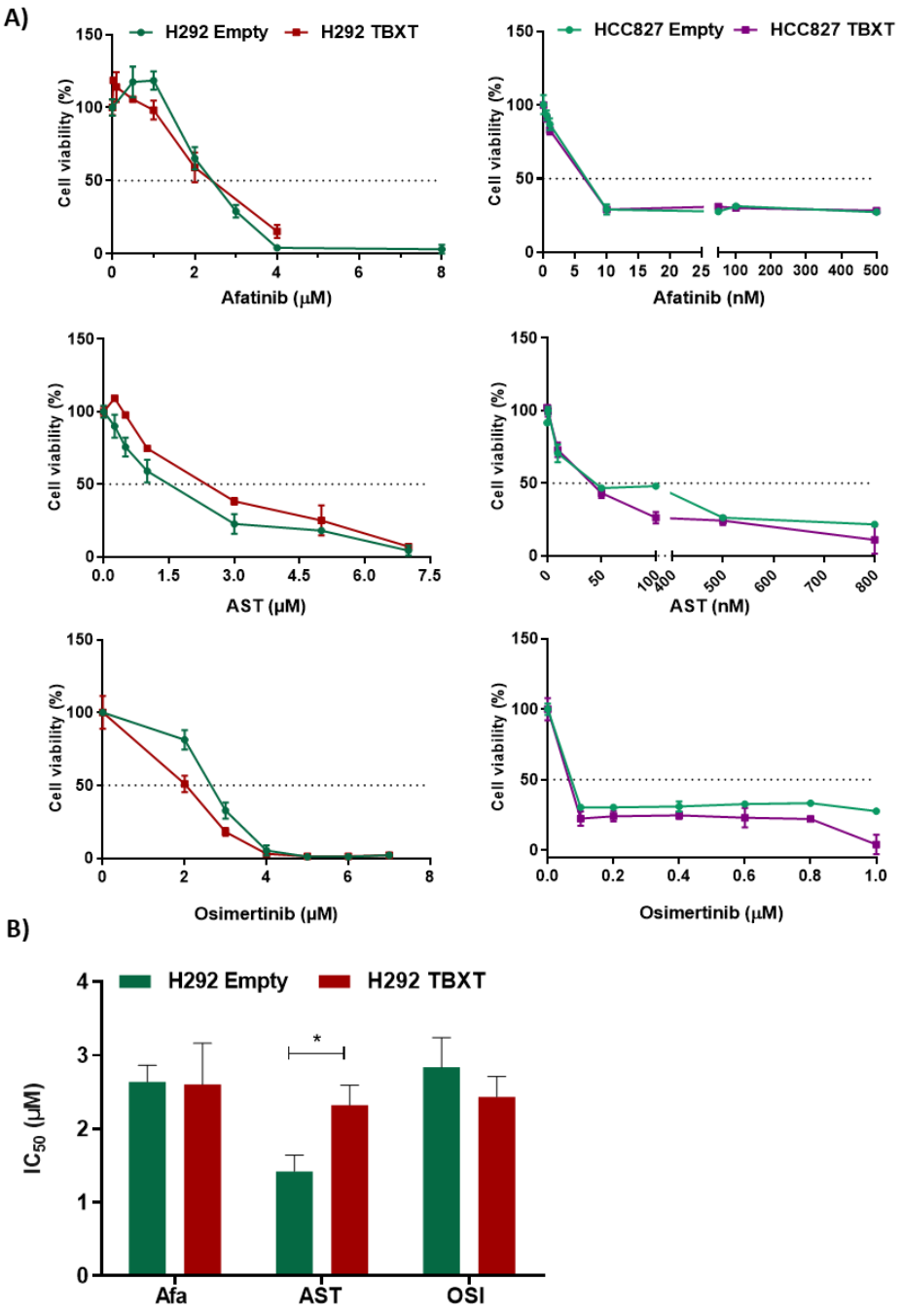

Figure 18. Evaluation of our genetically modulated H292 and HCC827 LC models response to EGFR targeted therapies. A) To assess the cytotoxicity of afatinib, AST1306 and Osimertinib, Empty and TBXT cells were treated with increasing concentrations of these drugs for 72 hours, and the cell viability was measured by MTS assay. The graphs are represented as the mean±SD (relative to DMSO alone) and are representative assays of three independent assays for H292 cells, done in triplicate. B) Comparative analysis of EGFR inhibitors IC_{50} values for Empty and TBXT H292 cells.

Given the differences of response between Empty and TBXT cells, in particular with AST1306 in H292 cells, we next aimed at giving some insight regarding the mechanisms underlying this divergent response, by determining the basal activation levels of EGFR signaling, namely AKT and MAPK pathways, in our genetically modulated H292 and HCC827 models and under different culture conditions (Figure 19). As expected, in FBS and EGF stimulating conditions the levels of p-AKT, p-MEK1/2 and p-ERK1/2 were upregulated compared to basal conditions (0.5% FBS medium). Furthermore, as previously mentioned, HCC827 cell is *EGFR* mutant, having a constitutive activation of both EGFR (Figure 15B) and its signaling compared to H292 cells, *EGFR* WT (Figure 19). Focusing on H292 cell line results, it can be noted that TBXT had higher levels of AKT, MEK1/2 and ERK1/2 activation, even at basal conditions. When looking at HCC827 cells results, they also present the same tendency concerning p-ERK1/2 and p-MEK1/2 , but herein TBXT cells did not present increased levels of p-AKT when compared to the control Empty cells (Figure 19). Thus, the overactivation of AKT found in H292 TBXT, but not in HCC827 TBXT cells, suggests us that AKT pathway overactivation could be one of the major players underlying the lower responsiveness of H292 TBXT cells to AST1306 inhibitor.

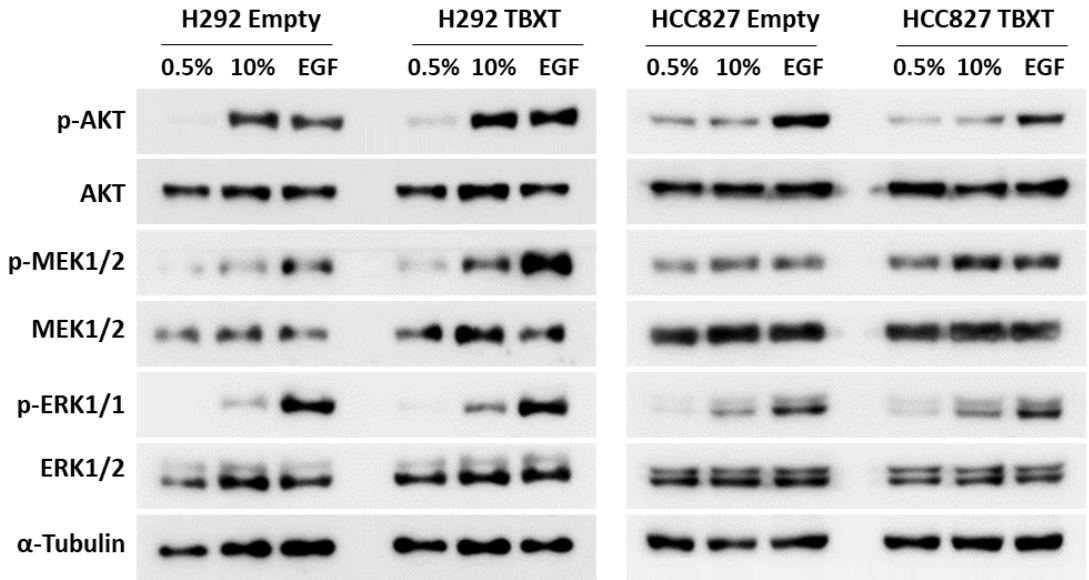

Figure 19. Characterization of EGFR signaling in H292 and HCC827 transfected cell lines. The activation of some AKT and MAPK pathways proteins, namely AKT, MEK1/2 and ERK1/2, was analyzed by western blot at 0.5% FBS, 10% FBS and EGF (10 ng/ml) stimulating conditions (15 minutes). Representative assays of two independent experiments. Tubulin was used as loading control.

