Universidade de Aveiro Departamento de Biologia
2016

DIANA ROBERTA DA CRUZ RIBEIRO

SOX2 a master gene regulating progression in prostate cancer?

SOX2 um gene essencial na regulação da progressão do cancro da próstata?

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Professora Doutora Joana Tavares de Oliveira, Professora Auxiliar da Faculdade de Medicina Veterinária da Universidade Lusófona de Humanidades e Tecnologias e da Professora Doutora Maria de Lourdes Gomes Pereira, Professora Associada com Agregação do Departamento de Biologia da Universidade de Aveiro e co-orientação da Professora Doutora Rita Barros, Professora Afiliada da Faculdade de Medicina da Universidade do Porto.

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agradecimentos

Gostaria de agradecer:

À Professora Joana de Oliveira e Professora Rita Barros, por me terem aceite no instituto e por todo o apoio fornecido ao longo do último ano.
À Professora Maria de Lourdes Pereira, por toda a disponibilidade no decorrer de todo o meu período de mestrado.
Ao Professor Cláudio Sunkel e ao Professor Manuel Sobrinho-Simões, diretores do i3S e IPATIMUP respetivamente, por permitirem a oportunidade de desenvolver o meu trabalho de mestrado na instituição.
À Professora Raquel Almeida, por me ter aceite no grupo Differentiation in cancer.
A todos os membros do Differentiation in cancer, um grupo ao qual sou grata por fazer parte, particularmente à Lúcia e Ana Luísa, por todo o apoio que me deram.
Ao Professor Manuel Teixeira e à Professora Paula Paulo por terem fornecido gentilmente as linhas celulares que foram usadas neste estudo.
Aos meus pais e irmão, agradeço todo o apoio e encorajamento durante o meu percurso académico e sonhos.
A todos os meus amigos, obrigada por estarem sempre comigo!

o júri

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Keywords Stem cell markers, SOX2, prostate cancer, migration, apoptosis

Abstract Stem cell markers are powerful prognostic tools to predict cancer progression and cancer-specific survival in patients with metastatic prostate cancer (PC). Among these is the SRY (sex determining region y)-box 2 (SOX2) gene. SOX2 is expressed in metastatic lesions of androgen resistant human PC. In primary PC, SOX2 was overexpressed and found to be as useful as Gleason and prostate specific antigen (PSA) for prognosis in a small series of 30 patients. Experimental models in mice suggest that SOX2-expressing basal cells in p63 and Pten-null mice contribute to the luminal population and tumorigenesis. SOX2 is an androgen receptor (AR) repressed gene found to promote castration resistant PC (CRPC) phenotypes. Moreover, it is involved in paclitaxel resistance of the PC cell line PC-3 via the PI3K/Akt pathway. Despite its apparently crucial role in metastasis, the actual role of SOX2 and whether or not it might be modulated in bone metastatic lesions of PC has not been well documented.

After obtaining a biopsy positive for PC, patients face the option of waiting or performing a radical prostatectomy (RP) or radiotherapy. RP is a high morbidity associated surgery involving also large economical burden to the healthcare system. Indeed, the available therapies regarding the management of PC significantly interfere with the patient wellbeing and thus the development of novel diagnostic and therapeutic tools is mandatory. As such, this project aims at developing SOX2-based diagnostic criteria and to assess its possible role in PC progression. More precisely, the main purpose of this study, using a series of PC cases and cell lines is to study SOX2 and accurately assess its role in invasion. Here, we successfully validated SOX2 as a pivotal player in the early progression and invasion of prostate carcinomas. We have demonstrated that SOX2 silencing mediated by siRNAs stemmed a significant decrease in the migration capacities and an increase in the apoptotic process of SOX2 knocked-down PC3 cells. These results further support the hypothesis that SOX2 might take part of an important transcription program certainty

responsible for modulating the establishment of metastasis. We also found that SOX2 was overexpressed in basal-like cells of human PC tissue, and not in normal adjacent tissue, further supporting the possibly involvement of SOX2 in the tumorigenesis of the prostate. In addition, we also evaluated the relationship of the expression of SOX2 with a series of clinical and histopathological criteria and found that it was significantly associated with an increased risk of relapsing after primary therapy, pointing to its possible role in tumour recurrence. In conclusion, we believe that the characterization of the SOX2 activities throughout the development, progression, and relapse of prostate carcinomas will become a major step forward towards understanding the biology underpinning this disorder. Ultimately, such understandings can hopefully lead to the discovery of a SOX2 repressor which could potentially be used for anti-cancer therapy.

palavras-chave Marcadores de células estaminais, SOX2, cancro da próstata, migração, apoptose

Resumo Os marcadores de células estaminais constituem excelentes ferramentas de prognóstico para determinar a progressão e sobrevivência de pacientes diagnosticados com cancro da próstata metastático. Dentro deste grupo de marcadores encontra-se o SOX2, um fator de transcrição expresso em lesões metastáticas de carcinomas da próstata resistentes aos androgénios. Em neoplasmas primários da próstata, a expressão de SOX2 encontra-se frequentemente elevada, apresentando uma utilidade semelhante ao fator Gleason, e ao antigénio específico da próstata para o prognóstico numa pequena serie de 30 pacientes. Modelos experimentais em ratinhos apontam que células basais $SOX2^{+}$ contribuem para a população de células luminais e para a tumorigénese em ratinhos mutantes para a p63 e o PTEN. O SOX2 é um gene cuja expressão é reprimida pelo receptor de androgénios, possuindo um papel essencial na aquisição de fenótipos de resistência à castração. Além disso, encontra-se igualmente envolvido nos processos de aquisição de resistência ao paclitaxel na linha celular PC3 através da via de sinalização PI3K/Akt. No entanto, apesar do seu papel no aparecimento de metástases ser cada vez mais evidente, a sua função e a forma como é modulado neste tipo de lesão permanece ainda por identificar. Após confirmação de um diagnóstico positivo para cancro da próstata, ao paciente podem ser apresentadas várias vias de tratamento, incluindo opções de monotorização ativa, prostectomia radical ou radioterapia. Atualmente, a prostectomia radical constitui uma cirurgia associada a elevadas taxas de mortalidade e a elevados gastos económicos para o sistema de saúde. Além disso, as terapias habitualmente direcionadas para o cancro da próstata interferem significativamente com a qualidade de vida do paciente. Assume-se assim, que o desenvolvimento de novas ferramentas terapêuticas, assim como de

diagnóstico, será de extrema urgência. Este projeto tem como objetivo avaliar a possível aplicação do SOX2 no desenvolvimento de novos critérios de diagnóstico, assim como determinar o seu possível envolvimento na progressão de carcinomas da próstata. Especificamente, o principal objetivo deste estudo é compreender qual o papel exato do SOX2 nos processos invasivos, utilizando uma série de casos e linhas celulares de cancro da próstata. Assim, foi possível demonstrar que o silenciamento do SOX2 mediado por siRNAs fomentou uma redução significativa das capacidades migratórias, e ainda, um aumento da morte celular programada nas células da linha PC3, onde a expressão de SOX2 foi silenciada. Verificamos que a expressão de SOX2 em células basais de amostras de cancro da próstata, encontrava-se aumentada em comparação ao tecido adjacente normal, corroborando a hipótese de que o SOX2 poderá estar envolvido na tumorigénese da próstata. Procedeu-se ainda a uma avaliação da relação existente entre a expressão de SOX2 e uma série de critérios clínicos e histopatológicos. Verificou-se que o SOX2 encontra-se significativamente associado a um maior risco de recorrência após tratamento primário, sugerindo que este fator de transcrição poderá desempenhar uma função importante nos mecanismos subjacentes à recorrência tumoral. De um modo geral, a caraterização das atividades do SOX2 ao longo do desenvolvimento, progressão e recorrência de carcinomas da próstata, tornar-se-á um importante passo no sentido de compreender os processos biológicos implícitos nesta condição. Em última análise, tal poderá conduzir à descoberta de novas estratégias promissoras para o tratamento de carcinomas da próstata, baseadas na repressão de SOX2.

LIST OF ABBREVIATURES

ADT	Androgen deprivation therapy
AFMS	Anterior fibromuscular stroma
AR	Androgen receptor
AS	Active surveillance
ASAP	Atypical small acinar proliferation
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
CKs	Cytokeratin's
CRPC	Castration-resistant PC
CSCs	Cancer stem cells
DAB	Diaminobenzidinetetrahydrochloride
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DRE	Digital rectal exam
ECM	Extracellular matrix
EDTA	Ethylediaminetetraacetic
EGFR	Epidermal growth factor receptor
ERBT	External-beam radiotherapy
EUA	European Association of Urology
FBS	Fetal bovine serum
GRP	Gleason score after radical prostectomy
H_2O_2	Hydrogen peroxide
HGPIN	High-grade prostatic intraepithelial neoplasia
HMG	High mobility group
iPSCs	Induced pluripotent stem cells
ISUP	International Society of Urological Pathology
LGPIN	Low-grade proliferative inflammatory atrophy
LHRH	Luteinizing hormone-releasing hormone
LSCC	Laryngeal squamous cell carcinoma
M	Metastasis

mCRPC	Metastatic castration-resistant PC
MMPs	Metalloproteinases
Na_3VO_4	Sodium orthovanadate
N	Regional lymph node metastasis
NE	Neuroendocrine
PAP	Prostatic acid phosphatase
PBS	Phosphate-buffered saline
PBT	Proton beam therapy
PC	Prostate cancer
PC_SV	Prostate cancer overall survival
PHI	Prostate health index
PI	Propidium iodide
PIA	Proliferative inflammatory atrophy
PMSF	Phenyl methyl sulfonyl fluoride
PR_SV	Disease survival periods
PSA	Prostate specific antigen
RNA	Ribonucleic acid
RP	Radical prostectomy
siRNA	Small interference ribonucleic acid
Sry	Mammalian testis-determining factor
SOCE	Store-operated calcium entry
T	Size of the tumor
TICs	Tumor initiating cells
TMA	Tissue microarray
TNM	Tumor, node and metastasis
TRUS	Trans-rectal ultrasound

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INTRODUCTION

1. Anatomy and histology of the human prostate.

The prostate is a walnut-sized fibromuscular gland of the male reproductive system sitting in the pelvis, directly below the bladder and surrounding the urethra. It as a pivotal role in producing a part of the seminal fluid and thus it is indirectly implicated in motility and nourishment of the sperm (McNeal, 1972; Marandola et al., 2004).

The anatomy of the prostate was surrounded by controversy for several years. Indeed, early descriptions of the human prostate envisage that, comparable to other mammals, the gland followed a lobar pattern of development (Lowsley, 1912). Nonetheless, after birth it is possible to denote that foetal lobes coalesce and give rise to a homogeneous structure. Thus, McNeal (1981) proposed the existence of several zones categorized into the peripheral (70 percent of glandular tissue), central (20 percent of the glandular tissue), and transitional zones (5 percent of glandular tissue), and also the anterior fibromuscular stroma (AFMS) (figure 1) (McNeal et al., 1980; McNeal, 1981; McNeal, 1981a).

The peripheral zone is the main location for the development of a subset of prostate associated lesions, including prostatitis and carcinomas, and extends around the posterolateral peripheral area of the gland from the apex to the base (Lee et al., 2011). On the other hand, the central zone is a cone-shaped region wherein less than 10 percent of carcinomas develop. This region embraces the majority of the prostate base and surrounds the ejaculatory ducts, which empty bilaterally into the urethra in a precise point, the verumontanum (Bhavsar and Verma, 2014). Lastly, the transition zone is composed by two small symmetrical lobules sideways to the urethra in the midland (Lee et al., 2011). The vast majority of benign prostatic hyperplasia (BPH) and up to 20 percent of prostate cancer (PC) develops in this region. Nevertheless, and despite some uncertainty, transitional carcinomas have been positioned as lower malignant potential when compared to the peripheral ones (Greene et al., 1991; Reissigl et al., 1997).

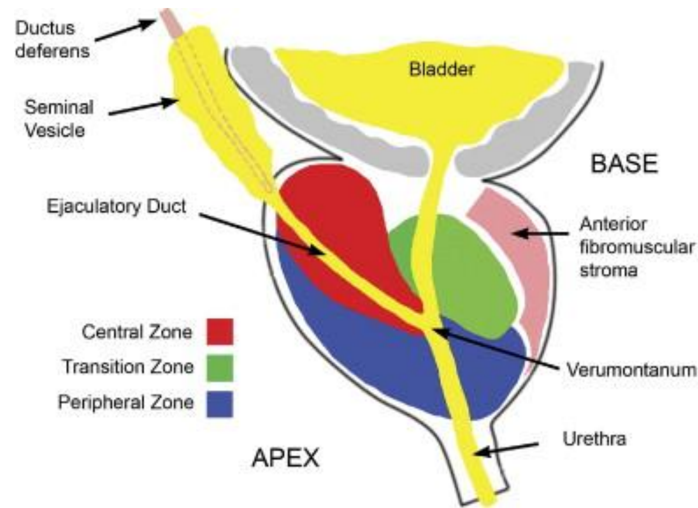


Figure 1 | Sagittal view of the human adult prostate gland elucidating the zonal division of the gland as described by McNeal in 1981. The prostate can be divided into several zones, i.e., peripheral, transition and central zones, which are surrounded by the AFMS (Cohen et al., 2008).

The epithelium of the prostate is organized as glandular acini consisting of basal, luminal, transient, and neuroendocrine cell groups (figure 2) (Isaacs and Coffey, 1989; Long et al., 2005). The luminal secretory cell layer is composed of tall columnar highly-differentiated androgen-dependent cells (Masai et al., 1990) frequently characterized by the expression of low molecular-mass cytokeratin's (CKs) (mostly CK8 and CK18) and the cell-surface marker CD57 (Sherwood et al.,1990; Sherwood et al., 1991; Okada et al., 1992; Liu et al., 1997). Luminal cells have a master role in the synthesis and secretion of several components of the seminal fluid, including PSA (prostate specific antigen), PAP (prostatic acid phosphatase) and human kallikrein-2 (Long et al., 2005). Contrarily, the basal compartment consists of undifferentiated androgen-independent cuboidal epithelial cells commonly characterized by the expression of high-molecular-mass CK5 and 14 and the cell-surface marker CD44 (Liu et al., 1997; Yang et al., 1997; van Leenders et al., 2000). Importantly, the presence of stemness subpopulations within this layer provides the basis for all the prostatic epithelial cells. Indeed, mitosis yields groups of intermediate cells (Wernert et al., 1987; Fry et al., 2000; Hudson et al., 2001) which ultimately differentiate into luminal cells as they migrate towards the luminal compartment (Isaacs and Coffey, 1989; Long et al., 2005). In addition, the prostate contains a less abundant

group of androgen-independent and non-proliferating terminally differentiated NE cells intermingled within the luminal and basal cell layers (Noordzij et al., 1995; Abrahamsson, 1996). The majority of NE cells are identified based on morphology and secretory products, i.e., serotonin (Abrahamsson et al., 1987; Abdul et al., 1994), synaptophysin (di Sant'Agnese, 1998) and chromogranin A (Huttner et al., 1991) and have an apparent enrolment in growth, differentiation and carcinogenic processes (di Sant'Agnese, 1998; Sciarra et al., 2003).
The prostate gland is further supported by the existence of the AFMS wherein smooth muscle cells, fibroblasts, various immune cells, autonomic nerve fibers, components of the extracellular matrix (ECM) and endothelial cells are positioned (figure 2) (Barron and Rowley, 2012).