Taken altogether, LC cells with Brachyury overexpression have an overactivation of important signaling pathways in this pathology, which can result in a lower sensitivity of these cells to EGFR inhibitors. However, these are preliminary studies that deserve to be dissected in the near future.

4.3. Targeting Brachyury expression in cancer

4.3.1. *In vitro* evaluation of the impact of small molecule inhibitors in Brachyury expression

Given the recent findings of two small molecule inhibitors, afatinib and THZ1, that can downregulate Brachyury expression in chordoma[183,237], we aimed to study whether this transcription factor could also be pharmacological inhibited in PCa and LC models, in order to revert its oncogenic potential in both tumor models.

To do so, we first used two colon cancer cell lines, SW620 and SW480, that endogenously express high levels of our molecule of interest[195,212,284], to validate the results obtained in chordoma[183,237]. These cell lines were treated with both drugs in three different concentrations, that were chosen accordingly to the literature, and Brachyury protein levels were evaluated by Western Blot. As reflected in Figure 20A, both THZ1 and afatinib significantly decreased Brachyury protein levels in SW620 cell line, while for SW480 THZ1 was far more effective comparing to afatinib, suggesting that the modulation of Brachyury expression by these drugs could be cell line specific (Figure 20A).

Next, we move on to our genetically modulated PCa and LC models to assess the specificity of these drugs in downregulating the ectopically-induced Brachyury expression (Figure 20B and 20C). Since our major aim from the beginning was to exploit the potential predictive role of Brachyury in the therapeutic response to antiandrogens and anti-EGFR drugs, being its role in the regulation of AR and EGFR expression always assessed, we herein decided to firstly proceed only with the cell lines that are strongly positive for those targets, LNCaP and HCC827, respectively. Focusing on our PCa model (Figure 20B), and as it can be noted, afatinib was more effective than THZ1 at targeting Brachyury, since that only the highest concentration of THZ1 used was able to decrease Brachyury expression levels. Looking at AR, it is possible to observe that these drugs can also affect its expression, both in Empty and TBXT cells, but more strongly in TBXT cells in which Brachyury was concomitantly inhibited. These findings can somehow validate that in fact Brachyury modulation has an impact in AR expression (Figure 20B).

Concerning our LC model (Figure 20C) and in contrary to the results obtained with LNCaP cells, Brachyury was markedly more efficiently targeted by THZ1 than afatinib, for which the effect was similar to the one obtained with LNCaP cells (Figure 20B). However, it is important to note that both drugs were more effective at downregulating the shorter isoform of Brachyury than its full-length form. Additionally, afatinib completely abrogated the activation of EGFR in both Empty and TBXT cells, as expected. Likewise, it seems that THZ1 downactivated EGFR, but only in TBXT cells when Brachyury expression was

completely abolished (Figure 20C). We have previously shown that TBXT cells had an overactivation of ERK1/2 and upregulation of Snail, compared to the Empty cells (Figures 17B and 19) and herein we validate those findings, and further verified that TBXT cells retained a basal p-ERK1/2 expression, even in depletion conditions upon afatinib treatment. Similarly, both drugs were able to downregulate Snail expression, with lower efficacy in Brachyury-overexpressing cells. Intriguingly, we can see that THZ1 treatment stimulated ERK1/2 activation in both Empty and TBXT cells, a phenomenon that was already evidenced by other authors[285].

Thus, with this analysis we were able to conclude that these small molecule inhibitors are capable to target Brachyury, expressed endogenously and ectopically, in other models apart from chordoma. Furthermore, the results obtained here demonstrated that the Brachyury targeting of both drugs is probably dependent on the cell line genetic background. Finally, we were able to validate that Brachyury upregulated EGFR signaling, Snail and AR expression, and demonstrate that even though not being Brachyury specific, THZ1 and afatinib can somehow be used to revert the Brachyury-induced phenotype in both PCa and LC.