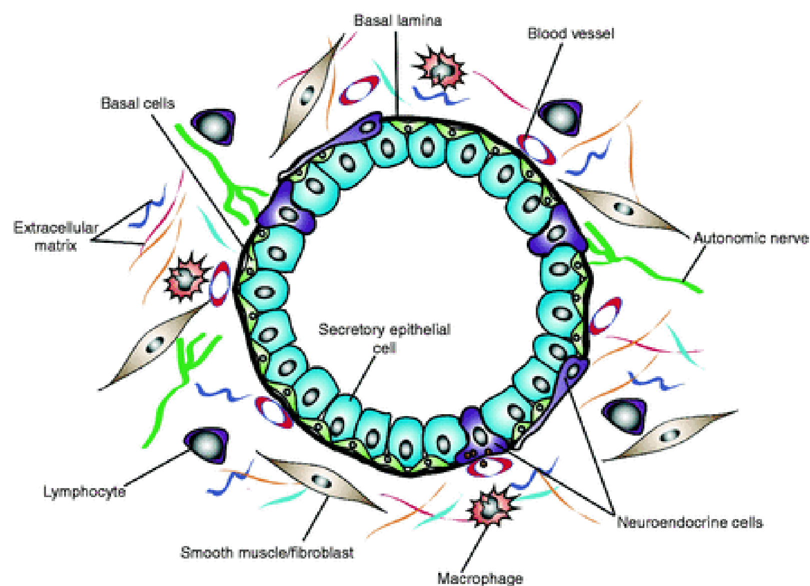


Figure 2 | Cellular components of the human prostate gland. Secretory epithelial cells are positioned on the basement membrane and secrete products into the acinar lumen. Basal cells and NE cells are also present in the prostate gland, which is further supported by a stromal compartment. The later is composed of smooth muscle, fibroblasts, blood vessels, autonomic nerve fibers, inflammatory cells, and ECM components (Barron and Rowley, 2012).

The role of stromal-epithelial interactions as well as the outcome engaged are not completely understood. However, a growing body of evidence advices that the stroma is capable of producing a myriad of growth factors whose function is

paramount for the development and growth of both normal and cancerous prostate (Ware, 1993; Chung, 1995).

2. Cancer: a disease of the genome

Cancer is a disease of the genome resulting from a successive accumulation of genetic and epigenetic modifications which ultimately prompt a cell to survive and proliferate in the very harsh microenvironment that defines a tumor. Millions of people currently live with a positive diagnosis of cancer. Acquisition of invasive and metastatic capacities is an intricate and coordinated series of events encompassing the main cause of cancer-related death. Indeed, nearby 14.1 million new cases were detected in 2012 of which 8.2 million deaths were reported (Ferlay et al., 2013). Notwithstanding recent progresses, the disease is growing at a frightening pace with 21.4 million cases and 13.2 million deaths expected to occur in 2030 possibly due to the continual growth, aging and improper lifestyle of the worldwide population (Ferlay et al., 2010).

PC is often a complex and heterogeneous pathology in many facets of the disease, i.e., epidemiological, biological, pathological and clinical criteria. As such, research in this field is extremely mandatory. Novel insights into the biology underlying its development and proliferation, the causes and, most importantly, how it can be prevented and treated are required. All of these topics will be discussed above.

3. Prostate cancer epidemiology

In human settings PC is recognized as one of the most common malignancies and cancer-related death among men. In fact, nearby 1.1 million cases were detected in 2012 and accounted for approximately 15 percent of the global cancer burden (Ferlay et al., 2015).

Based on geographic location, the global commonness of PC diverges impressively. These disparity is partly attributed to the routinely implementation of PC diagnosis techniques, which are capable of identifying even the clinically non-detectable neoplasms. Likewise, since European and North America nations possess highest rates of PC testing they exhibit highest rates of PC,

whereas countries that register low rates of PC testing, i.e., Asian and African nations, exhibit lowermost rates of PC (Marugame and Katanoda, 2006; Kvale et al., 2007; Shin et al., 2010). Yet, the Caribbean and African black population display higher mortality rates (figure 3) (Ferlay, 2010). Such variances have been accredited to the existence of genetic variants (Corder et al., 1995; Platz et al., 2000; Shook et al., 2007), the serum levels of sexual hormones (Winters et al., 2001) and growth factors (Scher et al., 1995; Tricoli et al., 1999; Winters et al., 2001).

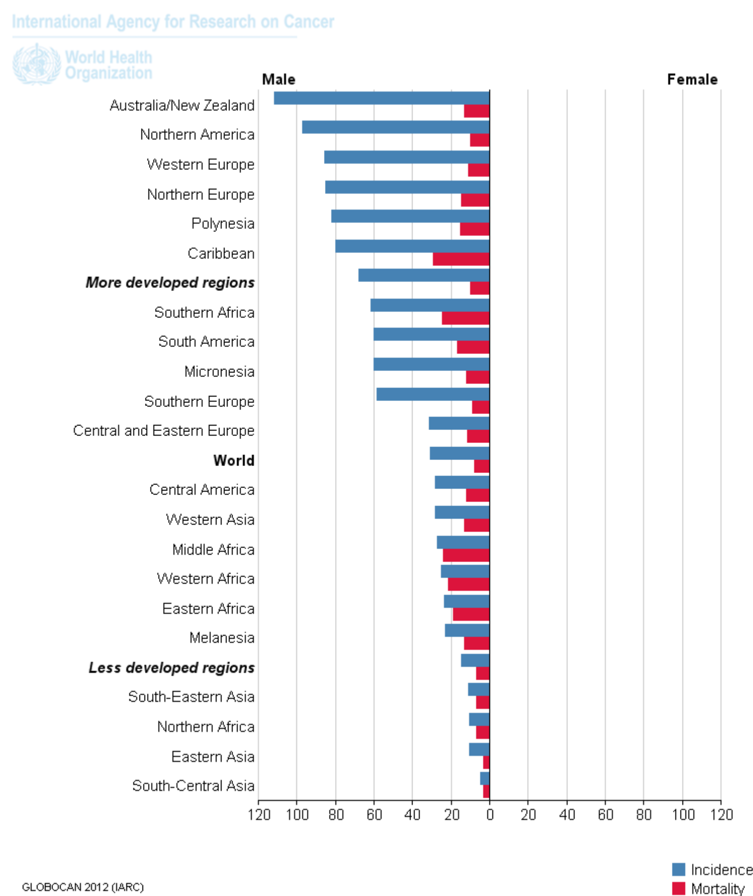


Figure 3 | Worldwide age-standardized PC incidence and mortality rates (Source: GLOBOCAN – IARC (2012). Available from http://globocan.iarc.fr).

In the Portuguese scenery, PC is the most common malignancy with 6622 cases being diagnosed in the year of 2012, of which 1582 deaths were reported (figure 4) (Ferlay et al., 2012).

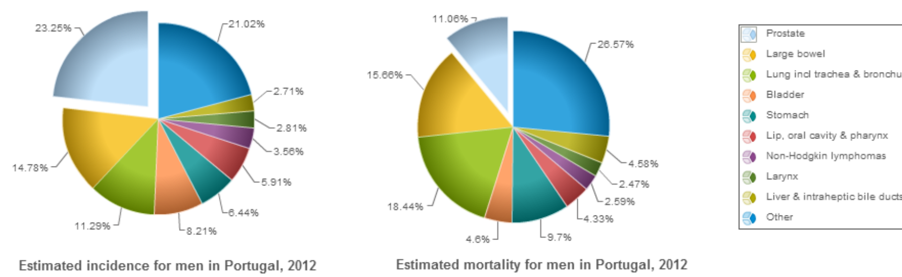


Figure 4 | Estimated incidence and mortality of cancer for men in Portugal. PC is the most frequent type of cancer found in males and the third leading cause of death in 2012, following stomach and lung cancer (Source: GLOBOCAN-IARC (2012). Available from http://globocan.iarc.fr

Prostate carcinomas are considered to be slow growing neoplasms (Virtanen et al., 1999) with incidence and mortality rates tending to escalate with age (Franceschi and La Vecchia, 2001). In fact, diagnosing PC in males younger than 50 in relatively scarce (Haas and Sakr, 1997). The majority of cases are diagnosed in males beyond the age of 50, with the average standing between 72 and 74 years of age (Grönberg, 2003). The relationship between aging and PC is not completely understood. In these respect, literature data highlights that aging impacts on the standard expression of numerous genes in the prostate, i.e., inflammation, senescence and oxidative stress associated genes, thus consenting the inception of the disease (Shen and Abate-Shen, 2010).

Family history is also a well-known PC risk factor (Goh et al., 2012). The risk of developing PC is higher in males whose first-degree relatives possess the disease. Indeed, if one or at least two first-degree relatives have PC, the risk of developing the disease is two and five to eleven times higher, respectively, in comparison to the broad population (Bratt, 2002). Moreover, if the diagnosis is performed in a patient with three or more cases running in the family, or at least two relatives develop PC at young age, their cancer is defined as truly hereditary, i.e., the cancer is caused by inherited high- and low-penetrance genes (Grönberg et al., 1994; Schaid, 2004; Heidenreich et al., 2014).

Regarding racial disparity, African Americans exhibit the highest rates of PC, followed by Caucasian, Hispanic, and Asian/Pacific Islander males (American Cancer Society, 2003; Crawford, 2003). Indeed, it was previously established that African Americans possess a 1.4 and 2 to 3 times higher risk, in

comparison to Caucasians, of developing and dying from the disease, respectively (American Cancer Society, 2003; Chornokur et al., 2011).

4. Etiology

The heterogeneity perceived in prostate neoplasms advocates that both genetic and environmental factors define the likelihood of developing clinical PC (Alvarez-Cubero et al., 2012; Heidenreich et al., 2014). Nonetheless, age, family history, and ethnicity are currently the only fully-acknowledged PC risk factors (Heidenreich et al., 2014). Several genes have been linked to familial PC and include *HOXB13*, *RNASEL, EPAC2, MSR1, CHEK2, CAPZB,* vitamin D receptor, and *PON1* (Deutsch et al., 2004; Porkka and Visakorpi et al., 2004; Wiklund et al., 2004; Dong, 2006; Ewing et al., 2012). In addition, rare germline mutations in *BRCA1* and *BRCA2* were established to boost the risk of PC (Thompson et al., 2001; Leongamornlert et al., 2012). For instance, *BRCA2* mutations have been associated with higher Gleason scores (Mitra et al., 2008), poorer prognosis (Castro et al., 2013), and responsible for triggering nearby 5 percent of young onset PC (Edwards et al., 2004; Kote-Jarai et al., 2011).
Geographical discrepancies have been attributed to both environmental and lifestyle associated factors (Imperato et al., 1996) namely diet, obesity, smoking and alcoholic habits, chemical exposure, transmitted infectious disease and vasectomy (American Cancer Society, 2014). For instance, it has been reported that diets rich in fat, animal proteins, processed meats and dairy foods have the potential to enlarge the risk of developing PC (Grönberg, 2003; Nelson et al., 2003; Deutsch et al., 2004). Contrarily, a number of nutritional elements, i.e., selenium, vitamin D and E, have been proposed to hold a protective effect against PC (Nelson et al., 2003; Deutsch et al., 2004; Damber, 2008).

4. Pathophysiology

The advent of solid neoplasms is considered to be a multistep process shaped by successive gathering of genetic and epigenetic events. PC is considered to be a complex, heterogeneous and multifocal condition with a long natural course. In fact, it partakes the capacity of dwell histological for numerous years

before progressing to clinical detectable disease (Bostwick, 1989; Sakr et al., 1994).

Pathological conditions affecting the prostate of adult males range from benign, premalignant to malignant lesions. Most of the benign prostatic hyperplasia (BPH) develops nearby the prostatic urethra in the transition zone (De Marzo et al., 2007). It is characterized by a non-malignant overgrowth of epithelial and stromal cells leading to the enlargement of the prostate, i.e., glandular and stromal tissues undergo hyperplasia. This condition is androgen-driven and ultimately leads to the onset of lower urinary tract symptoms in aging men (Sandhu and Te, 2004; Roehrborn, 2005; Bushman, 2009).

The notion that the development of PC occurs through a series of early and late histological modifications lead to the designation of proliferative inflammatory atrophy (PIA), high-grade prostatic intraepithelial neoplasia (HGPIN), and atypical small acinar proliferation (ASAP) as the precursor lesions of PC (Berney and Warren, 2013). PIA develops in the peripheral zone and is often associated with atrophic epithelia, i.e., cells divide at higher rates in regions of chronic inflammation (Bostwick and Cheng, 2012). In the case of PIN, it regularly develops in the peripheral zone and consists of architecturally pre-existing benign prostatic acini and ducts delimitated by atypical cells whose features resemble those of PC cells (Epstein, 2009; Berney and Warren, 2013). Based on cytological features PIN is further stratified into low-grade PIN (LGPIN) and HGPIN. In LGPIN the nuclei of cells is enlarged, vary in size, have normal or slightly increased chromatin content, and possess small or inconspicuous nucleoli (McNeal, 1986). On the other hand, HGPIN is characterized by the existence of nuclei enlarged cells, increased chromatin content, and prominent nucleoli resembling carcinoma cells. Importantly, only HGPIN is associated with disruption of the basal compartment and inverted epithelial proliferation, i.e., proliferation occurs in the luminal layer, being for that reason, the most likely precursor lesion of PC, probably arising from LGPIN and PIA in a time-dependent manner (figure 5) (Putzi and De Marzo, 2000; De Marzo et al, 2003; Joniau et al., 2005).

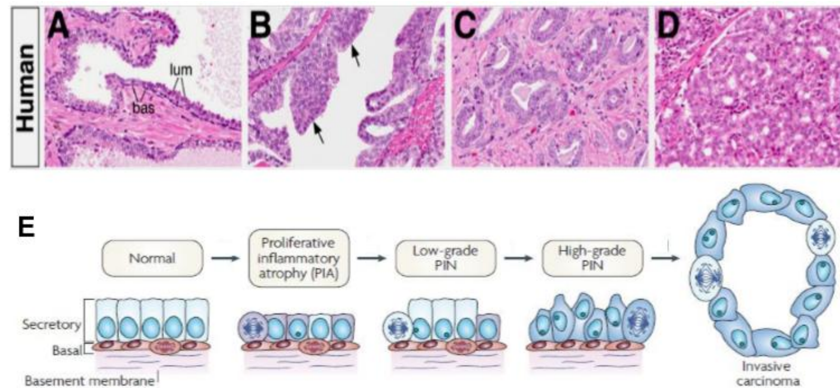


Figure 5 | Histopathology of human PC. Hematoxylin-eosin stained sections of prostate benign tissue with representative basal (bas) and luminal (lum) cells indicated (A). PIN tissue with arrows pointing to regions of hyperplastic epithelium (B). Well-differentiated adenocarcinoma (C). Poorly differentiated adenocarcinoma (D). Cellular model of early prostate neoplasia progression (adapted from De Marzo et al., 2007; Shen and Abate-Shen, 2010).

ASAP is defined as a focus of small acinar structures rising from atypical epithelial cells and includes a variety of lesions i.e., lesions of adenosis, atypical adenomatous hyperplasia, intraductal hyperplasia, and acinar atypical hyperplasia. These foci can be found in up to 5 percent of PC samples obtained by biopsy and are predominantly located at the peripheral zone (Cheville et al., 1997; Vis et al., 2001). Nevertheless, despite of the fact that the morphologic characteristics that the ASAP category exhibits are highly suggestive of cancer they are not sufficient to definitely confirm a diagnosis (Bostwick and Meiers, 2006; Montironi et al., 2006).

The identification of premalignant change is crucial for preventing the emergence of cancer. The majority of malignant histologies are initiated in the peripheral zone and are known as adenocarcinomas. Indeed, this heterogeneous and multifocal condition comprises 95 percent of the prostate malignancy and relies on androgens for progression and survival (Carroll et al., 2002; Culig and Bartsch, 2006). The clinical course of PC is variable and extremely difficult to predict. While some tumors remain organ-confined, others might metastasize, even in early stages of the disease, favourably to the bone but also to lymph nodes, lungs and liver. When invasion of periprostatic tissue occurs, symptoms associated with PC usually manifest. Nevertheless, at this point the disease is already incurable, underlining the need of curative early-stage PC diagnosis prior to the onset of symptoms (DeVita et al., 2008).

6. Diagnosis

In most patients PC was traditionally diagnosed once the disease was already incurable. Nevertheless, the number of early-stage cases presently diagnosed upon screening or even accidentally up surged. This considerable shift is attributed to the emergence of novel screening tools, namely the PSA blood test and trans-rectal ultrasound (TRUS)-guided biopsy albeit digital rectal exam (DRE) endures as the prime clinical screening tool for PC (Borley and Feneley, 2009).