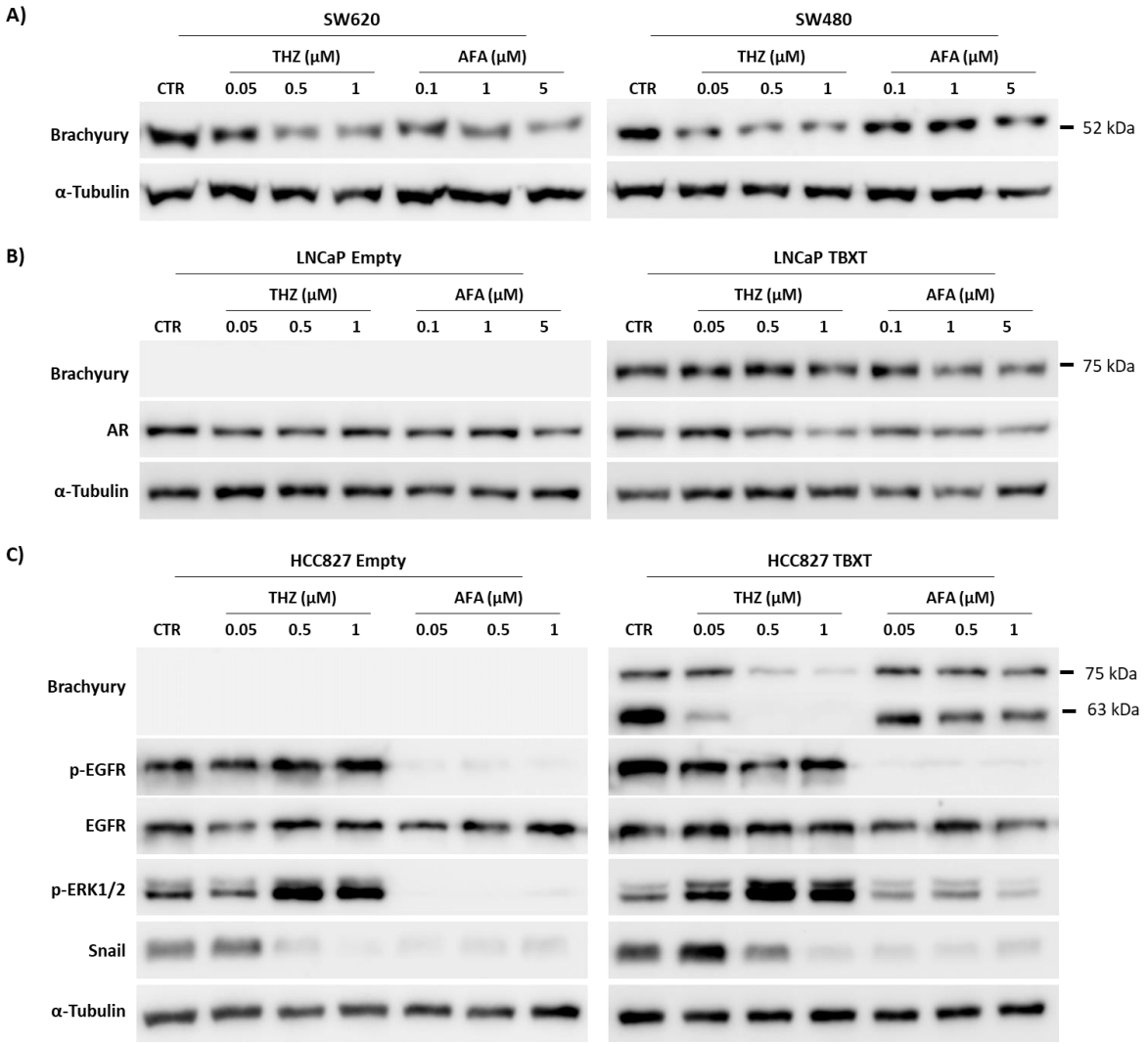

Figure 20. Analysis of Brachyury targeting by THZ1 and afatinib small molecule inhibitors in colon, prostate and lung cancer cells. A) Western blot analysis of endogenously Brachyury expression in the colon cancer cell lines SW620 (left) and SW480 (right) upon a 24-hour treatment with fixed concentrations of THZ1 and afatinib (AFA). B, C) Evaluation of ectopic Brachyury expression, and other relevant proteins associated with the Brachyury-induced phenotype (AR, EGFR, p-EGFR, Snail, p-ERK1/2), then again upon treatment with THZ1 and AFA in LNCaP (B) and HCC827 (C) cells. These are representative western blots of two independent experiments. Tubulin was used as loading control.

4.3.2. Development of zeolite and mesoporous silica based drug delivery systems

Due to the promising results obtained before, in this particular part of the work we intended to encapsulate both afatinib and THZ1 in zeolite and mesoporous silica, to improve the delivery of these drugs to cancer cells. For that, we chose three different silica porous materials, NaY, MCM-41 and SBA-15 with different size pores to evaluate which material would be capable to host higher quantities of the drug, in order to increase the efficacy of the respective DDS. First, we went ahead to evaluate the toxicity of the chosen parental materials in HCC827 cell line in a wide range of concentrations (0.025-0.75

mg/ml) and proved the biocompatibility of NaY, MCM-41 and SBA-15 materials, with no cytotoxicity observed (Figure 21A). It is noteworthy to mention that we started with limited available quantities of each drug and as such only two materials were further selected to function as matrixes for the development of THZ1 and afatinib DDS. Having in consideration the chemical structure of both drugs (Figure 11) and the porosity of the materials, we chose NaY and SBA-15 to entrap afatinib, which will allow to compare between zeolite and mesoporous silica structures; and the mesoporous silica materials MCM-41 and SBA-15 as hosts for THZ1, which in terms of chemical structure is larger than afatinib.

Following the development of those DDS we evaluated the cellular viability of HCC827 cells after 72 hours of treatment with increasing concentrations of THZ1 and afatinib DDS. Altogether, the results obtained demonstrated the successful development of these DDS, in other words, these host structures were able to entrap the selected drugs and were able to deliver them to the cells, ultimately resulting in a decrease of cellular viability (Figure 21B and 21C). The decrease of viability was higher with the afatinib DDS (Figure 21C) which can be due to the higher starter quantities of afatinib used in the development of the respective DDS or due to the higher sensitivity of HCC827 cell line to afatinib compared to THZ1. Given that we hadn't yet determined the real doses of afatinib and THZ1 entrapped in these DDS we can't conclude about which was the best host material and the efficacy of the DDS compared to the free drugs. So, further characterization work of the samples is needed in order to understand the host-guest interactions of the drugs and the silica porous materials and their viability as DDS for cancer therapy.