6.1. Digital rectal exam.

The use of DRE as a PC screening tool is grounded on the principle that the palpable asymmetry of the gland and, more importantly, hard nodular areas comprise clues of cancer. The vast majority of prostate carcinomas develops in the peripheral zone and can be screened by DRE when their volume exceeds 0.2 ml (Heidenreich et al., 2011). Indeed, abnormal DRE is closely associated with an enlarged risk of poorer Gleason scores and usually renders the performance of biopsy (Okotie et al., 2007; Gosselaar et al., 2008). However, the existence of certain DRE-inherent drawbacks ultimately deprive the exam of sensitivity, i.e., the bulk of palpable neoplasms are generally advanced in stage (Smith and Catalona, 1995) whereas other clinically relevant neoplasms may be located in regions unreachable by palpation (Basler and Thompson, 1998).

6.2. Prostate specific antigen blood test.

PSA is a serine protease produced by the epithelia and ducts of the prostate whose function relies on semen liquefication (Wang et al., 1981; Yousef and Diamandis, 2001). As such, the presence of higher levels of PSA in the semen is conceivable albeit some is capable of evading the gland and enter into circulation (Wang et al., 1981). Despite some uncertainty, ample evidence highpoints that the upsurge of PSA in the serum might be accredited to the disruption of the basal compartment, one of the earliest events of PC (figure 6) (Partin et al., 2002; Balk et al., 2003). Indeed, the notion that disease rises the serum content of this protein lead to the emergence of the PSA blood test as a fundamental screening tool who has revolutionize the management of PC. However, the test remains largely deprived of specificity, i.e., the occurrence of

non-cancerous disorders also increases PSA (Andriole et al., 1994), with higher serum levels merely expressing a greater probability of effectively possessing PC. The traditional threshold for an abnormal PSA test was demarcated at 4.0 nanograms per millilitre (ng/ml) (Mettlin et al., 1991; Brawer et al., 1992; Catalona et al., 1993; Crawford et al., 1996) with the occurrence of cancer and even other prostatic disorders being often associated with PSA values oscillating between 4 to 10 ng/ml. Indeed, almost 75 percent of tumors are spotted within this range and match potentially organ-confined and curable neoplasms. Conversely, the amount of localized tumors droplets to less than 50 percent in patients harbouring total PSA values superior to 10 ng/ml (Catalona et al., 1994)

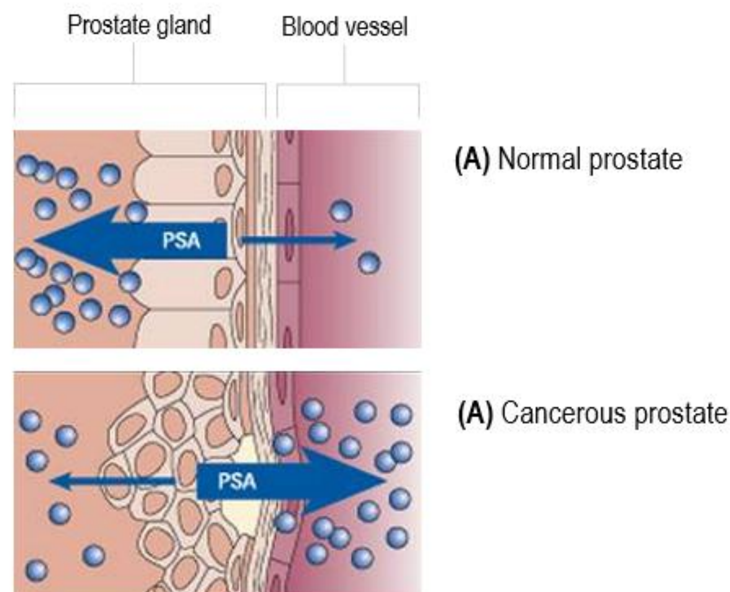


Figure 6 | Presence of PSA in normal (A) and cancerous (B) prostate. In (A) the cells of the prostate are tightly arranged with a trivial quantity of PSA leaking into circulation. In the presence of PC the basement membrane becomes disrupted and a greater amount of PSA leaks into the bloodstream (B) (adapted from http://www.johndonohue.info/psa.html).

Overall, the European Association of Urology (EAU) guidelines indorse TRUS-guided systemic biopsy performance in cases whose serum PSA exceeds 4 ng/ml. Among these, in nearby 20 to 30 percent cases the diagnosis will actually be confirmed (Emiliozzi et al., 2001; Wolf et al., 2010). Contrarily, patients whose PSA is beneath 4 ng/ml should not be admitted for biopsy. However, there is mounting clinical evidence that PSA values lower than 4

ng/ml may also be harbouring relevant disease (Catalona et al., 1999; Roehl et al., 2002; Heidenreich et al., 2011). Despite this, lowering the PSA threshold should not be considered otherwise over-diagnosing and over-treating clinically insignificant cancers will become a reality. Instead, aiming to improve the accuracy of the test, novel PSA-related approaches were developed and involve the assessment of the PSA density, velocity and doubling time, as well as the free/total PSA ratio and the prostate health index (PHI) test (Mottet et al., 2016).

6.3. Transrectal ultrasound-guided biopsy.

In PC settings, the utility of TRUS as a first-line screening tool was proven to be valueless due to specificity, informative and expense concerns. Actually TRUS is rather used as an aid-tool who provides visual guidance for the performance of biopsies (Terris, 2002).

Core needle biopsies comprise the mainstay approach for diagnosing PC. Abnormal DRE, elevated or increasing PSA, and earlier identification of premalignant change are some of the indications for performing a biopsy (Borley and Feneley, 2009). Briefly, a hollow needle is inserted into the prostate *via* the wall of the rectum and removes 10 to 12 small cores of tissue (Eichler et al., 2006; Hara et al., 2008) which will be further analysed to give information concerning the grade and extension of the tumor. However, the absence of sufficient tissue for analysis and also the heterogeneous character of this disease may comprise a hurdle for distinguish whose glands are malignant from those who are benign (Epstein, 1995).

7. Prognostic factors

Besides PSA, grade and stage are the most usual prognostic factors for PC (Kattan et al., 1998; Epstein et al., 2006). The malignant potential of a certain neoplasia is determined by analysing the degree of differentiation exhibit by cancer cells. In 1966 Donald Gleason and collaborators proposed what is now the universal standard system for grading prostate carcinomas, which was recently updated by the International Society of Urological Pathology (ISUP) (figure 7) (Gleason, 1966; Gleason and Mellinger, 1974; Epstein et al., 2016).

The Gleason grading system is entirely based on the degree of histological deviation displayed by hematoxylin-eosin stained PC tissue sections under low to intermediate magnification. Five distinct patterns of grades can be assigned to the most (primary) and second-most (secondary) predominant patterns found in a tissue on a scale from one (well differentiated pattern) to five (poorly differentiated pattern) (Gottipati et al., 2012). Ultimately, the morphological heterogeneity perceived in the neoplasia can be evaluated by summing the primary and secondary grades and thus establish the final score. The Gleason score ranges from two to ten (Gleason and Mellinger, 1974). Briefly, Gleason 2 to 6 tumors are considered to be well differentiated and thus possess a fairly good prognosis. Conversely, Gleason 7 to 10 tumors are less differentiated and, for that reason, associated to a poorer prognosis (Humphrey, 2004; Gottipati et al., 2012).

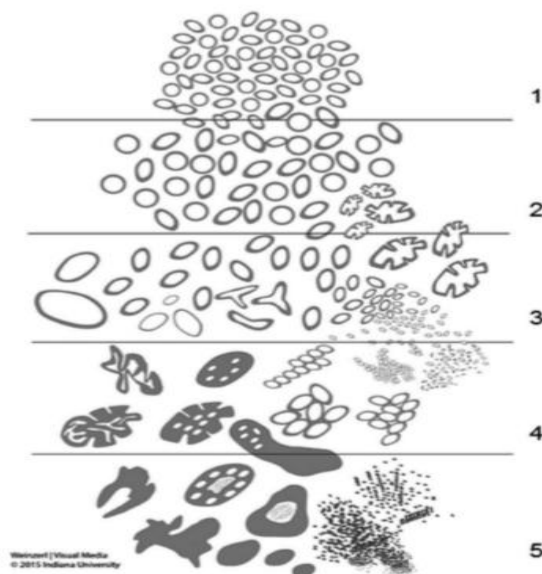


Figure 7 | 2015 modified ISUP Gleason grading system. Five distinct patterns of grades can be attributed the two most dominant patterns found in the tissue. Pattern 1 matches closely-packed, uniform, rounded to oval glands. Pattern 2 to more loosely arranged glands with smooth ends that minimally invade non neoplastic tissue. Patten 3 to irregular size and shape glands with more infiltrative margins. Pattern 4 to fused, cribriform or ill-defined glands. Finally, pattern 5 to almost no glandular differentiation (Epstein et al., 2016)

One of the most important phases in the process of diagnosing a cancer is defining its extension or spread and, therefore, determining its behaviour,

choosing the most suitable therapy and predicting the resultant outcome. For most cancers the tumor, node and metastasis (TNM) staging system is the most widely used by clinicians (table 1) (Cheng et al., 2012). As the name implies, the system is organized in three main categories: a T (T1 to T4), an N (N0 or N1) and an M (M0 or M1) segment. The T describes the degree of extension of a primary tumor. Concisely, T1 and T2 refer to organ-confined neoplasms, whereas T3a and T3b symbolise extra-prostatic extension, with or without seminal vesicle invasion, respectively. Lastly, T4 tumors signify invasion of neighbouring organs (Yarbro et al., 1999). On the other hand, the N and M components specify whether or not a cancer had metastasized to the lymph nodes or distant sites, respectively. As soon as this process is completed a stage ranging from 0 (in situ) to IV (most advanced disease) can be assigned.

Stage	T	N	M	PSA (ng/ml)	GS
I	T1a-c	N0	M0	<10	≤ 6
	T2a	N0	M0	<10	≤ 6
	T1-2a	N0	M0	X	X
IIA	T1a-c	N0	M0	<20	7
	T1a-c	N0	M0	≥ 10 and < 20	≤ 6
	T2a	N0	M0	<20	7
	T2b	N0	M0	<20	≤ 7
	T2b	N0	M0	X	X
IIB	T2c	N0	M0	Any PSA	Any Gleason
	T1-2	N0	M0	≥ 20	Any Gleason
	T1-2	N0	M0	Any PSA	≥ 8
III	T3a-b	N0	M0	Any PSA	Any Gleason
IV	T4	N0	M0	Any PSA	Any Gleason
	Any T	N1	M0	Any PSA	Any Gleason
	Any T	Any N	M1	Any PSA	Any Gleason

Table 1 | American Joint Committee on Cancer (AJCC) stage grouping (2010 edition). M (Metastasis), N (node), PSA (prostate-specific antigen) T (tumour), x (unknown) (adapted from Cheng et al., 2012).

Prognostic factors have been used to determine the risk of relapsing after therapy (D'Amico et al., 1998). In fact, the discrimination of patients into low, intermediate or high-risk groups (table 2) consents urologists the opportunity of selecting the treatment that better suits a certain tumour (Graefen et al., 2004; Greene et al., 2006; Damber and Aus, 2008). Nonetheless, this system is not flawless and, as such, the identification of novel diagnostic and prognostic biomarkers for a better therapeutic resolution is mandatory.

Risk-group	PSA level (ng/ml)	GS	Stage
Low	≤ 10	≤ 6	T1c to T2a
Intermediate	10-20	7	T2b
High	≥ 20	≥ 8	T2c-3a

Table 2 | Stratification of PC patients into distinct risks groups. Patients can be categorized into low-, intermediate-, and high-risk groups based on the serum level of PSA, GS, and stage (adapted from D'Amico et al., 1998 and Mottet et al., 2015).

8. Treatment

8.1. Treating localized neoplasms.

One of the major struggles regarding the management of PC is the discrimination between men whose tumour is expected to remain quiescent from those who harbour aggressive neoplasms and thus require radical therapy (Attard et al., 2015). In the presence of low-risk tumours patients can be cured and face the option of either surgery or radiotherapy (Cooperberg et al., 2004; Galper et al., 2006; Bill-Axelson et al., 2011). The surgical removal of the prostate and seminal vesicles, also known as radical prostectomy (RP) (open laparoscopic, or robotic-assisted), comprises a high morbidity associated surgery involving ample economic burden to the healthcare system. Of every 38 performed surgery only one case truly benefit from it, with the remaining 37 reporting substantial short or long term adverse events, including sexual dysfunction and urinary incontinence (Sanda et al., 2008; Carter et al., 2015). This procedure is particularly recommended for males whose life expectancy is greater than 10 years and whose comorbidities are low (Albertsen et al., 2011).

Generally, patients beyond 70 years of age are not suitable for surgery (Albertsen et al., 1998).

Radiotherapy can be delivered by external-beam radiotherapy (EBRT), brachytherapy or, more recently, proton beam therapy (PBT), and can be used as monotherapy or combined as an adjuvant post-RP (DeVita et al., 2008; Heidenreich et al., 2016). In comparison to RP fewer side effects are reported, with both possessing similar survival rates (Jani and Hellman, 2003). ERBT embodies a conventional non-invasive approach that delivers beams of high-energy x-rays to gland and surrounding milieu, including the seminal vesicles (Duchesne, 2001; Moule and Hoskin, 2009). Conversely, PBT resorts to protons as an alternative to x-rays to eliminate neoplastic cells. To date no clinical trial comparing ERBT to PBT is concluded. Yet, PBT appears to consent a superior control of the disease as well as dosimetry and toxicity advantages over conventional radiotherapy (Wisenbaugh et al., 2014). Brachytherapy refers to the inclusion of small radioactive seeds directly into the prostate, either permanently or temporarily, thus protecting the surrounding tissues from the effects of radiation (Moule and Hoskin, 2009).

A prominent number of radically-treated tumours are being reported to be seemingly harmless (Cooperberg et al., 2011). For this reason, reducing overtreatment in men whose tumours do not pose a real harm and are likely to remain quiescent has become a priority for urologists (Loeb et al., 2014). The emergence of surveillance strategies, i.e., active surveillance (AS) or watchful waiting, offers patients the opportunity of escaping needless, possibly harmful treatment and refer to any approach that is used to delay or avoid therapy *via* surveillance after a positive diagnosis (Attard et al., 2015; Chung and Lee, 2016). AS encompasses a management strategy involving close monitoring of the course of the disease by repetitive testing (Filson et al., 2015). As soon as early signs of progression start to be perceived curative intended therapy is introduced (Choo et al., 2012). Comparable to AS, watchful waiting also embroils meticulously monitoring of PC albeit the expectancy is to deliver palliative treatment once the manifestation of symptoms is imminent (Chung and Lee, 2016).

8.2. Treating advanced neoplasms

In most patients PC presents itself as an organ-confined disease which can be effectively treated by surgical, radio, or *surveillance* options. Nonetheless, some eventually progress or present disseminated and incurable disease with the available treatments being merely palliative. The balance between cell proliferation and cell death is crucial for preserving the authenticity of tissues. In fact, deviations in the normal operation of these processes have been allied to the onset, progress and maintenance of a number of pathologic conditions, including cancer. Prostate carcinomas rely on androgens for growth and survival, i.e., androgens stimulate proliferation and inhibit apoptosis of cancer cells. Therefore, androgen deprivation therapy (ADT), either by chemical or surgical castration, emerged as the standard of care for advanced or recurrent PC (Harris et al., 2009). While surgical castration, or bilateral orchiectomy, eradicates testosterone by removing the testis, chemical castration is usually achieved by the use of luteinising hormone-releasing hormone (LHRH) agonists and antagonists. These drugs are able to diminish the amount of testosterone in circulation by blocking the pituitary synthesis of LH *via* mechanisms of negative feedback or competitive inhibition, respectively, and may be used in combination with surgical or radiation options (Perlmutter and Lepor, 2007; Ramsay et al., 2009; Heidenreich et al., 2011). Anti-androgens have also been used, i.e., flutamide, bicalutamide and nilutamide, and operate as competitive inhibitors thus avoiding the stimulation of the androgen receptor (AR) (Monnet et al., 2015).

ADT produces a temporary therapeutic response that is usually followed by relapse and disease progression in one to two years, a status known as castration-resistant PC (CRPC). In these cohort the occurrence of metastatic CRPC (mCRPC) embraces the leading cause of death (Gupta et al., 2014). The molecular mechanisms underlying mCRPC are not entirely understood but largely reliant on the signalling governed by AR (Knudsen and Scher, 2009; Chandrasekar et al., 2015). The acquisition of numerous genetic alterations leading to AR amplification, binding of alternative AR ligands, intratumoral steroidogenesis, and enhanced activity of several pathways, including the Wnt/β-catenin signalling are some of the related mechanisms (Chandrasekar et al., 2015; Katzenwadel and Wolf, 2015). As such, several research teams are

now focused on developing strategies to block AR and their immediate downstream targets. Indeed, the management of mCRPC, i.e., administration of docetaxel plus prednisone (Carles et al., 2012) is being strongly modified due to the development of novel medicines that ultimately improve overall survival rates (Gilson et al., 2015). They include the two next-generation AR targeting abiraterone acetate and enzalutamide but also the sipuleucel-T vaccine, carbazitaxel, and radium 223 (Kantoff et al., 2010; Fizazi et al., 2012a; Fizazi et al., 2012b; Sher et al., 2012; Parker et al., 2013). However, and regardless of such improvements, survival rates remain low, with the average standing in 3.5 years (James et al., 2015).

Overall, currently available PC therapies significantly interfere with the patient wellbeing and thus the development of novel diagnostic and therapeutic tools is extremely mandatory.

9. Role of the stemness SOX2 gene in prostate cancer.

9.1. SOX2, a stem cell transcription factor.

A group of stem cell reprogramming factors is emerging as oncogenes in a myriad of cancers (Jeter et al., 2009; Karoubi et al., 2009). Their expression is capable of prompting large scale alterations in both gene expression and cell behaviour, raising the question of whether or not stemness signatures intervene in malignant transformation (Takahashi et al., 2006). Likewise, insights into their expression and function in cancer is paramount to better understand the mechanistic underlining its development.

The core embryonic stem cell transcription factor machinery is comprised by distinct factors whose orchestration is essential for maintaining pluripotency and preventing adequate cell differentiation (Chambers, 2009). Indeed, several studies reported that not only stemness genes support cell survival and proliferation, but also interfere with regular differentiation processes. Thus, it is feasible to contemplate that they are capable of promoting hallmarks of tumorigenesis and disease progression *via* mechanisms resembling their role in stemness cells (Ye et al., 2008). In 1990 the mammalian testis-determining factor (*Sry*) gene on the Y-chromosome (Sinclair et al., 1990) was discovered. This gene is characterized by the presence of a highly conserved high-mobility

group (HMG) domain whose function relies on deoxyribonucleic acid (DNA) recognition and binding. Proteins with amino acid similarity of 50 percent or higher to the *Sry* HMG domain are termed SOX. In the vertebrate genome the SOX family is composed by 20 distinct members which, based on HMG sequence homologies, are further distributed throughout eight subfamilies (Cao et al., 2015). Furthermore, they lack adequate affinity for DNA binding and thus require the recruitment of protein partners, including Nanog, OCT4, and Sall4 (Otsubo et al., 2008). In human, the SOX2 gene is positioned in the chromosome 3 and comprises three main domains: an N-terminal, a HMG, and a transactivation domain (figure 8) (Weina and Utikal, 2014).

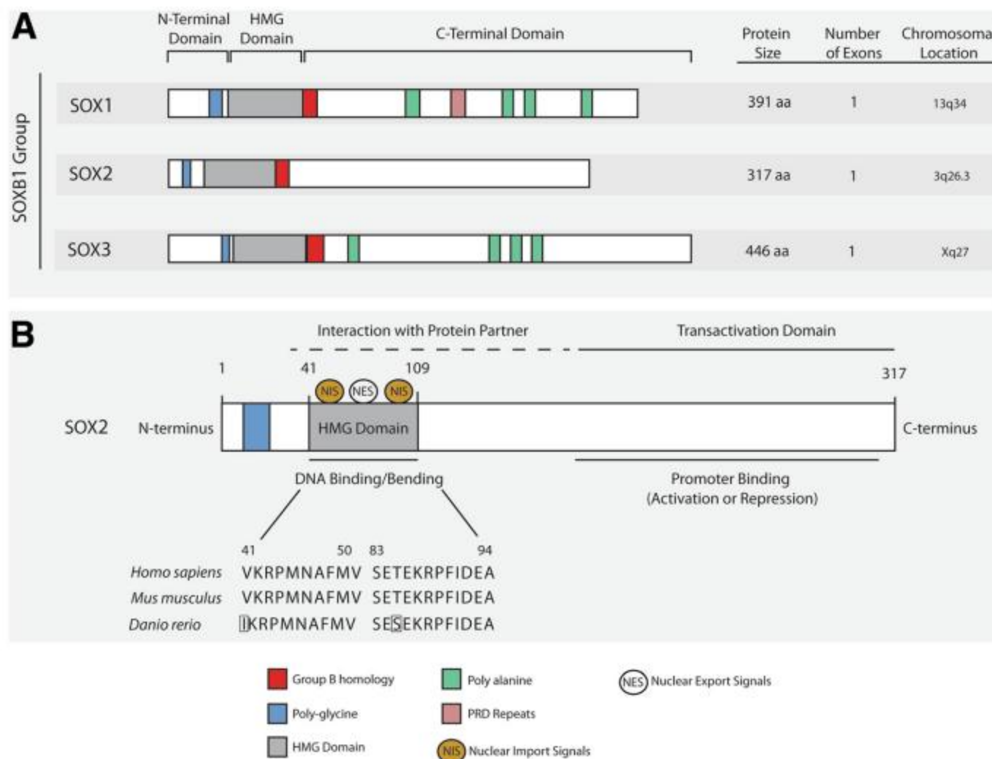


Figure 8 | SOX2 homology, structure, and protein function. SOX2 belongs to the SOXB1 group of SOX proteins. The elements of these group share large homology due to the fact that they all possess the N-terminal, the HMG, and the C-terminal domains (Weina and Utikal, 2014).

Data on the literature deeply envisage the pivotal role of SOX2 in embryonic stem cells pluripotency and self-renewal properties maintenance (Masui et al., 2007; Adameyko et al., 2012; Sarkar and Hochedlinger, 2013). In addition, SOX2 is one of the master transcription factors responsible for reprogramming

differentiated somatic cells to induced pluripotent stem cells (iPSCs) (Sarkar and Hochedlingerm, 2013). More recently, numerous efforts have been made in order to establish the link between SOX2 and malady, particularly cancer. In fact, several lines of evidence highpoint that SOX2 is deregulated in a variety of cancers, and capable of disturbing the physiology of cancer cells *via* involvement in intricate signalling pathways and protein-protein interactions (Weina and Utikal, 2014). However, the molecular network sustained by this transcription factor and the resultant clinical pathological outcome has not been well documented.

9.2. Role of SOX2 in the development and progression of prostate cancer.

SOX2 is expressed in the basal compartment of both normal and neoplastic epithelium of the prostate (Jia et al., 2011; Ugolkov et al., 2012; Russo et al., 2015). Importantly, it has been postulated that the expression of SOX2 in neoplasia is significantly deregulated. Indeed, the published outcomes concerning expression of SOX2 in diseased versus normal prostate are conflicting. While one discloses that the expression of SOX2 diminishes significantly in organ-confined PC *via* gene promotor methylation (Russo et al., 2015), others found SOX2 to be overexpressed in cancerous tissue (Jia et al., 2011). Notably, it appears that SOX2 expression is correlated with the clinical progress of PC, i.e., strong expression of SOX2 was uniquely perceived in tissues with augmented histologic grade and Gleason score (Jia et al., 2011). Analysis of p63 and Pten-null mice reveals that basal expression of SOX2 contributes to the luminal population and enhances tumorigenesis (Wang et al., 2014). SOX2 was also found to promote tumorigenesis and survival by sustaining the epidermal growth factor receptor (EGFR)-mediated self-renewal of stemness PC cells. In essence, EFGR signalling enhances SOX2 expression whereas SOX2 knock-down results in EGFR signalling eradication (Rybak and Tang, 2013). Moreover, SOX2 governs the expression of cyclin E, p27, and survinin (Lin et al., 2012) and it is involved in paclitaxel resistance of the PC3 cell line *via* the PI3K/Akt pathway (Li et al., 2014).

SOX2 is emerging as a powerful prognostic tool to predict cancer progression and cancer-specific survival in patients with metastatic PC (Fujimura et al.,

2014). Experimental models proposed SOX2 as an AR repressed gene, capable of promoting the emergence of CRPC phenotypes (Kregel et al., 2013). In fact, the inhibition of AR *via* enzalutamide treatment is responsible for prompting a robust increase in the expression of SOX2 (Kregel et al., 2013). Moreover, upregulation of SOX2 in the LNCaP cell line is seen concomitantly to ADT resistance (Seiler et al., 2013; Wang et al., 2014). SOX2 overexpression stemmed resistance to the depletion of androgens and facilitated the establishment of xenograft tumours in castrated mice (Seiler et al., 2013). Indeed, the number of $SOX2^+$ cells after progression and metastasis was found to be enlarged (Kregel et al., 2013). Such increase was seen as part of a reprogramming of primarily non-metastatic PC cells, which attained the potential to colonize and grow in bone. Nonetheless, the precise role of SOX2 and whether or not it might be modulated in PC bone metastasis is poorly understood.

AIMS

The prevalence of PC in man is very high. The probability of obtaining a biopsy positive for prostate carcinoma within a given population has been estimated to be around 30 percent. A Gleason score is established taking into account histopathological criteria. Although of prognostic value it does not distinguish those tumours which would benefit from aggressive therapy from those which would be of no real harm to the patient on the long run (Attard et al., 2015)
The present study aimed to investigate the effects of SOX2, a stem-cell associated transcription factor in order to better understand whether it might play a putative role during PC progression. Thus, our specific objectives in order to evaluate the effect of SOX2 inhibition in the biological behaviour of PC cell lines were:

a) To assess SOX2 expression in several PC cell lines cultured *in vitro* assembled into a tissue microarray by immunohistochemistry and in human PC cell lines:

- Fluorescence analysis
- Western blot analysis

b) To ascertain the role of SOX2 in PC progression by assessing the effect of SOX2 knock-down with siRNAs in *in vitro* studies:

Cell morphology
Apoptosis
Migration capacity, wound-healing

c) To assess SOX2 pattern of expression in a human PC series comprised exclusively of Gleason 8 and 9 graded cancer cases

MATERIALS AND METHODS

1. Prostate Cancer Tissues

Patients

PC samples were obtained from patients submitted to surgery at the Centro Hospitalar São João, Porto. Both incisional and excisional biopsies were removed and fixed in 10% neutral buffered formalin. After being dehydrated and embedded in paraffin, a section of 3 µm was obtained from each representative paraffin block for staining with hematoxylin and eosin and immunohistochemistry.

2. Cell lines and culture conditions

PNT2, PNTA1, 22RV1, DU145, MDA-PCa-2b, LNCaP, VCaP, RWPE-1 and PC3 cell lines, kindly provided by Professor Manuel Teixeira, IPO, were used in initial studies (Table 3 and 4). Cells were cultured at 37ºC in a humidified 5% CO_2 incubator (Thermo Scientific) and maintained in complete media supplemented with 10% of fetal bovine serum (FBS) (Gibco Life Technologies) and 1% Penicillin Streptomycin (Gibco Life Technologies) for a confluence of 70 to 80%. PNT2 and 22RV1 cell lines were cultured in RPMI 1640 (Gibco Life Technologies) and PC3 cells in F12 medium (Gibco Life Technologies). Re-plating of cells was accomplished by using trypsin followed by resuspension of cells into fresh medium.

Table 3 | Main characteristics of human prostate cell lines. All these cell lines are androgen independent.

Line	Type	Origin and Tumorigenicity	PSA expression
PNT2	Epithelial; adherent	The primary culture was derived from a prostate of a 33-year-old male at post mortem. The cells are non-tumorigenic in nude mice.	N/A
PNTA1	Epithelial Adherent	The primary culture was derived from a prostate of a 33-year-old male at post mortem The cells are non-tumorigenic in nude mice.	N/A
DU145	Carcinoma; epithelial; adherent	Established from a brain metastasis of a 69-year-old Caucasian male. The cells are tumorigenic in nude mice	-
PC3	Grade IV adenocarcinoma; epithelial; adherent	Initiated from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year-old Caucasian male. The cells are tumorigenic in nude mice.	-

Table 4 | Main characteristics of human prostate cancer cell lines. All these cell lines are dependent of androgens.

Line	Type	Origin and Tumorigenicity	PSA expression
22RV1	Carcinoma Epithelial Adherent	Established from a xenograft that was propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft. The cells are tumorigenic in nude mice.	+
MDA-PCa-2b	Adenocarcinoma Epithelial Adherent	Derived from a bony metastasis of an androgen-independent carcinoma of a Black male with 63 years of age. The cells are tumorigenic in nude mice.	+
LNCaP (clone FGC)	Carcinoma Epithelial Adherent.	Initiated from a left supraclavicular lymph node metastasis of a Caucasian male with 50 years of age collected from a needle aspiration biopsy. The cells are tumorigenic in nude mice.	+
VCaP	Epithelial adherent	Established from a vertebral bone metastasis from a 59 year-old Caucasian men CRPC diagnosed. The cells are tumorigenic in nude mice.	+
RWPE-1	Epithelial Adherent	The cells were established from a normal human prostate of a 54 years old male	+

3. Tissue microarray construction

Nine PC cell lines were arrayed in duplicates into one tissue microarray (TMA) block. The first requirement for construction of the TMA is to obtain a large number of cells cores. Likewise, each cell line was grown and collected from culture flasks by scrapping. Afterwards, cells were washed three times with phosphate buffered saline (PBS), ressuspended in 10% Neutral-buffered formalin and fixed for 1 hour in gently agitation. The cell pellet was processed with the Thermo Scientific Richard-Allan Scientific HistoGel^{TM} (Thermo Scientific). Briefly, the HistoGel must be liquefied by heating in a water bath. Each cell pellet was embedded in the liquefied gel and vortex to allow effective mixing. The samples were refrigerated at -20ºC to allow complete solidification and transferred to a cassette. Following overnight fixation, the samples were processed, embedded in paraffin, sectioned and haematoxylin-eosin stained. The Beecher manual tissue microarrayer (Manual Tissue Arrayer, Beecher Instruments, Inc, Wisun, USA) was used to construct the TMA. A core of tissue was removed from a donor block and implanted into a recipient block following a previously established order. Non-neoplastic tissue from the prostate as well as mammary cancer cell lines were included as control. Once this process was completed, the recipient block was melted to promote the binding of the cores with the block. Melting was performed by overnight incubation at 37ºC. In the next day, the blocks were submitted to cycles of resting plus incubation at 37ºC for 1 hour. This process was repeated twice. Afterwards, the TMA block was incubated at 60ºC for homogenization of its surface and sectioned into 2 to 3 µm tissue sections in coated glass slide (Superforst Plus, Gerhard Menzel, Braunschweig, Germany).

4. Immunohistochemistry

The expression of SOX2 was assessed in paraffin-embedded PC samples and the assembled TMA by immunohistochemistry following a standard protocol. Briefly, the slides were deparaffinised in xylene and rehydrated in an alcohol/water gradient. Antigen retrieval was performed in ethylediaminetetraacetic (EDTA) for 40 minutes following a resting period of 20

minutes at room temperature. The activity of endogenous peroxidase was blocked by incubating the sections in 3% hydrogen peroxide (H_2O_2) for 10 minutes. Afterwards, sections were incubated overnight at 4ºC with monoclonal anti-SOX2 (1:50 dilution, SP76 clone, Cell Marque, Rocking, CA, USA), washed in tris-buffered saline-tween 20 (TBS-T) and developed using the Dako REAL™ Envision™ Detection System Peroxidase/DAB + (Envision-DAKO, Glostrup, Denmark). The reaction was developed using 3,3′-diaminobenzidinetetrahydrochloride (DAB) (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Sections were then counterstained with haematoxylin, dehydrated and mounted using histologic mounting media (Thermo Scientific Richard-Allan). All stained sections were examined using a Zeiss Optical Microscope and reviewed by two observers.