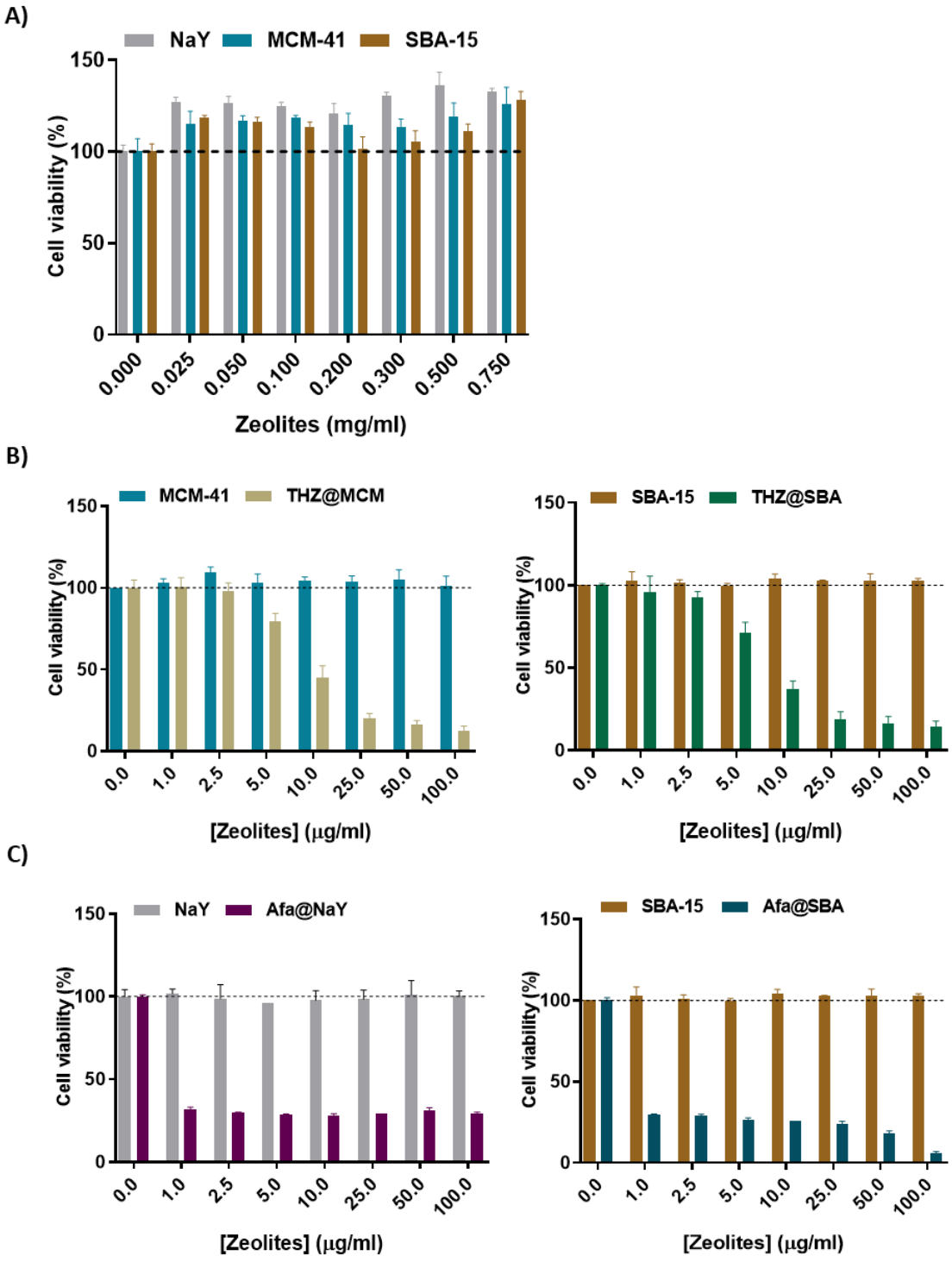

Figure 21. Biocompatibility of NaY, MCM-41 and SBA-15 materials and development of THZ1 and afatinib DDS. A) The toxicity of each parental material, NaY, MCM-41 and SBA-15, was assessed in HCC827 cell line by MTS assay upon a 72-hour incubation with increasing doses of the respective nanomaterial. B, C) THZ1 (THZ@MCM and THZ@SBA) and afatinib (Afa@NaY and Afa@SBA) DDS were developed. Their impact on cell viability was evaluated in HCC827 cells by MTS assay after a 72-hour treatment with both THZ1 (B) and afatinib (C) DDS. These are representative assays of at least four independent assays done in triplicate, being the graphs represented as the mean±SD, relative to medium alone (100% viability).

CHAPTER 5:

General Discussion

The T-box transcription factor Brachyury was first described and extensively studied in embryonic development context[156-158,162-168], but more recently has been implicated in cancer as well[176-183]. Brachyury is overexpressed in a variety of human tumors, including lung, breast, colon, prostate, hepatocellular and oral squamous cell carcinomas, chordomas, hemangioblastomas, GISTs and high-risk testicular germ cell tumors[181,185-197,207,209,210]. This transcription factor was described as an EMT driver[186,187,189,190,193,196,197,204,205,207,208,210,211] and associated with metastization and radio- and chemotherapy resistance in several malignant neoplasms[186,187,190,193,198,203-205,210,213,214]. Furthermore, Brachyury is preferentially expressed in human tumors vs. normal adult tissues[184-189], and high levels of this molecule associate with poor prognosis in a number of neoplasms such as HCC, GISTs, high-risk testicular germ cell tumors, oral squamous cell carcinoma, colorectal, breast, lung and prostate cancers[185-187,189-194,207,209,210]. In the specific case of PCa, our group provided some evidence that this transcription factor might be a regulator of AR[213] and regarding LC, it was suggested that Brachyury could be associated with resistance to EGFR targeted therapy[185]. With this in mind, the major aim of this work was first to dissect whether Brachyury can in fact be a predictor of response to antiandrogens and EGFR targeted therapies in PCa and LC, respectively, and secondly determine the potentiality of this transcription factor to be a therapeutic target in these two models.

PCa is one of the most incident cancers and is responsible for a great number of cancer-associated deaths among men worldwide[1]. The gold standard treatment for advanced PCa is ADT or CAB[28,32,47], and even though these hormonal therapies are effective in the vast majority of cases, especially at an initial phase, it is not curative and therapy resistance is developed ultimately leading to CRPC[32,51]. CRPC is a lethal form of PCa, and despite all the efforts made by the scientific community to the development of newer drugs, most of the patients eventually relapse[63]. In this sense, biomarkers that can predict the emergence of these therapy resistance mechanisms are extremely needed as well as new therapeutic targets to potentially prevent CRPC progression. As previously mentioned, given the role of Brachyury in PCa reported by our group[190,213], namely in PCa aggressiveness *in vitro*, therapy resistance and poor prognosis, it is clear that this transcription factor comprises an interesting new potential biomarker and therapeutic target in this malignancy.

Our group[190] and other study[195], already reported a characterization of Brachyury expression in a panel of PCa cell lines, in which PC3, DU145 and LNCaP were positive for Brachyury expression and the primary tumor derived 22RV1 cell line was negative. Given that we aimed to study the impact of this transcription factor in the cells response to antiandrogen therapies we chose two androgen-dependent

models, LNCaP and 22RV1, and genetically modulated these cells to overexpress Brachyury (TBXT cells). First, we began to validate the success of this OE by evaluating the Brachyury expression post-transfection, showing that indeed an OE of our molecule of interest was accomplished, becoming restricted to the nucleus. As previously stated, in this work we used a different expression vector, than of the previous reports[190,213], to attain the OE Brachyury. Given this, and in a way to validate the functionality of our genetic modulation we tried to recapitulate the previous findings regarding the Brachyury-mediated EMT in 22RV1 cells[190] but this time at a protein level, through a western blot analysis. Unfortunately, this epithelial cell line does not express much EMT-associated proteins, and our results evidenced that TBXT cells have diminished levels of the epithelial markers E-cadherin and β-catenin, which indicate, even at a low extent, a possible EMT induction, which is in accordance to the group's previous work[190]. Overall, the 22RV1 results together with the Brachyury's nucleus location obtained for LNCaP TBXT cells, are suggestive of a functional transfection.

Secondly, given the association between Brachyury and EMT in PCa[190,213] and other malignancies[186,187,189,193,195-197,204,205,207,208,210,211], we analyzed the expression of EMT- and focal adhesion (FA)-related molecules, at protein and mRNA levels, in the LNCaP cell line, a model in which Brachyury was never genetically modulated by our group or others. This specific cell line expresses both epithelial (E-cadherin and β-catenin) and mesenchymal (Vimentin) traits, and in fact the EMT program in cancer is strongly linked to EMT partial states in which cells gain a more mesenchymal phenotype while retaining some epithelial characteristics[69,70,81], and as such our analysis in the metastatic LNCaP cell line suggested that TBXT cells might represent a partial EMT state. When comparing Empty and TBXT clones we came across some interesting findings. β-catenin was slightly upregulated at mRNA and protein levels in Brachyury-overexpressing cells and despite being considered an epithelial marker it is also an effector of the Wnt/β-catenin signaling pathway that has been associated not only with the EMT process[69,70,286] but also with Brachyury[216,217]. It should be noted that Brachyury is linked to Wnt pathway in normal embryonic development[216,217], which might happen in a neoplastic context as well, however to the best of our knowledge no study addressed this association in cancer. However, we hypothesized that higher levels of Brachyury might lead to an overactivation of Wnt/β-catenin signaling, which explains the observed increase in β-catenin. It would be valuable to evaluate the cellular localization of β-catenin in Empty and TBXT cells given that if an increase of β-catenin at a cytoplasmic level, acting with E-cadherin in cell to cell adhesion, rather than in the nucleus was registered our hypothesis of Brachyury/β-catenin/EMT association would be discarded. In addition, Wnt/β-catenin and TGF-β are crosstalking pathways[70,287,288], and interestingly Larocca et al., using prostate and lung cancer cell lines, described an autoregulatory

positive feedback loop between Brachyury and TGF-β1 that induces EMT[214]. Given this report, we can also hypothesize that the upregulation of Brachyury in LNCaP cells leads to a higher secretion of TGF-β1 cytokine leading to the activation of the TGF-β signaling and due to the crosstalk with Wnt/β-catenin pathway ultimately results in an increase and/or stabilization of β-catenin, and the underlying EMT induction.