5. Protein extraction and Western Blot

Cells were scrapped and washed with PBS 3 times at 4ºC. Next, cells were incubated with lysis buffer (RIPA (20 mM Tris, pH7.2, 10mM EDTA, 0.3 M NaCL, 0,1% Triton X-100, 0,005% Tween-20), protease inhibitors (1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 mM sodium orthovanadate (Na_3VO_4) and complete protease inhibitor cocktail (Roche Applied Science)) on ice for 30 minutes. The lysates were centrifuged for 15 minutes at 12000 G, the supernatants collected, and the protein content quantified using the BCA Protein Assay Reagent (Thermo Scientific, USA), according to the manufacturer's instructions. Proteins were run on Mini-PROTEAN TGX 4-20% ge**ls** (Bio-Rad, USA) and transferred by electro blotting to nitrocellulose membranes (Amersham, Biosciences). The membranes containing the proteins were blocked with 5% non-fat dry milk diluted in TBS (50mM Tris, pH 7.6, 150mM NaCl, 0,005% Tween 20) and incubated with agitation at 4ºC overnight with anti-SOX2 (1:500 dilution, Cell Maque, Rockling, CA, USA) and anti-actin (1:4000, Santa Cruz Biotecnology) diluted in 5% non-fat dry milk in TBS. In the day after, the membranes were washed with TBS-tween and incubated with the secondary antibody goat anti-rabbit (1:2000 dilution, DAKO) at room

temperature for 1 hour. Immunolabeling was performed by using the enhanced chemiluminescence reagent (ECL; Amersham Biosciences).

6. Fluorescent immunocytochemistry

PC3 cells were cultured in glass coverslips for approximately 24 hours. Cells were washed with PBS and fixed with cold methanol for 30 minutes. Following fixation, cells were re-hydrated with PBS and incubated with normal rabbit serum in PBS with 10% bovine serum albumin (BSA) for 20 minutes to block nonspecific staining. Next, sections were incubated with primary antibody for SOX2 (1:50 dilution, SP76 clone, Cell Maque, Rockling, CA, USA) in PBS with 5% BSA overnight at 4ºC. After washing, the slides were incubated with secondary goat anti-rabbit antibody (1:100 dilution, DAKO) for 45 minutes, washed twice, incubated with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, USA) for 15 minutes. For fluorescence analysis, slides were mounted in Vectashield mounting medium (Vector Laboratories, USA) and analysed in a Carl Zeiss fluorescent microscope (Carl Zeiss Microscopy, Germany).

7. Transient transfection assays: inhibition of SOX2 by siRNAs

PC3 cell line was transfected with a commercial set of three small interference ribonucleic acid (siRNA) duplexes targeted against human SOX2 (#HSS144045: sense 5'-GCGUGAACCAGCGCAUGGACAGUUA-3'; #HSS186041: sense 5'-CCUGUGGUUACCUCUUCCUCCCACU-3'; #HSS186042: sense 5'-CCAAGACGCUCAUGAAGAAGGAUAA-3'; Invitrogen, Life Technologies, Carlsbad, CA, USA) or scrambled controls (1: sense 5'-GCCGAAAUGGCGACGUCCAGAAUAU-3'; 2: sense 5'-GCGCAGCGAAGUCGGUCGAUACGU-3'; 3: sense 5'-GAUGCGGACGGAGGAUCHAUGUCA-3', Invitrogen, Life Technologies, Carlsbad, CA, USA). The cell line ($6x10^5$) was firstly seeded into six well plates. In the following day the inhibition of SOX2 was performed with each well containing 1.67 μl of either scrambled controls or siRNA duplexes. Master mixes for each transfection condition were prepared. Four distinct tubes were prepared to which we added (i) 1.67 μl of the plasmid DNA (ii) 1.67 μl of the

universal control (iii) and (iv) 5 µl of Lipofectamine 2000 reagent (Invitrogen), plus 125 µl of Opti-MEM Reduced Serum Media (Invitrogen). After 10 minutes of incubation at RT, the contents of tubes (iii) and (iv) were added to the first ones, mixed gently and incubated for 20 minutes at room temperature. The cells were washed with phosphate-buffered saline (PBS) and 750 µl of Opti-MEM plus 250 µl of the complex mixture were pipetted carefully to each well. The plates were mixed by shaking and incubated at 37ºC for approximately 24 hours. The culture medium was changed to standard medium and the cells collected in the next day.

8. Wound-healing Assay

The wound-healing assay was carried out in a time-lapse microscope and image acquisition was performed for 18 hours. Briefly, 3 x 10^5 cells were plated in triplicates in 24 wells culture plate for a confluence of 100%. An artificial wound was done by scratching each well with a pipette tip. The culture medium was replaced by fresh medium and the migration rate of cells was assessed considering their healing capacity for 18 hours.

9. Annexin V/PI Assay

Each cell line was harvested and transfected according to the previously conditions described. Cells were pelleted by centrifugation, the culture medium was complete removed, and cells were ressuspended in 400 µl of binding buffer (Annexin V Apoptosis detection kit, eBioscience, San Diego CA, USA). 195 µl of each sample was aliquot and firstly stained with 5 µl of annexin V for 10 minutes and then with 10 µl of propidium iodide (PI). The incubations were performed at room temperature in the dark. The percentage of apoptotic cells was measured in the flow cytometer BD Accuri C6 (BD Biosciences, San Diego CA, USA).

10. Statistical analysis

Whenever adequate, the results were presented as mean ± standard deviation. Statistical analysis was performed using One Way ANOVA (Analysis of

variance) test and for multiple comparisons Dunnett and Tukey's tests with $p<0,05$ as the level of significance, in GraphPad Prism 5.02 version.

RESULTS

Androgen receptor positive vs negative human prostate cancer cell lines morphology

PNT2 is a human epithelial normal prostate cell line, whereas PC3, DU145 and 22RV1 comprise human PC cell lines.
PC3, DU145 and PNT2 are three well-established AR negative human PC cell lines. In morphologic terms, DU145 cells appear more circular and smaller in comparison to the highly metastatic PC3 cell line. The later further displays a dendritic-like morphology. In the case of PNT2 cell line, it mostly resembles the appearance of DU145 cells. However, the shape of PNT2 cells appears less circular and the cells are capable of establishing a few more processes. Furthermore, the PNT2 line possess a slightly larger cell size than DU145 cells (figure 9).
22RV1 is an AR positive human PC cell line. Regarding its morphologic features, and in comparison to the previously described cell lines, it exhibits a more polygonal shape and the cells growth appears more clustered. In addition, 22RV1 cells are smaller than PC3 cells (figure 10).

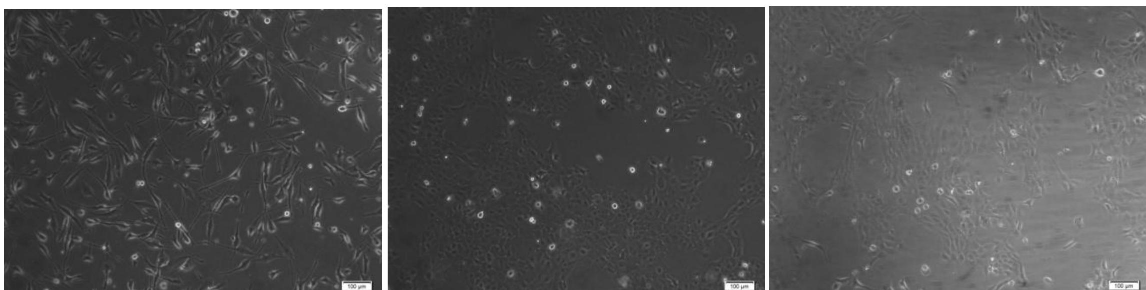


Figure 9 | Phase contrast microscopy pictures of human androgen independent cancer and normal prostate cell lines. Pictures of the highly metastatic PC3 (A), moderately metastatic DU145 (B) and normal PNT2 (C) cell lines. Cellular morphology was inspected using a Zeiss inverted microscope at 10x magnification (Scale = 100 μm)

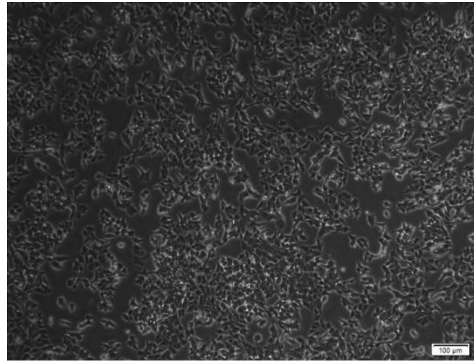


Figure 10 | Phase contrast microscopy pictures of the human AR positive PC 22RV1 cell line. Cellular morphology was inspected using a Zeiss inverted microscope at 10x magnification (Scale = 100 µm).

Androgen receptor positive and negative human prostate cancer cell lines TMA assembly

The TMA comprises an extremely versatile methodology that consents large scale measurements of either ribonucleic acid (RNA) or protein expression in various sections of tissue simultaneously (Kononen et al., 1998). The construction of TMA blocks is generally made from tissues embedded in paraffin. Nevertheless, novel methods that enabled the construction of TMAs from small volumes of cells in suspension were developed (Waterworth et al., 2005). The whole technique is performed in a centrifuge tube and produces an outstanding preservation of both cytomorphology and immunoreactivity of the cellular cores included in the TMA block, contrarily to what would occur in cytospins. The latter is often used to restrain cells onto glass slides for staining procedures. However, it generally prominently affects the quality and morphological features of the cell prep (Methods in Enzymology, 2013).

We started by selecting the most representative areas of each cell core, previously separately embedded in paraffin, using hematoxylin-eosin staining. Afterwards, each cell core was transferred in duplicates to a recipient block, following a defined array of coordinates previously established in an excel datasheet (figure 11), to better capture the heterogeneity of each core. To include all samples, i.e., controls and nine prostate cell lines, one tissue array was constructed. As described in figure 11, non-neoplastic tissue of the normal

prostate was included as positive control in position 1. Mammary cancer cell lines were included in positions 28, 29, 30, 33 and 34 also for control purposes. The remaining positions were occupied by normal and cancer cell cores of the prostate as well as paraffin to assure the stability of the block during sectioning. Multiple 3 μm sections were cut and stained by a specific antibody for immunohistochemistry analyses.

TMA prostate cancer cell lines

Prostate	Paraffin	Paraffin	Paraffin	Paraffin	Paraffin	Paraffin
Paraffin	PC3 (1)	PNT2 (1)	MDA-Pca-2b (1)	VCaP (1)	RWPE-1 (1)	RWPE-1 (2)
Paraffin	PC3 (2)	PNT2 (2)	MDA-Pca-2b (2)	VCaP (2)	CMM115	CMA07 (1)
Paraffin	22RV1 (1)	DU145 (1)	LNCaP (1)	PNT1a (1)	CMM26	CMA07 (2)
Paraffin	22RV1 (2)	DU145 (2)	LNCaP (2)	PNT1a (2)	CMTU27	Paraffin

Figure 11 | Construction of the PC cell lines TMA block. A TMA grid was firstly generated in an Excel data sheet containing the coordinates of the block and the cell cores identification. Normal prostate tissue as well as mammary cancer cell lines were included as controls.

SOX2 expression in the Androgen Receptor positive and negative human prostate cancer cell lines TMA

All prostate cell lines were almost negative. PC3 cell line was lightly positive, when compared with the positive control; basal cell lines of the normal human prostate tissue as well as mammary cancer cells were included as controls (figure 12, 13 and 14).

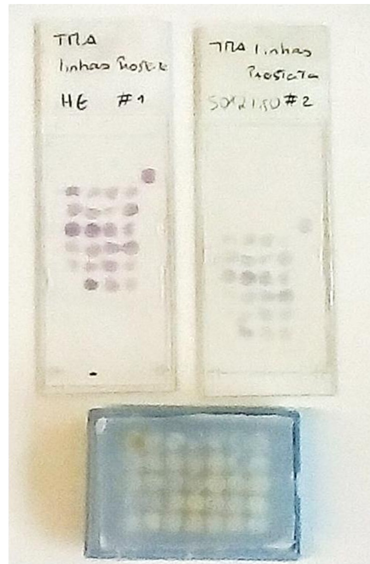
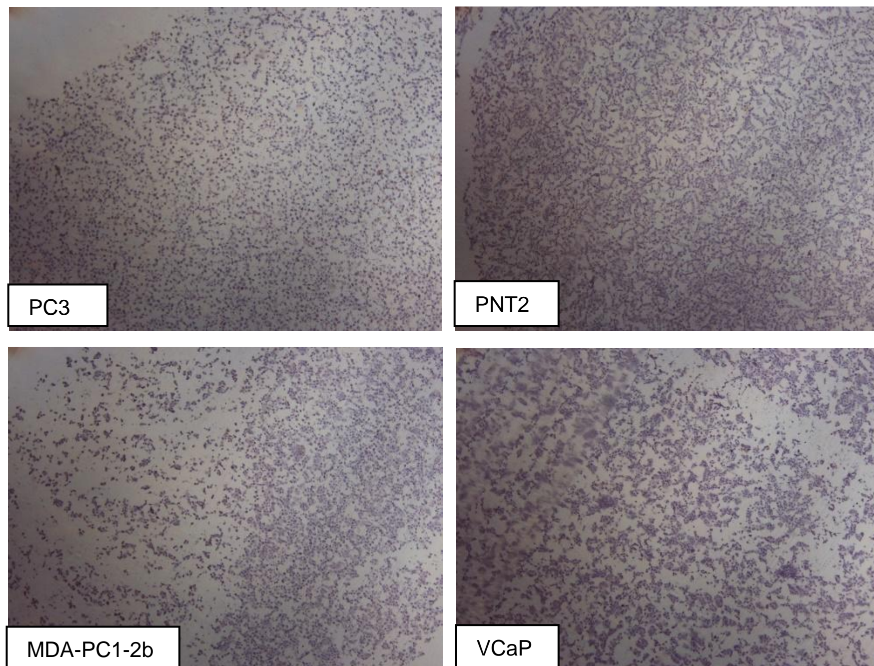


Figure 12 | Macroscopic pictures of the PC cell lines TMA block and sections. The image shows hematoxylin & eosin and SOX2 stained sections and the paraffin embedded cell cores block. Normal human prostate tissue as well as mammary cancer cells were included as controls.



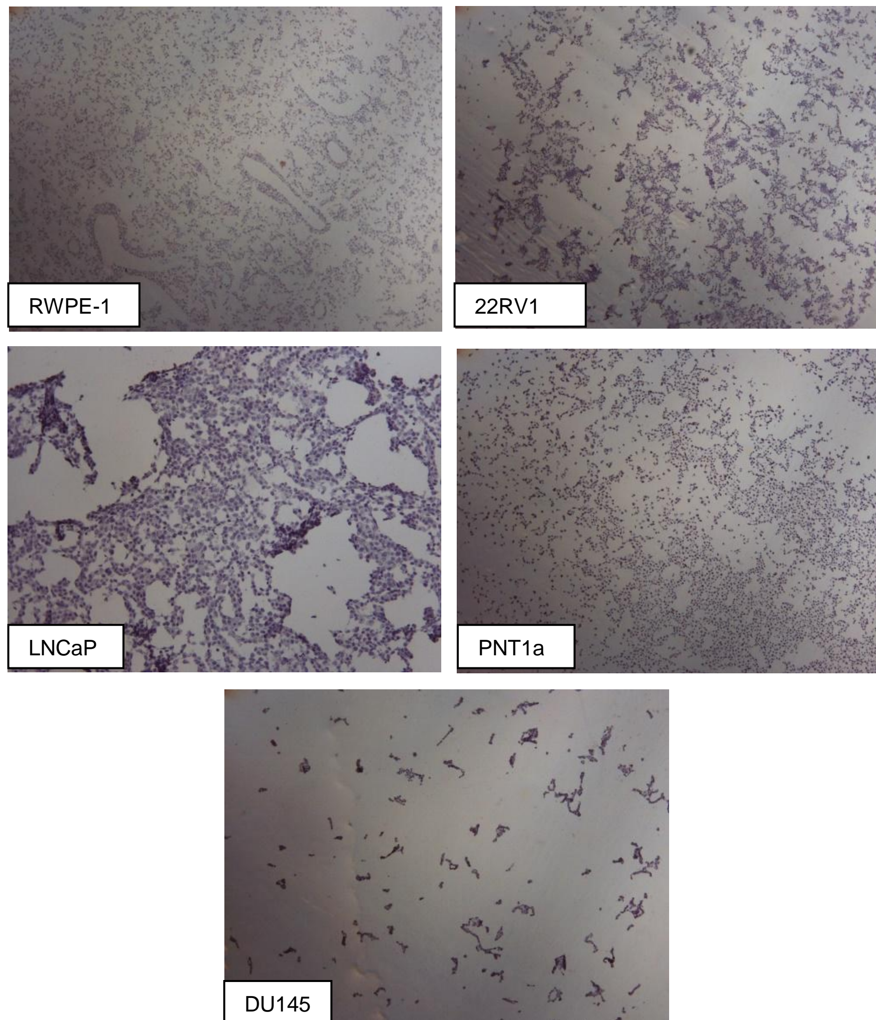
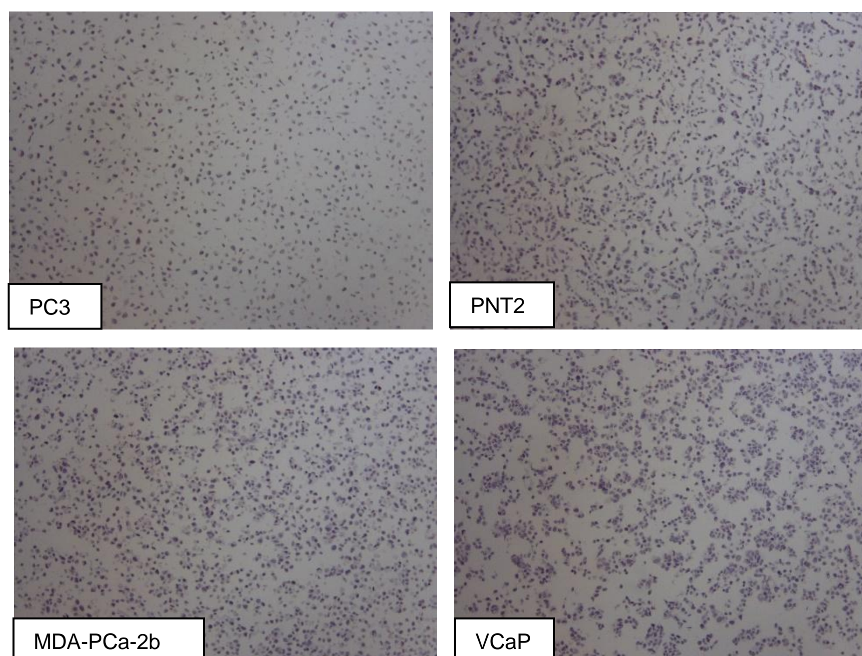


Figure 13 | Haematoxylin & eosin sections in PC cell lines assembled in the TMA.



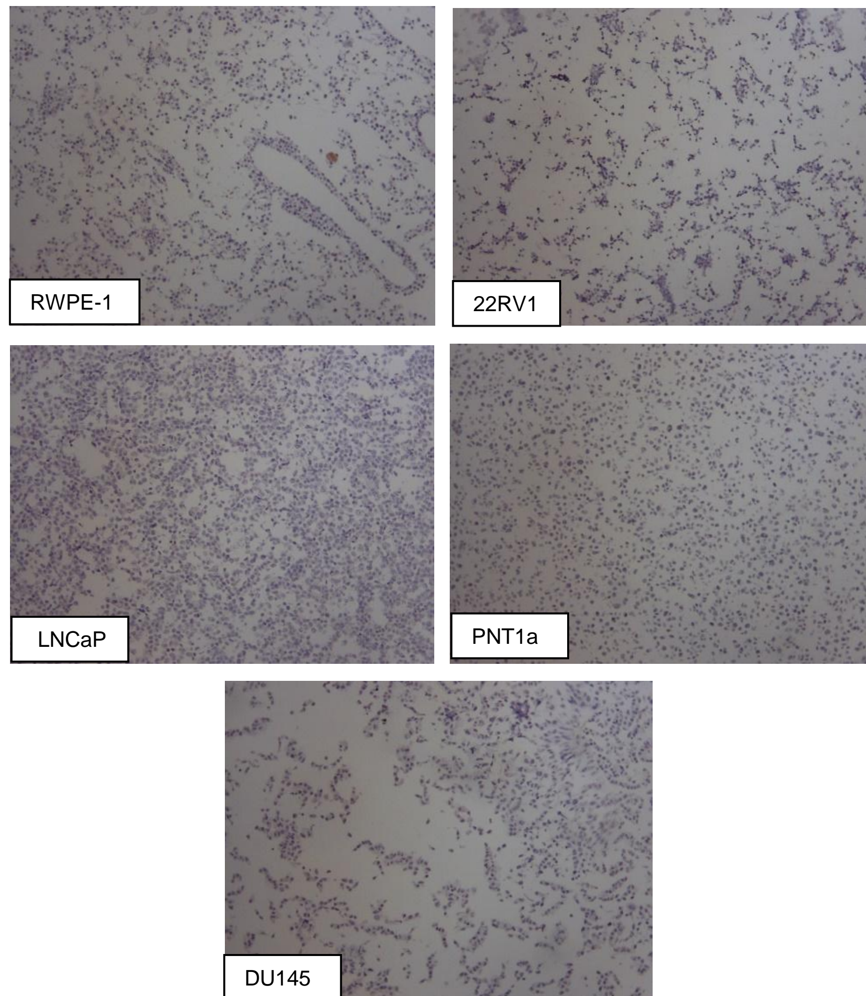


Figure 14 | Expression of SOX2 in PC cell lines assembled in the TMA by immunohistochemistry. All prostate cell lines were negative with the exception of PC3 which was lightly positive. Normal human prostate tissue as well as mammary cancer cells were included as controls (data not shown).

SOX2 expression in prostate tumor cell lines.

The expression level of SOX2 in the PC3 cell line was confirmed by Western Blot analysis and fluorescent immunocytochemistry. PNT2 and 22RV1 were included as negative controls. The 3D6 colon cancer cell line was used as a positive control. As indicated in figure above expression of SOX2 was only detected in PC3 cell line pointing to a possible involvement of SOX2 in the tumorigenesis of PC3 cells (figure 15).

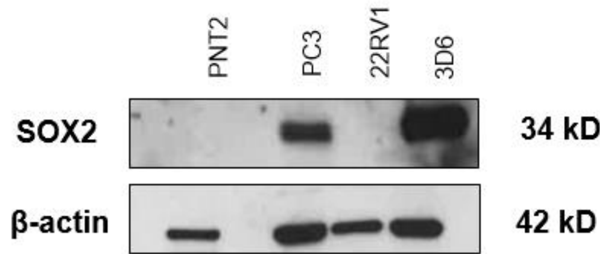


Figure 15 | SOX2 expression in prostate tumor cell lines by Western blot analysis. SOX2 protein expression is significant in PC3 cell line and non-detectable in PNT2 and 22RV1 cell lines. The 3D6 colon cancer cell line was used as a positive control.

Regarding immunocytochemistry on methanol-fixated PC3 cells, despite most cells being negative, a few cells were strongly positive (red fluorescence). Subcellular localization points to a predominantly nuclear and/or cytoplasmic expression of SOX2 in this cell line (figure 16).

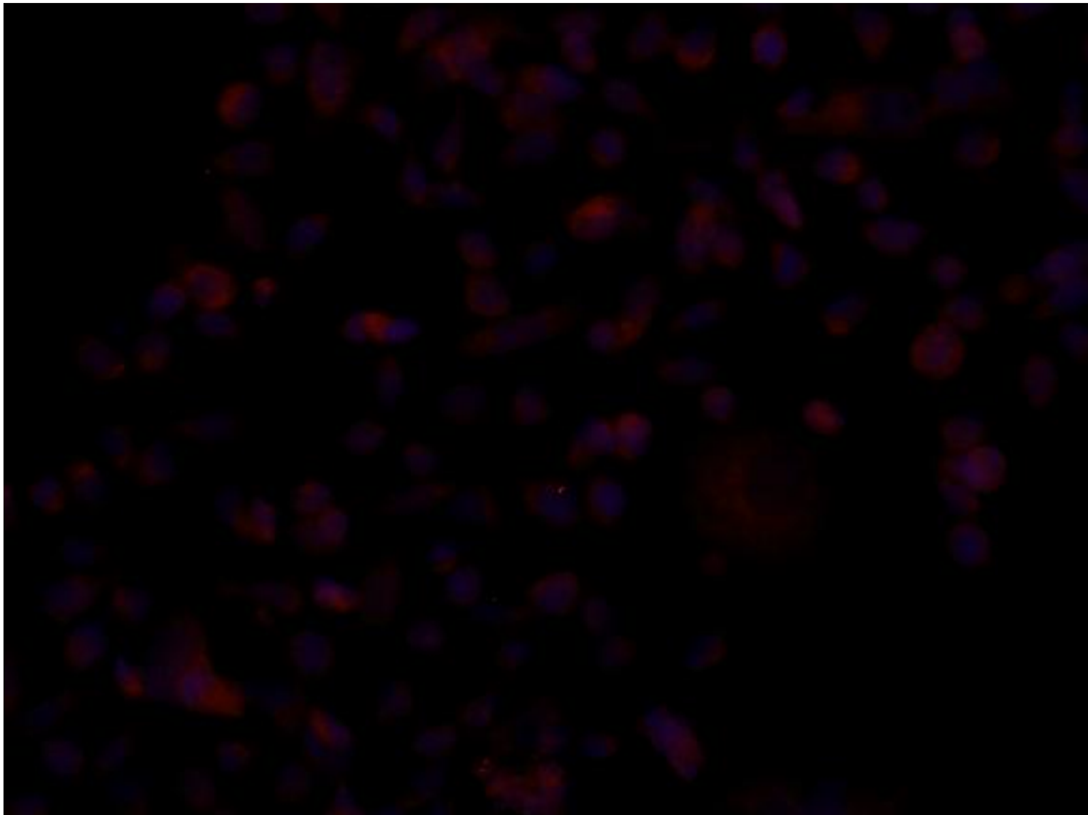


Figure 16 | SOX2 protein expression in the PC3 cell line by immunocytochemistry. The majority of cells do not show SOX2 protein expression. Only a few cells were strongly positive for SOX2 (red fluorescence) and are predominantly positioned in the nucleus and/or in the cytoplasm.

Down-regulation of SOX2 in prostate derived cell lines

In order to perform down-regulation experiments of SOX2 in neoplastic settings, we used metastatic PC3 cell lines which were cultured for 48 hours in siSOX2 or scrambled conditions (normal PNT2 cell lines were included for further assay control purposes). Protein expression of SOX2 was determined by Western Blot analysis. As shown in figure 17 the expression of SOX2 was diminished in protein extracts from PC3 cells where siRNAs were used when compared to the scrambled transfected cells thus confirming the efficiency of the transfection experiments. Thus, SOX2 targeting siRNAs effectively knocked-down its expression in PC3 cells.

Figure 17 | SOX2 expression in prostate tumor cell lines transfected with siSOX2 and scrambled controls by Western Blot analysis. SOX2 protein expression decreases in protein extracts from PC3 cells transfected with siRNA's for SOX2 when compared to scrambled controls. In PNT2 protein extracts the expression of SOX2 remained non-detectable so we use this as cell as a control for the experiments (data not shown).

Effect of SOX2 knockdown on the morphology of PC3 cells

Cell clustering and morphology in PC3 cell lines before transfection with siSOX2 showed no major differences when compared with cell clustering and morphology in PC3 cell lines transfected with siSOX2. The slight differences found occasionally were attributed to culture conditions (time, cell confluence) (figure 18).

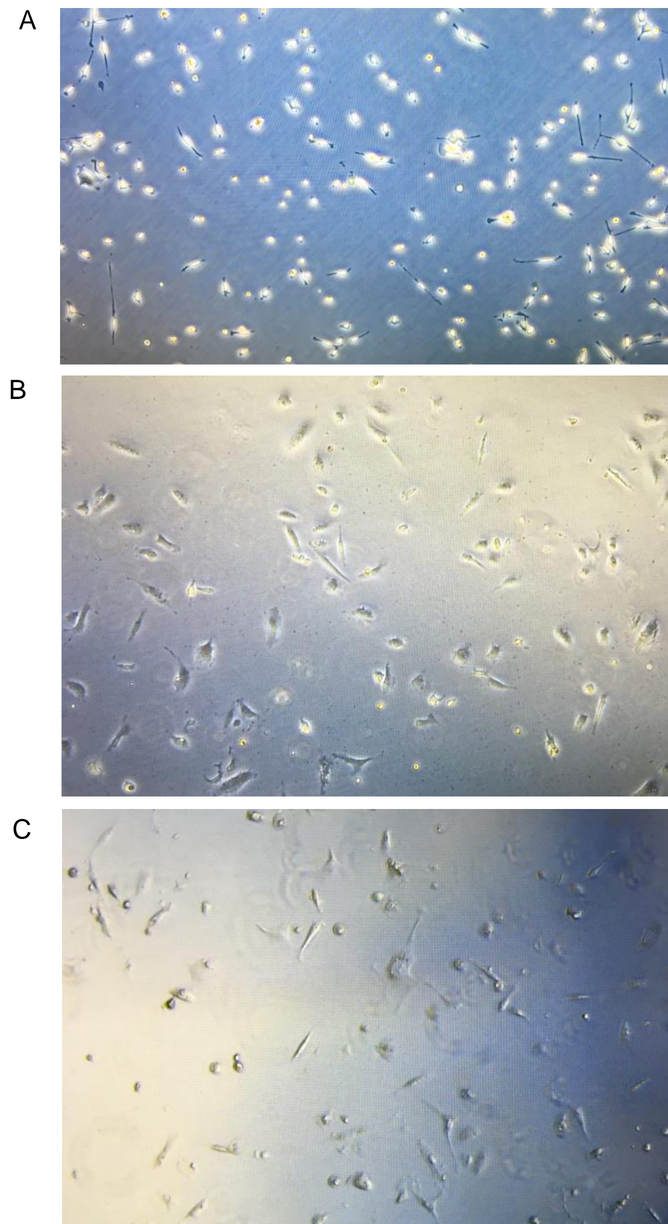


Figure 18 | Contrast phase microscopic pictures of PC3 cells pre- and post- transfected with siRNAs for SOX2 or scrambled controls. No major differences were detected in clustering and morphology parameters when the PC3 cell line (A) was transfected with siSOX2 (B) when compared to scrambled controls (C).

Effect of SOX2 knock-down in programmed cell death of PC3 cell line.

The influence of SOX2 silencing on programmed cell death was evaluated by the *in vitro* assays below. To assess the effect of SOX2 silencing on the PC3 cells line programmed cell death the Annexin V/PI assay was performed. To this end, we cultured cells under silencing and scrambled conditions for 48 hours.

As previously shown, expression of SOX2 in the normal PNT2 cell line was not detectable by Western Blot. As such, we used this cell line as an internal control for the experiment. We found that forty-eight hour SOX2 silencing significantly increased the percentage of apoptotic cells of PC3 (p=0.0115) and not PNT2 (p=0.6194) cell lines when compared to scrambled controls for SOX2 silenced cells. These results suggest promotion of programmed cell death of the PC3 cell line by SOX2 inhibition (figure 19).