Furthermore, our results demonstrated an increase in YY1 levels upon Brachyury OE. YY1 is a transcription factor often dysregulated in cancer, including PCa, and it has been associated with tumor progression, therapy resistance[289] and more importantly in the case of our work is connected to EMT[290,291]. Tong Yang et al, using a LNCaP subline, C4-2, showed that YY1 overexpression is associated with EMT-associated changes, such as upregulation of Vimentin, N-cadherin and Twist1[291]. Even more curiously, YY1 has been associated with TGF-β-mediated EMT in other tumor types[292,293], which might occur in PCa as well, however this issue was not addressed yet.

Taken altogether, our results demonstrating an upregulation of β-catenin, YY1 and Vimentin (in this last case only noted in the CTR group) upon Brachyury OE, and the possible mechanisms that could explain these results, namely the hypothesized Wnt/β-catenin pathway activation and the crosstalk with TGF- signaling, strongly point for an EMT partial state in LNCaP cells retaining the epithelial marker E-cadherin. In this regard, an increase in migration and invasion capabilities is expected, however since LNCaP cells do not form uniform monolayers the *in vitro* assays were not performed yet.

As abovementioned we also explored the expression of some FA-associated proteins, namely talin, FAK, paxillin and α-actinin in LNCaP transfected cells. It should be noted that FAs are clusters of integrins, ECM and cytoskeletal proteins, that are assembled and disassembled in a dynamic manner and are intimately connected with cancer migration, invasion and metastasis[294]. Talin, FAK, paxillin and α-actinin are indeed important proteins involved in FA formation, and interestingly enough this latter was already described as a Brachyury target in embryonic development of *Ciona intestinalis*[295]. Focusing in our results, we found a marked downregulation of talin in TBXT cells while FAK, paxillin and α-actinin levels remained unchanged. Talin is responsible for the link of integrins to the ECM through actin binding, has a role in the activation of integrin signaling and functions as a transducer of intra- and extracellular signals, being ultimately involved in mechanisms of cell adhesion, migration, invasion, anoikis, proliferation and survival[294]. As such, our results regarding talin, FAK, paxillin and α-actinin expression in TBXT cells suggest that these cells might have a faulty ability to form the mechanical link between integrins and the ECM rather than an impaired FA formation. Moreover, across the literature, the reports

regarding the specific role of talin in cancer are not concordant, in which some evidenced that its overexpression has been described in several malignancies and correlates to increased migration, invasion and resistance to anoikis[296], whilst others described the opposite findings[297,298]. Despite these contradictory observations and bearing in mind the connection of talin in cell migration and adhesion, our results of the Brachyury-mediated loss of talin in LNCaP cells might have some implications: first, regarding cell migration, our overall results are suggestive of an EMT partial state in LNCaP TBXT cells which could consequentiality be associated with an inherent higher migratory ability, so the lower levels of talin in these cells are in agreement with the reports that described that higher levels of talin are associated with impaired cell motility[297,298]; second and as abovementioned, talin serves as a mechanotransducer for intra- and extracellular signals, and the evidenced Brachyruy-mediated talin downregulation might imply a more insensitive state for those signals.

In agreement to the previous findings of the group[213], we demonstrated that not only 22RV1, but also LNCaP Brachyury-overexpressing cells, had upregulated levels of AR expression. Herein we further proved that Brachyury, in addition to upregulate the full-length AR in 22RV1 cells, it increases mainly the expression of a ARv, which was not assessed in the previous study[213]. Overall, these first results gave strength to our initial hypothesis and we confidently proceeded to evaluate the impact of Brachyury in the response of these PCa cells to flutamide, bicalutamide, enzalutamide and abiraterone treatment. Flutamide and bicalutamide belong to the 1st generation of anti-AR and possess relatively weak affinity to the AR compared to the 2nd generation of antiandrogens such as enzalutamide[49]. Abiraterone, with a different mechanism of action, is an inhibitor of the CYP17A enzyme, and is capable of preventing adrenal and intratumoral androgen synthesis[32,34]. Given the differences between the mechanisms of action of these drugs, it was indeed expected a higher sensitivity of the cells to the newer therapies enzalutamide and abiraterone. Additionally, as previously mentioned, 22RV1 is an androgen-dependent model that co-expresses the full length AR and a ARv[282], which is associated to a lower responsive phenotype[32,51,64]. In accordance, our results in general mirror the expected sensitivity differences between the treatment with the various drugs and also between LNCaP and 22RV1 cells, except in the case of abiraterone, for who 22RV1 cell line was more sensitive, as described before[299]. Moreover, despite the general higher sensitivity of LNCaP cells to antiandrogen therapies, this cell line was, as 22RV1 cell line, resistant to flutamide which is in agreement to the information provided by the literature[32]. LNCaP is a T877A *AR* mutant which converts the AR antagonist flutamide in an agonist that stimulates and activates AR[32]. Unfortunately, and in contrary to what we were expecting, Brachyury OE did not interfered with the cells response to the therapies used here, with no differences found between Empty and TBXT cells in terms of IC_{50} values. By

western blot assay we were able to see that all drugs inhibited AR, mainly the DHT-induced AR expression, in both cell lines, being less efficient in TBXT cells, which retained higher levels of AR expression upon inhibition than the Empty ones. The lack of cytotoxic impact by Brachyury OE in response to antiandrogens might imply that despite the positive association of expression between this transcription factor and AR as well as the physical binding of these proteins that occur *in vitro*[213], is not sufficient to trigger therapy resistance at least *in vitro* and in a short timeframe of exposure. To further validate the lack of predictive value of Brachyury in antiandrogen therapies response it would be extremely relevant to perform *in vivo* assays. Furthermore, and even though our results demonstrated that the initial Brachyury levels does not impact the response of PCa cells to antiandrogen therapies it does not fully pin out the involvement of Brachyury alongside with other resistance mechanisms in the overall resistance that occurs in PCa patients. Indeed, our group already implicated Brachyury in PCa therapy resistance, specifically to the chemotherapeutic drug, docetaxel[213], and herein we expanded those findings in our novel PCa model, LNCaP in response to antiandrogens.

The case of LC is even more concerning than PCa, being the former the most diagnosed and the most fatal cancer worldwide[1], with the lower 5-year survival rate[92-94]. As a consequence of the incredible advances regarding molecular targeted therapies and personalized medicine in the field of oncology and specially in LC, most NSCLC patients have their tumor tested for molecular aberrations in several oncogenic drivers and have their treatment adapted to their molecular characteristics[95,101,105,107-110]. The most common oncogenic drivers in NSCLC, and in particular in ADC tumors, are *KRAS*, *EGFR* and *ALK* rearrangements[101,110]. In this work we focused on EGFR and the respective targeted therapies. Currently, three generations of EGFR inhibitors exist in a clinical context and their development improved indeed the OS and PFS of *EGFR* mutant patients when compared to the previous standard treatment, conventional chemotherapy[101,125-130,133,134]. Unfortunately, patients eventually acquire resistance to these molecular targeted therapies and relapse, and a plethora of underlying resistance mechanisms were unraveled[67,101,107,116,121,123,131,136,138,141,142,147,300,301]. As such, biomarkers of response to these kind of therapies, that would be valuable in the selection of the optimal population of patients that would greatly benefit treatment, and that might make possible the identification of patients more prone to develop resistance comprise an urgent unmet need. Furthermore, new therapeutic targets that can be used for combined therapeutic modalities in order to target and prevent the resistant phenotypes constitute another attractive endeavor in cancer research. In this regard, the molecule of interest of this work, Brachyury, comprises an attractive potential predictive biomarker and therapeutic target in NSCLC.

Brachyury (mRNA and protein) expression has been detected at different percentages, intensities and even in different subcellular localizations in lung tumors (summarized in Table 2), and several reasons might be behind these events, such as the specific cohort analyzed, the specific antibody and dilution used and even the technical approach. Altogether, these reports demonstrated that high Brachyury expression in LC, specially NSCLC, is associated with higher tumor stage and grade, lymphatic permeation, vascular invasion and poor prognosis[193,195,205,210]. Interestingly, Roselli and colleagues conducted one of the first and only studies regarding Brachyury in therapy resistance in a context of molecular targeted therapy, in particular anti-EGFR therapies[185]. This report, asides from the oncogenic properties of Brachyury and its role in EMT induction, was in fact the one that led us to explore the role of this transcription factor in resistance to EGFR-TKIs.

We started by characterizing our panel of NSCLC cell lines for Brachyury expression, and from the seven cell lines tested, all expressed very low levels of Brachyury protein, except H1975 cell line that was negative. From our panel of cell lines only A549 has been characterized for Brachyury expression, with some contradictory results, one study conducted by Hamilton et al[284] reported A549 as being negative for Brachyury mRNA expression, while three other studies reported this cell line as positive for Brachyury mRNA and/or protein expression at low levels[185,195,204]. For further experiments, we chose two cell lines, H292 and HCC827, for genetically overexpress Brachyury, because they are genetically dissimilar and both express high levels of total EGFR. Curiously, we observed that in these LC cell lines the genetic modulation resulted in upregulated levels of two Brachyury isoforms. Due to alternative mRNA splicing events, Brachyury has different isoforms (data from Ensembl), however until now the potential different roles of each isoform in cancer have not been extensively addressed[302]. Additionally, our results demonstrated a differential expression of Brachyury isoforms in H292 and HCC827, while H292 expressed higher levels of the long isoform, HCC827 showed higher levels of the short Brachyury isoform, which could be interesting to further dissect about its differential roles in the future.

Before moving to the main aim of this part of the work, first we dissected the biological role of this transcription factor in the chosen LC cell lines, given that were never used before in studies regarding Brachyury. By performing *in vitro* biological assays, and in agreement to other reports using LC cell lines[193,195,204,205], we demonstrated that Brachyury upregulation in H292 cell line resulted in a statistically significant increase in cell viability, migration and colonies formation. Furthermore, we evaluated the cyclin kinase inhibitor (CKI) p21 expression levels, which was reported as a direct target of Brachyury in LC, being Brachyury described as a repressor of p21[204]. As such, and in a way to further validate the

functionality of the transfection in our LC cell lines, we found the reported negative association between Brachyury and p21 in H292 TBXT cells, while in HCC827 TBXT cells no association was evidenced. Cyclin kinases are proteins required for cell cycle progression, and the CKI p21, a master p53 target is well known for its role as a negative cell cycle regulator[303]. The decreased levels of p21 of H292 TBXT cells, could suggest a positive effect in cell cycle progression and possibly explain the advantage in cell viability of these cells, however a cell cycle and apoptosis analysis would give us more insight regarding this matter especially since dissimilar results of Brachyury role in cell cycle has also been described[195,204,218].

Following this, as for PCa, we analyzed the expression of EMT- and FA-associated molecules at mRNA and protein levels upon transfection and while only an upregulation of Snail protein was found for HCC827 cell line, some expected and unexpected results were obtained for H292 cells. Given the significant increase of migration of H292 TBXT compared to Empty cells, it would be expected an induction of an EMT state, in other words, a decrease of epithelial markers and an increase of mesenchymal ones as well as EMT related transcription factors. In agreement to this, in H292 TBXT cells, in comparison to Empty cells, it was observed an increase in Snail and Slug mRNA and in Vimentin protein in the 10% FBS culture condition, while vimentin mRNA levels remained unchanged. Indeed, a positive association between Brachyury, Snail and Slug mRNA levels in NSCLC cell lines[195,204] as well as in tumor specimens[193] was already evidenced.

Additionally, we also evaluated the expression of Nanog, Oct4 and Sox2 at mRNA level, which are stem-related and pluripotent transcription factors highly associated with CSCs as well as with tumorigenesis, metastasis and resistance to therapy, including to EGFR-TKIs in ADC tumors[304,305]. More interestingly, Brachyury has been associated with these markers in several malignancies[211,212], including LC[200]. In accordance with these findings, in H292 TBXT cells there was an increase of mRNA levels of these CSCs markers, specially Nanog and Oct4, compared to Empty cells. These results imply that Brachyury might have a role in an induction of stemness in LC tumors, that could be a consequence of the induction of the EMT program itself or these pluripotency transcription factors could possibly be direct targets of Brachyury or only targets of its transcriptional program. Moreover, Chiou et al. reported a role of Nanog and Oct4 in EMT induction, besides their well-known role in stemness, which tightens even more the connection between stemness, EMT and the pluripotency transcription factors[306]. The fact that Brachyury has been associated with all these latter processes, makes it possible to realize that altogether our results are suggestive of an EMT induction, an increase in stemness and a more aggressive phenotype in H292 cells upon Brachyury OE. However, we had some unexpected results as well, such as no

alterations regarding N-cadherin protein levels, and also an increase in E-cadherin mRNA expression, which given that this cell line does not express this marker at a protein level and that the mRNA analysis was done only once, those results should be carefully analyzed and further validated. Unfortunately, we were not able to conduct the biological assays in HCC827 cell line, to confirm whether the aggressive Brachyury-mediated phenotype is dependent on p21 downregulation, or even EMT and stemness induction.

Finally, considering our interesting preliminary results and in order to study our hypothesis of Brachyury playing a role in EGFR-TKIs resistance, we explored the impact of Brachyury in the modulation of LC cells response to EGFR inhibitors. First, we evaluated EGFR expression and EGFR activation (p-EGFR) levels after transfection in three different culture conditions, for both H292 and HCC827, but no differences in total and phosphorylated EGFR levels were noted. As previously mentioned, a group of researchers already demonstrated the role of Brachyury in the modulation of response to EGFR targeted therapies[185], however it should be noted that in the mentioned study it was used two *KRAS* mutant NSCLC cell lines (A549 and H460), which are not the more adequate models to study EGFR targeted therapy given the *KRAS* predictive value of poor response to EGFR inhibitors[307,308]. In this work we intended to use more adequate cell lines, such as the *EGFR* WT H292 and the *EGFR* mutant HCC827, that constitute better models to study EGFR targeted therapy, specially HCC827 given that nowadays only *EGFR* mutant patients are clinically directed to this kind of treatment. Moreover, in the work conducted by Roselli and collaborators[185] it was used the AG1478 EGFR inhibitor, that possesses a similar structure and mechanism of action of the 1st generation EGFR inhibitors gefitinib and erlotinib, and then again to achieve more translational results, in this work we used more clinically relevant EGFR inhibitors, such as 2nd and 3rd generation EGFR inhibitors, namely afatinib, AST1306 (allitinib) and osimertinib. Even though allitinib is not widely used in LC at a clinical context, this inhibitor belongs to the 2nd generation of irreversible EGFR inhibitors and therefore is more potent than AG1478[307].