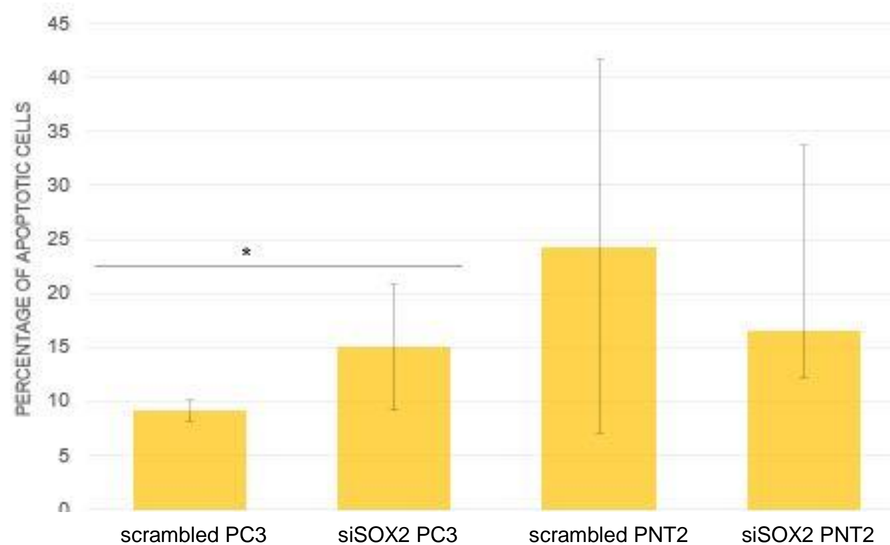
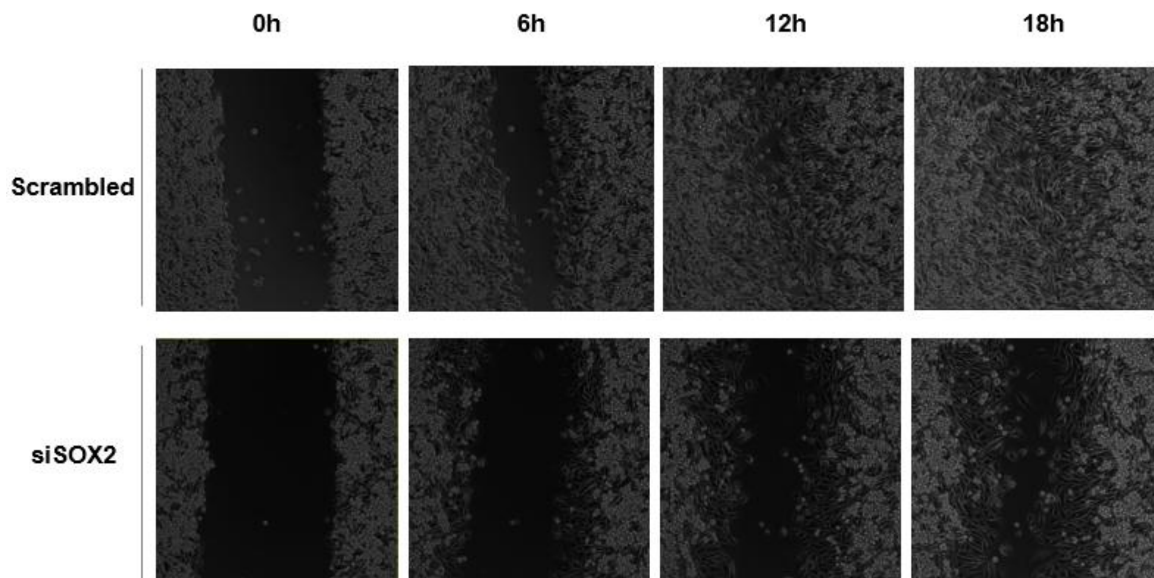


Figure 19 | Percentage of apoptotic PC3 and PNT2 cells after 48 hours treatment with siRNAs for SOX2 and scrambled controls. SOX2 knockdown mediated by siRNAs significantly increased programmed cell death for the PC3 cell line (p=0.0115) and not for the PNT2 cell line (p=0.6194).

Effect of SOX2 knockdown on migration capacity of PC3 cell line

In order to investigate the effect of SOX2 silencing on the migration capacities of prostate tumor cells, wound-healing assays were performed. The knockdown of SOX2 expression in the PC3 cell line decreased cellular migration, as proven by an increase in the time required to close the artificial wound when compared to scrambled controls (figure 20). The migration rate decrease was statistically significant (p<0.01) using the T-test for the PC3 cell line. No differences were observed in the rates of cell migration for the PNT2 line after silencing SOX2

(figure 21). Thus, our results indicate that the migrating ability of PC3 cells is impaired upon SOX2 knockdown.



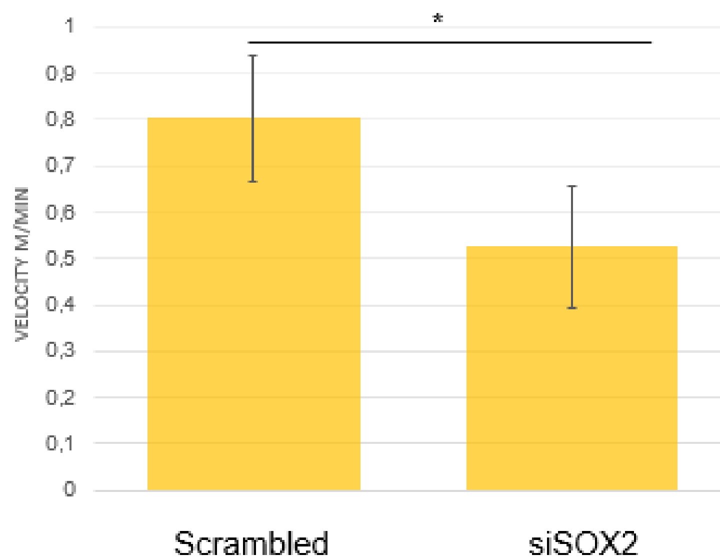
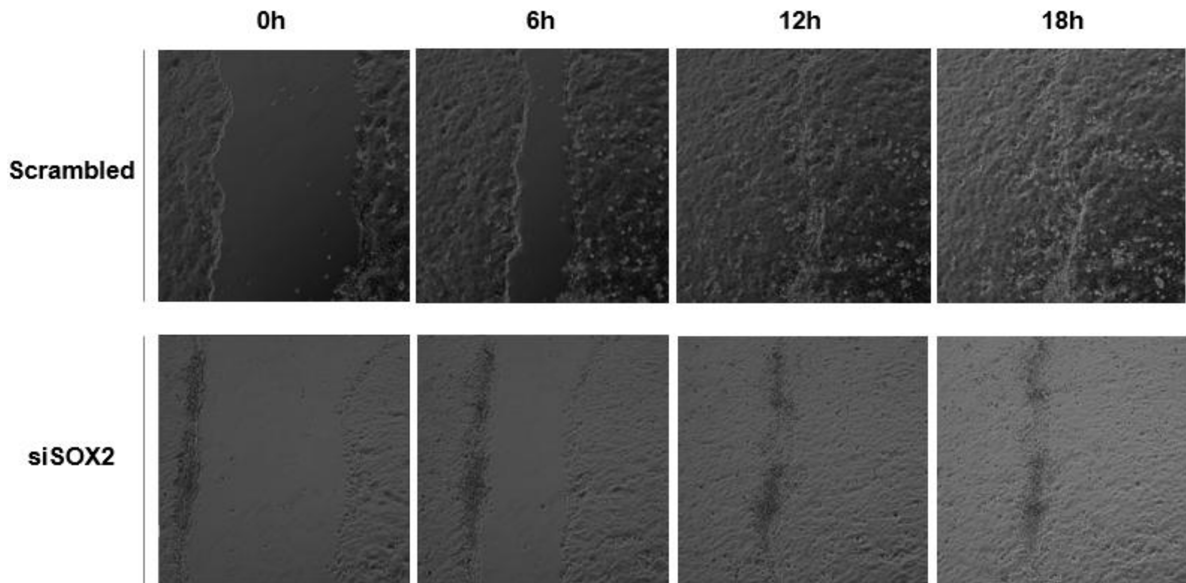


Figure 20 | Wound-healing assay in PC3 cell line transfected with siSOX2 or scrambled control. Cells transfected with siSOX2 moved slower for closing the artificial wound when compared with cells transfected with scrambled controls. This decrease in migration rates was statistically significant ($p<0.01$). The results were obtained by time-lapse microscopy for 18 hours.



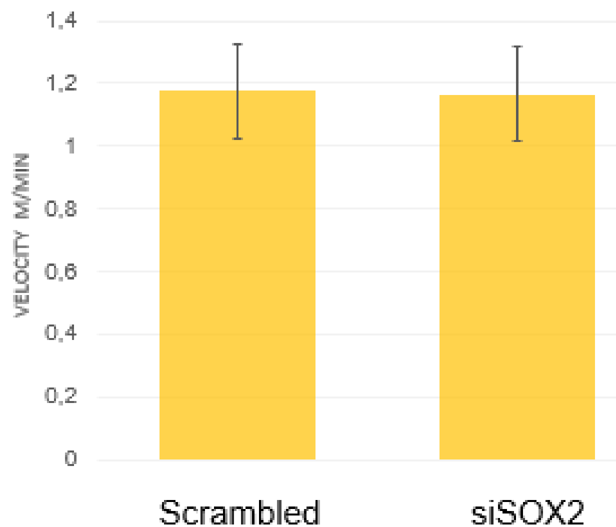


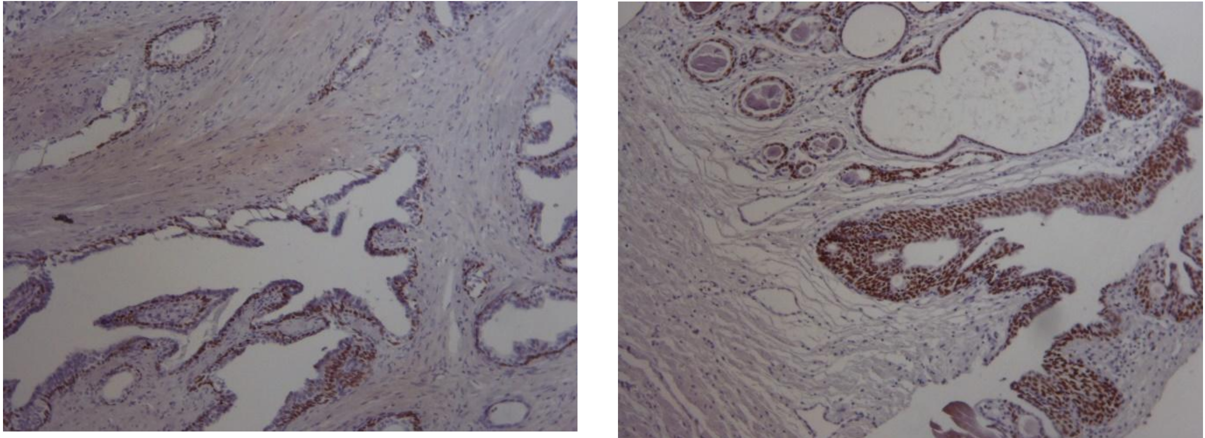
Figure 21 | Wound-healing assay in PNT2 cell line transfected with siSOX2. No considerable alteration in the migration rate of cells transfected with siSOX2 and scramble for closing the artificial wound was observed. The results were obtained by time-lapse microscopy for 18 hours.

Evaluation of SOX2 expression in a Gleason 8 and 9 PC series

In order to investigate if SOX2 would relate to a specific aggressive cell subtype in invasive PC cells, a small series of Gleason 8 and 9 human PC samples was examined by immunohistochemistry. SOX2 was expressed in basal-like cells

both in primary tumors and normal-adjacent tissue (figure 22). These results further point to a role of SOX2 possibly associated to cancer aggressiveness.

A



B

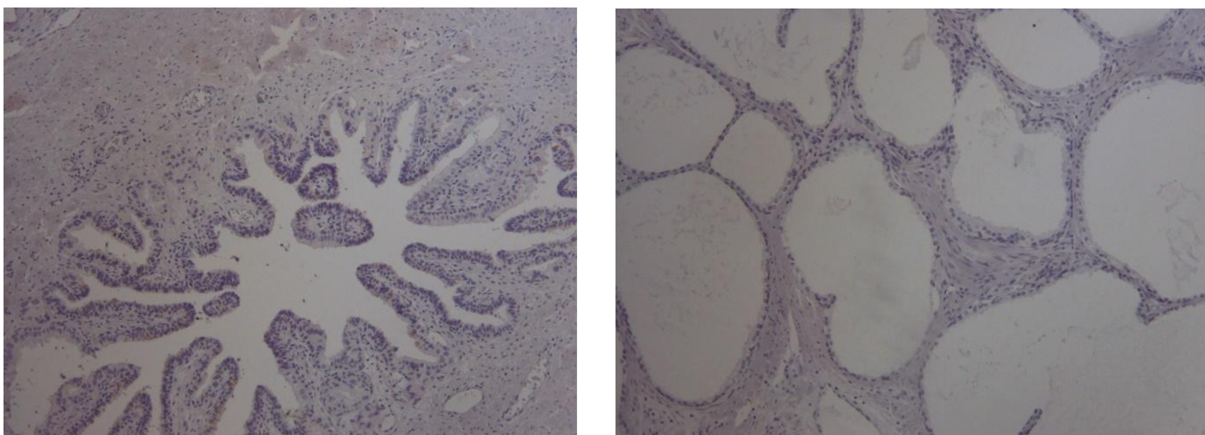


Figure 22 | SOX2 expression in Gleason 8 and 9 PC series by immunohistochemistry. Panel A and B shows the positive and negative pattern of expression of SOX2, respectively, in PC samples.

In order to assess the link between SOX2 expression and PC tumorigenesis we proceeded to a statistical analyses focusing on SOX2 relationship with a series of clinical and histopathological guidelines described in table 5 and 6. We stratified patients onto two categories based on SOX2 negativity (N=0) and positivity (N≥1) of PC specimens. Hence, the clinical pathological outcomes were compared between samples with and without SOX2 staining; a total of ten

patients who underwent surgery were analysed with five cases (50%) in the N0 category and the remaining five (50%) in the N1 category. We found no significant correlation between SOX2 expression, and tumor size (p=1) or Gleason score (p=0,141112); both SOX2 negative and positive tumors fall into either T1 or T2 and displayed a Gleason of 8 and 9. Nevertheless, in what regards the status of regional lymph nodes (N) we found that SOX2 negativity is closely associated to the presence of metastases in the lymph nodes (p<0,01).
However, when we compared SOX2 positive and negative tumors regarding recurrence criteria, i.e., D'Amico risk, disease and disease-free survival, we found that SOX2-positive tumors were significantly associated to an increased D'Amico risk in comparison to SOX2 negative tumors (p=0,0399698524). Furthermore, we also denoted a statistical tendency and as such a possible correlation between SOX2 and disease-free survival (p=0,06775149). Indeed, SOX2-positive tumors possessed shorter disease-free survival duration. However, the results did not reach statistical significance. Nevertheless, these data point to a probable SOX2 involvement in tumor recurrence.
In what concerns overall PC survival periods, no statistical differences were found (p= 0,662875818). Finally, a possible relationship between SOX2 expression and the basal PSA was also addressed. We found no statistical correlation between SOX2 and PSA, though SOX2 positive tumors were apparently associated with increased levels of PSA (p=0,208109336).

Table 5 | Clinical pathological data of the PC samples used in this study by SOX2 negativity (pN0), n=5 prostate carcinomas. Several criteria were used in order to ascertain a possible relationship between SOX2 and PC, including stage, D'Amico risk, disease-free survival (PR_SV free) and disease survival (PR_SV), PC overall survival (PC_SV), size of the tumor (T), regional lymph node (N) and bony (M) metastasis, Gleason score after RP (GPR) and basal PSA.