Focusing first in the H292 cell line, we were able to demonstrate that despite not being *EGFR* mutant, it is still very sensitive to these drugs with IC_{50} values ranging from 1.4-3.2 μM. Comparing the response between Empty and TBXT clones, our experiments demonstrated that Brachyury upregulation increased the resistance to AST1306 treatment with a statistically significant higher IC_{50} value in the TBXT clone, but this was not the case of afatinib and osimertinib. Regarding HCC827 cell line, as expected, these cells were highly sensitive to EGFR-TKIs compared to H292 cell line, with IC_{50} values in the nanomolar range (not determined in the osimertinib case). Bearing in mind that even though our results

came from only one assay and the drug scales of afatinib and osimertinib should be further optimized, this allowed us to speculate that Brachyury does not modulate the response to EGFR inhibitors, in *EGFR* mutant cell lines.

These dissimilarities between the effect of Brachyury in our two models led us to examine some possible underlying reasons, and to do so, we evaluated the expression of proteins involved in important oncogenic pathways such as, PI3K/AKT and MAPK/MEK/ERK, that constitute part of the downstream EGFR signaling. In H292 cell line it was clear that TBXT cells had elevated levels of p-AKT, p-MEK1/2 and p-ERK1/2, which suggests that Brachyury leads to an overactivation of PI3K/AKT and MAPK/MEK/ERK pathways. Furthermore, since these pathways are known for their role in growth, proliferation, migration and so on, these last results are in agreement with the previous observed increase in cell viability, migration and colony formation abilities upon Brachyury OE. Regarding HCC827 model and differently from what it was seen with the H292 cell line, in this *EGFR* mutant model, Brachyury OE resulted only in a slight increase in MAPK activation at basal and stimulating conditions. Curiously, when in depleting conditions, upon afatinib treatment, HCC827 TBXT cells still expressed basal levels of p-ERK1/2 in contrary to Empty cells despite the total abrogation of EGFR activation, corroborating the finding that Brachyury sustains MAPK activation. Furthermore, comparing H292 and HCC827 cells, in the last case, Brachyury OE did not increased the levels of p-AKT when compared to the control Empty cells, as it happened in H292 cell line. Thus, the overactivation of AKT found in H292 TBXT, but not in HCC827 TBXT cells, suggest us that AKT pathway overactivation could be the one of major players underlying the lower responsiveness of H292 TBXT cells to AST1306 inhibitor, as was already described before[309]

Altogether, the results showed that Brachyury OE induces aggressiveness, modulation of p21, EMT, stemness and EGFR signaling activation, which could be behind the lower responsiveness of the cells to AST1306 inhibitor in the *EGFR* WT cell line used here, while the same phenotype was not observed in HCC827 cell line. Regarding this subject, it is important to integrate our results of the relative expression of Brachyury isoforms in HCC827 TBXT cells, as well as the genetic and inherent characteristics of this specific cell line. In other words, the fact that these cells express the short isoform over the longer one, which have been proved to potentially have different roles in cell cycle and prognosis at least[302], could have had an impact on the different responses regarding EGFR inhibition we observed here, a finding that was the opposite of H292 TBXT cell line. In addition, we must keep in mind that we were limited in the number and type of assays done in HCC827 cell line, and as such we cannot totally exclude the possibility of a similar phenotype in *EGFR* mutant cell lines.

Transcription factors are often dysregulated in cancer and as such targeting these molecules that govern gene expression is a fundamental anticancer treatment. Nevertheless, given the subcellular location and lack of ligand binding domain/pocket of these molecules, strategies to specifically and effectively target most of transcription factors remained elusive until few years ago[310]. Even though no small molecule to direct target Brachyury has been developed yet, two studies reported two drugs that indirectly target this transcription factor in chordoma cell lines[183,237]. The first one to be reported was afatinib, a pan-ErbB inhibitor that besides targeting EGFR, as abovementioned it is also capable to target all ErbB receptors[101]. Magnaghi and his coworkers performed a screening of several TKIs in a panel of chordoma cell lines and showed that afatinib was the only inhibitor that showed activity in 6 out of 7 cell lines. After this screening the authors demonstrated that this EGFR inhibitor downregulates Brachyury protein possibly being the reason behind the great activity of afatinib in chordoma cell lines[237]. The second drug to be described as able to target Brachyury was THZ1, a covalent CDK inhibitor that targets CDK7, and CDK12/13 though only at higher doses[311].

The last main aim of this work was to study the potential of Brachyury as a therapeutic target in PCa and LC, mainly in those with high AR and EGFR expression, respectively. First, we intended to expand the abovementioned findings regarding afatinib and THZ1 effects on Brachyury expression in other models besides chordoma, using for that cell lines that endogenously express high levels of this transcription factor. Specifically, we chose two colon cancer cell lines, SW480 and SW620, that were reported in the literature as positive for Brachyury expression[195,212,284]. THZ1 treatment demonstrated a great ability to downregulate the expression of our molecule of interest in both cell lines, while afatinib was effective in SW620 and SW480 cell lines, but in this latter only at high concentrations.

Regarding PCa, in LNCaP cells afatinib was much more effective on decreasing Brachyury protein levels compared to THZ1, in which only at a concentration of 1 μM was able to downregulate this transcription factor. Looking at AR, it is possible to observe that these drugs can also affect its expression, both in Empty and TBXT cells, but more strongly in TBXT cells in which Brachyury was concomitantly inhibited. Rasool and his colleagues shown that CDK7, the main target of THZ1, acts downstream of AR[312]. CDK7 inhibition by small interfering RNA (siRNA) or pharmacologically through THZ1 results in the inhibition of AR transcriptional program but not in the levels of AR itself in LNCaP and VCaP cell lines[312]. Even though the degree of downregulation of both Brachyury and AR was only modest, it occurs both with THZ1 and afatinib, which somehow validate that in fact Brachyury modulation has an impact in AR expression[213].

Concerning our results in the LC model, even though afatinib being able to abrogate EGFR activation, was only able to slightly decrease the short isoform of Brachyury whilst no effect on the long isoform expression was observed in HCC827 cell line. The results suggest that Brachyury inhibition by afatinib doesn't occur in concomitance with EGFR activation inhibition. In contrary, THZ1 was very effective at targeting both Brachyury isoforms in this cell line.

Overall, our results strongly suggest a cell line dependent mechanism of the tested drugs to downregulate endogenously and ectopically expression of Brachyury, as it was observed for chordomas[183,237]. Magnaghi and collaborators conducted some assays with proteasome and autophagy inhibitors (MG-132 and bafilomycin) demonstrating that in these conditions afatinib was not capable to downregulate Brachyury protein in chordomas, which led them to hypothesize that the underlying mechanisms of afatinib to target Brachyury involve these pathways of protein degradation, more than EGFR inhibition[237]. In fact, our results are concordant with their findings given that LNCaP[313] and SW620[314] cells are p-EGFR and EGFR negative, respectively, and afatanib was very effective at targeting Brachyury expression in these models.

Concerning our THZ1 results, in the study conducted by Sharifnia and her coworkers it was shown that the underlying mechanism of the THZ1-mediated Brachyury downregulation in chordoma was the existence of a super enhancer region associated to this transcription factor *locus*[183], and indeed this CDK7 inhibitor has been described for its effects in targeting transcriptional addictions in cancer and genes associated with super enhancers[315-320]. Moreover, through an experiment in which Brachyury was ectopically expressed under the regulation of an exogenous promoter rather than Brachyury's endogenous regulatory elements, it was shown that THZ1 treatment barely had an impact on the ectopic protein levels[183]. Intriguingly, even though our PCa and LC models lack a *TBXT* associated super enhancer, THZ1 was still able to downregulate Brachyury expression in particular in the HCC827 cell line. The reason behind this downregulation of ectopically expressed Brachyury could be a post-translational mechanism or we can also hypothesize that the CDK7/12/13 inhibitor downregulates Brachyury through the inhibition of an upstream regulator of this transcription factor, which deserves further exploitation.

Given these interesting results, we move further to our objective of using silica porous materials as nanocarriers to improve the delivery of these drugs to cancer cells. Having in mind the chemical structures of afatinib and THZ1, we chose two different zeolite and/or mesoporous silica materials for each drug, which would allow us to compare and conclude about the potentiality of these hosts. So, we used the zeolite NaY and the mesoporous silica SBA-15 to entrap afatinib and the mesoporous silica

materials MCM-41 and SBA-15 to encapsulate THZ1, which when used alone all showed to be biocompatible with no cytotoxic effects in HCC827 cell line. After the development of these DDS we performed cytotoxic assays again and demonstrated the successful entrapment of afatinib and THZ1 in the respective materials, and to the best of our knowledge there are no studies reporting the development of DDS using these porous materials as matrix to host any of these drugs. To conclude about the potential of the developed DDS, additional experiments are needed, such as the determination of afatinib and THZ1 load in the porous materials, that would allow us to compare the efficacy of these DDS with the free drugs and further assess if these DDS maintained the drug's specificity to target Brachyury expression.

Overall, our results demonstrated that indeed the small molecule inhibitors, afatinib and THZ1, are able to downregulate Brachyury protein levels not only in chordoma, as first reported, but in several models and curiously in both endogenously and ectopically expressed Brachyury. Furthermore, as far as we are aware our study was the first to demonstrate the inhibition of more than one of Brachyury isoforms by afatinib and THZ1. Interestingly, our results concerning the inhibition of Snail protein levels upon afatinib and THZ1 treatment point out to a possible way to revert the Brachyury-associated phenotype, albeit these drugs not being Brachyury specific. In this sense, we can in the future treat our genetically modulated models with these drugs, free or in DDS, and assess their effects on the reversion of Brachyury-induced EMT, stemness, viability, migration, invasion, colony formation as well as use it in combination with other drugs used for PCa and LC treatment.

CHAPTER 6:

Conclusions and future perspectives

In this work the role of Brachyury in the biological behavior, therapy resistance as well as its targeting in PCa and LC models was assessed. First, we were able to expand the previous findings of the group to an androgen-dependent cell line never used before, LNCaP, and explore our hypothesis of Brachyury mediating resistance of the cells to antiandrogen drugs, concluding that this was not the case, having demonstrated that the overexpression of this transcription factor did not impact the cells response to these kind of therapies. In regard to our LC model, using two cell lines with different genetic backgrounds, never applied by others groups, the oncogenic role of Brachyury in one of these models was evidenced by the advantages in cell survival, migration and colonies formation upon Brachyury OE. In addition, since Brachyury was already reported to confer resistance to an EGFR inhibitor, we intended to further explore this using more clinically relevant models and EGFR-TKIs. We proved indeed, that in one of our LC models Brachyury conferred resistance to one out of three of the chosen EGFR inhibitors that we further hypothesized to be mediated by an hyperactivation of the AKT pathway, that we observed in these cells. Finally, we were able to demonstrate that both afatinib and THZ1 are capable to target Brachyury in other models besides chordoma and gave some insight on the potential of these drugs to reverse the Brachyury-associated phenotype. Finally, given these interesting results we move on to develop novel DDS to entrap the above cited small molecule inhibitors, but unfortunately, we were only able to prove their successful development and not their efficacy.

In conclusion, even though Brachyury has shown to not confer resistance to antiandrogen therapies in PCa it was also proved to be a potential predictive biomarker to EGFR targeted therapies in LC, which needs further addressing. Furthermore, since the demonstrated efficacy of afatinib and THZ1 small molecule inhibitors at targeting Brachyury we hypothesized that the usage of these drugs could be a strategic tactic to sensitize CRPC to docetaxel and also LC to EGFR targeted therapy, as well as to attenuate the Brachyury-associated aggressive phenotype.

Despite the interesting results obtained here, some limitations of this specific work should be acknowledged. First, to further complement these results several assays that we intended to perform from the beginning of this work, such as the biological *in vitro* studies in LNCaP and HCC827 cells, as well as additional cytotoxic assays for the EGFR-TKIs in HCC827 cell line, but that we were not able to do

it, due to the limited time and difficulty to work with these cells, should be performed. Second, it is of vital importance to have in mind that our mRNA analyses were done only once, being necessary to do more and different assays, as to study other EMT and FA proteins expression. Also, both EMT and FA processes are reversible, highly dynamic with a complex and tight regulation, which make them tremendously difficult to study and indeed the standard analyses of expression used here function almost as a snapshot at a single timepoint, and as such do not represent the overall process of these mechanisms. In other words, the fact that in a specific timepoint the cells do not express an epithelial and/or mesenchymal marker do not exclude them from being under an EMT or FA process. Third, it is worth mentioning that *in vitro* models, despite being of extreme significance in cancer research, they do not recapitulate the general complexity of a tumor, which results in observations that might not be translatable to either *in vivo* models and cancer patients, and such results should be further validated with more complex models.

Given our promising results that left several aspects open for discussion as well as the abovementioned limitations, we intend to further explore our initial hypotheses and new ones that arose from this work and as future perspectives we seek to:

- Deepen the role of Brachyury (both isoforms when applicable) in PCa and LC models, performing a comprehensive study of its impact in several signaling pathways mentioned here, namely TGF-β, Wnt/β-catenin, PI3K/AKT, MAPK, etc, and also cell cycle and apoptosis analyses;
- Conduct *in vivo* studies to fully validate the lack of Brachyury predictive value to antiandrogenic therapies in PCa;
- Recapitulate the results obtained here in regard to the biological behavior and in the response to EGFR-TKIs of LC cells, through more *in vitro* studies and moving further for *in vivo* models;
- Explore the possible underlying mechanisms of afatinib and THZ1 actions in Brachyury expression as well as their implications on the Brachyury-associated biological behavior of PCa and LC cells, *in vitro* and *in vivo*;
- Perform a thorough characterization of the DDS developed here to determine its efficacy in comparison to the free drugs, and being this the case we hope to validate the potential, safety and efficacy of these DDS on Brachyury-targeting and the potential phenotype reversion, using *in vitro* and *in vivo* models.

CHAPTER 7:

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