Case	SOX2	State	Stage	D'Amico risk	D'Amico risk p	PR_SV free	PR_SV	PC_SV	T	N	M	GRR	PSA basal
2420/95B3	0	3	2	2		1	1	11	33	1	0	9	9.9
8220/01 B27	0	3	1	2	3	127	127	135	33	1	0	9	16
6894/01 B28	0	0	1	2	2	85	85	142	23	1	0	9	18.4
10709/01 35	0	0	1	2	3	98	98	141	33	1	0	9	14.7
H05/13469 B24	0	0	1	2	3	78	79	89	32	1	0	9	6.4

Table 6 | Clinical pathological data of the PC samples used in this study by SOX2 positivity (pN≥1), n=5 positive carcinomas. Several criteria were used in order to ascertain a possible relationship between SOX2 and PC, including stage, D'Amico risk, PR_SV free and PR_SV, PC_SV, T, N M, GPR and basal PSA.

Case	SOX2	State	Stage	D'Amico risk	D'Amico risk p	PR_SV free	PR_SV	PC_SV	T	N	M	GRP	Basal PSA
5092/93B	2	3	1	2	3	19	53	56	33	0	0	8	13
2101/94 #4	2	1	2	2		1	50	106	32	0	0	9	103.4
7042/94 #3	1	3	1	3		15	105	120	23	1	0	9	47
104/01 B3	1	0	1	3	3	57	57	148	23	0	0	8	12
3352/00/F3 2	4	0	1	3	3	49	49	158	33	0	0	9	12.5

DISCUSSION

The natural history of prostate carcinomas remains elusive thus far, whether on how they evolve, disseminate and how they can be treated based on their unique features. Therefore, numerous efforts have been made and research has been particularly focused on identifying novel molecular markers or alterations that underline the ignition and progression of prostate neoplasms and can hopefully improve the accuracy of both diagnosis and therapeutic approaches.

In recent years, the notion that a small group of stem cells intervenes in the process of neoplastic transformation lead to the emergence of a novel theory regarding cancer. Indeed, cancer stem cells (CSCs) or tumor initiating cells (TICs) are believed to have the ability to generate an entire tumor. Among their features is the unlimited capacity of proliferation, self-renewal and also the extraordinary resistance to either chemotherapeutic or radiotherapy regimens (Reya et al., 2001). Hence, this small subset of cells persevering in the tumor microenvironment has been acknowledged to be a dominant determinant in the processes of development, progression, metastasis and recurrence of a tumor (Akunuro et al., 2011). In this regard, a group of stem cell reprograming factors is emerging as oncogenes in a myriad of cancers (Jeter et al., 2009; Karoubi et al., 2009). Among these, is the human transcription factor SOX2. This gene participates in cellular fate determination, differentiation and also proliferation processes (Takahashi et al., 2007; Chew et al., 2009). It is highly expressed in embryonic stem cells (Fong et al., 2008). More recently, SOX2 has been linked to the anomalous growth of numerous types of human solid tumors and to a promising contribution to CSCs (Gu et al., 2007; Kasper, 2008). In fact, expression of SOX2 has been shown in melanoma (Laga et al., 2011), digestive tract (Otsubo et al., 2008; Saigusa et al., 2009), breast (Chen et al., 2008), liver (Huang et al., 2011), pancreatic (Sanada et al., 2006) and lung carcinoma (Hussenet and Manoir, 2010). In prostate settings, SOX2 was previously established to be an AR repressed gene (Kregel et al., 2013). Its expression closely associates to the progression and aggressiveness of PC (Jia et al., 2011; Liu et al., 2013; Weina and Utikal, 2014; Russo et al., 2015).

Nevertheless, the dynamics and regulation of its activities and, most importantly, the ultimate clinical pathological impact lingers to be unveiled. As such, significant insights into the mechanist underlining the expression and role employed by SOX2 will provide a major step forward towards understanding the biological processes prevailing in the development and progression of prostate carcinomas.

In the present work we used a series of PC cell lines and patient samples to assess the effect of SOX2 on the biological behaviour of PC cells. To elucidate the role of SOX2 in prostate carcinomas, we started by screening a panel of prostate tumor cell lines for SOX2 by Western blot and immunohistochemistry analysis. We showed that the androgen independent and highly metastatic PC3 cell line expressed the protein. These findings further suggest that SOX2 possibly contributes to the tumorigenicity of PC. Moreover, immunofluorescence analysis revealed that SOX2 is located mostly in the nucleus of PC3 cells though a slight cytoplasmic staining was also observed. The mechanism involved in SOX2 cellular location regulation is poorly understood. While its nuclear localization points to a possible engagement in the transcriptional activity regulation of several genes, SOX2 ultimate function in the cytosol is unknown. Our results are in agreement with a previous study who also report a dual location, but mostly nuclear, for SOX2 in the PC3 cell line by immunofluorescence analysis (Jia et al., 2011).

To assess the effect of SOX2 inhibition by siRNA-mediated knock-down in the biological behaviour of PC cell lines, we proceeded to several *in vitro* studies. We performed a cell morphology assay during 3 days and it was observed that SOX2 knock-down did not affect cell morphology. At day 3, PC3 cells transfected with siSOX2 showed the same phenotype when compared with scrambled control cells. Thus, it is feasible to contemplate the trivial impact of SOX2 in what regards the morphological features of PC cells. Prior studies dedicated to SOX2 expression in PC cell lines did not explore morphologic features in detail. Nonetheless, in a study performed by Lundberg and colleagues (2016) the human colon cancer cell line Caco2 expressing high levels of SOX2 displayed a less adherent and spheroid growth pattern when compared to the wild type counterparts (Lundberg et al., 2016). Moreover, lung squamous cell carcinomas expressing SOX2 were found to display a higher

grade of nuclear atypia, with larger and often abnormal nuclei with or without nucleoli (Brcic et al., 2012). SOX2 elevation was also found to promote physiological changes to more differentiated phenotypes in the medullobastoma DAOY cell line (Cox et al., 2012).

We also evaluated cell death in both PNT2 and PC3 cell lines. As expected for the PNT2 cell line, the percentage of apoptotic cells was not significantly affected by SOX2 knock-down when compared to the control conditions. Nevertheless, we observed a significant increase in the number of PC3 siSOX2 transfected cells undergoing apoptosis. Hence, SOX2 appears to be a pivotal intervenient for human PC cells survival processes. The role of SOX2 in programmed cancer cell death was addressed by some studies. Jia and collaborators (2011) found that SOX2 is capable of improving the anti-apoptotic and chemotherapeutic resistance features of PC cells (Jia et al., 2011). Similar deductions were accomplished in distinct tumor models. In ovarian cancer settings, the apoptotic resistance capacities of cancer cells were boosted after SOX2 overexpression (Bareiss et al., 2013). In addition, SOX2 knock-down in human gastric cancer cell lines led to a significant stimulation of the apoptotic process (Otsubo et al., 2011). Research thus far leads to the consensus that SOX2 primarily acts as an anti-apoptotic factor in several types of human solid cancers. Furthermore, and despite some uncertainty, SOX2 activities have been documented to be cancer specific, i.e., SOX2 regulates distinct sets of apoptotic genes depending upon the organ affected. For instance, in PC backgrounds SOX2 overexpression diminished the store-operated calcium entry (SOCE) activity that further enhanced the anti-apoptotic properties of SOX2 (Jia et al., 2011). Furthermore, SOX2 silencing was found to induce apoptosis by up-regulating p27, down-regulating cyclin E and suppressing the expression of survinin (Lin et al., 2012). Conversely, in lung cancer settings, Chou and colleagues (2013) reported that SOX2 knock-down stimulated apoptosis and autophagy through BCL2L1 down-regulation (Chou et al., 2013). In summary, the concept that CSCs possess higher-resistance properties when compared to more differentiated cancer cells (Mimeault and Batra, 2006; Mimeault and Batra, 2007; Baumman et al., 2008) is on the basis cancer recurrence. A better understanding into this subject may lead to the development of novel effective approaches capable of inducing cancer stem cells death and thus avoid cancer

relapse. To this end, targeting the presumable anti-apoptotic SOX2 gene is emerging as a promising strategy for cancer therapy; however the processes and pathways where it operates and ultimately promotes the survival of PC cells requires further investigation.

The clinical course of PC is variable and difficult to predict. While some tumors remain organ-confined, others might metastasize even in early stages of the disease. The metastatic process comprises the main cause of cancer associated mortality (Chambers et al., 2002). Through specific migratory and invasive processes human PC cells preferentially metastasize to the bone (Harada et al., 1992; Bubendorf et al., 2004). Indeed, the conception of bony PC colonies encompasses an intricate and coordinated process involving i) displacement from a primary site; (ii) resistance to anoikis during circulation; (iii) binding to bone marrow cells and (iv) survival and growth in the bone microenvironment (Ibrahim et al., 2010). However, the molecular mechanisms underlying the establishment of these niches are still poorly understood but of pressing need due to the overwhelming mortality to them associated. The initial stages of metastasis are largely reliant on an eclectic range of mobility and invasive machineries (Friedl and Wolf, 2003). Indeed, primary tumor mass cells commitment to malignancy requires loss of differentiated features and consequential gain of mesenchymal properties, including increased motility and invasiveness (Kalluri and Weinberg, 2009). This process has been known as epithelial to mesenchymal transition (EMT) (Micalizzi et al., 2010). As in other types of cancer (Weina and Utikal, 2014), in prostate carcinomas SOX2 is closely associated with invasion largely through protein overexpression (Jia et al., 2011; Kregel et al., 2013). Taking these findings into consideration, we decided to perform *in vitro* wound-healing assays to determine whether or not SOX2 knock-down would also affect the migratory capacity of PC cells. Results obtained by *time-lapse* acquisition allowed the observation of cell migration in both PNT2 and PC3 cell lines during a wound healing of 18 hours. Cell migration was evaluated. Regarding the PNT2 cell line, not surprisingly, siSOX2 transfected cells migrated in a similar manner as scrambled controls in order to close the wound. However, in the PC3 cell line, it was clearly observed that siSOX2 transfected cells migrated to close the wound in a minor extent and moved slower, when compared to scrambled transfected cells. These

observations suggest that the knock-down of SOX2 expression promoted a significant decrease in cell migration rates, resulting in an impaired ability to close the wound. To our knowledge there is no report addressing the migration capability of PC cell lines after SOX2 inhibition by wound healing. Nevertheless, our results are in agreement with a recent paper published by Russo and collaborators (2015) wherein SOX2 overexpression in PC3 and 22RV1 cell lines significantly enlarged their invasive and migratory abilities when compared to empty vector transfected cells (Russo et al., 2015). Several studies support the probable contribution of SOX2 in the proliferation, migration, invasion and metastatic processes in various cellular models including glioblastomas (Alonso et al., 2011), colorectal cancer (Han et al., 2012; Li et al., 2013), breast cancer (Leis et al., 2012), lung cancer (Hussenet et al., 2010) and osteosarcomas (Basu-Roy et al., 2012). Indeed, the expression profile of SOX2 in human solid tumors has been positively correlated with their invasiveness and metastatic potential. To our knowledge, the orchestration of complex biological responses, such as cell motility, by SOX2 is largely unknown. The opening rounds of metastasis embroil loss of adhesion between tumor cells and subsequent shedding and invasion to other locations. Metalloproteinases (MMPs), namely MMP-2 and MMP-9, role in this process is paramount; by promoting the degradation of the ECM and decreasing cellular adhesion they stimulate the migration of cells during metastasis (Mook et al., 2004). Some studies had focused on the actual role of SOX2 in human cancer cell motility. SOX2 overexpression significantly increased the number of cells undergoing migratory and invasive processes in the SOX2-negative glioma cell line U-87 (Alonso et al., 2011). Furthermore, in colorectal cancer cells, SOX2 was found to be involved in the migration and invasion process *via* MMP-2 (Han et al., 2012). Similarly, SOX2 was able to induce migration and invasion *via* MMP2 and the PI3K/AKT/mTOR pathway in laryngeal squamous cell carcinoma (LSCC) (Yang et al., 2014). SOX2 overexpression was found to promote migration and invasion of ovarian cancer cells by directly regulating fibronectin 1 (FN1), a protein involved in cell motility processes, which in turn induces the expression of both MMP2 and MMP9 (Lou et al., 2013). FN1 was also found to induce the expression of MMP2 in human PC cells (Moroz et al., 2012) but to our knowledge there is no study aimed at assessing the role employed by SOX2 in

the regulation of MMPs in progression of prostate carcinomas. In summary, our results are in agreement with several studies supporting a SOX2 pivotal role in promoting mobility capacities of cancer cells. Nevertheless, the molecular mechanisms underlining this activity on PC requires further elucidation.

The participation of SOX2 in distinct facets of cancer biology is more than proven. Nevertheless, the clinical importance of this transcription factor in terms of prognosis, recurrence and resistance to therapy is also supreme for a better understanding of the disease. A host of human solid tumors have been acknowledged to express SOX2 thought its role and concomitant clinical value is notoriously debatable. Nevertheless, the expression of SOX2 has been positively associated to tumor grade and poorer prognosis in the majority of human cancers types, including hepatocellular carcinoma (Sun et al., 2013), colorectal cancer (Neumann et al., 2011), lung cancer (Yung et al., 2013), gastric cancer (Zhang et al., 2010) and LSCC (Tang et al., 2013).

To assess if SOX2 plays an important role in the development of prostate neoplams we proceeded to immunohistochemistry analysis of human PC tissue in a small series of ten patients. We found that expression of SOX2 was higher in cancerous in comparison to normal adjacent tissues. These findings were consistence to those of various publications (Jia et al., 2011; Bourguignon et al., 2012; Schorck et al., 2013; Ye et al., 2013) where an increase in the expression of SOX2 is perceived in the tumor, but not in normal-adjacent tissue. In PC settings, protein expression of SOX2 has been described to be both increased (Jia et al., 2011; Ugolkov et al., 2011) and decreased (Yu et al., 2014; Russo et al., 2015) in organ-confined tumors, when compared to their normal counterparts. Nevertheless, based on our results, we believe that SOX2 increased expression may be related to the tumorigenesis of the prostate. As such, we decided to evaluate the actual link between SOX2 expression and the tumorigenic process. We performed a statistical analysis to ascertain the possible relationship of SOX2 with a series of clinical and histopathological guidelines. Concerning pathological grade, the T tumor size, and stage we did not find any association to SOX2 expression. Both SOX2-positive and negative tumors displayed similar grade, size and stage. We believe that the discrepancy observed in our results might be accredited to the small cohort of samples analysed. These results do not corroborate the available literature. Indeed, the

expression of SOX2 in PC tissue was previously related to tumor grade, i.e., the percentage of SOX2-positive cells increases with Gleason score (Jia et al., 2011; Kregel et al., 2013). Similar results were obtained in breast cancer models wherein the expression of SOX2 had positively correlated with the TNM stage and histological grade (Stolzenburg et al., 2012). Furthermore, in esophageal cancer SOX2 co-localization with OCT3/4 was found to be closely associated with higher stage (Wang et al., 2009). In ovarian cancer settings, SOX2 overexpression has been related to poorer clinical prognosis (Ye et al., 2011; Zhang et al., 2012), i.e., lymphatic and vascular invasion, poorly differentiated neoplasms and incomplete resection (Lou et al., 2012). SOX2 expression was also associated with clinical stage in patients diagnosed with small cell lung cancer (Yang et al., 2013) and LSCC (Tang et al., 2013).

Concerning the regional status of lymph nodes we surprisingly found that patients bearing SOX2-negative tumors were closely associated to the presence of metastasis in the lymph nodes. It seems that SOX2-negative tumors are more prone to develop regional metastasis to the lymph nodes than those of SOX2-positive tumors. These results are in disagreement with the majority of data available on the literature. For instance, SOX2 overexpression was previously found to be closely associated to the presence of metastasis in the lymph nodes in PC (Russo et al., 2015), small cell lung cancer (Yang et al., 2013) and gastric cancer (Matsuoka et al., 2012).

Regarding the relationship of SOX2 with tumor recurrence, we found that patients bearing SOX2-positive tumors were significantly associated with an increased D'Amico risk. Although a potential correlation between SOX2, disease-free, and disease survival was also observed, i.e., patients bearing SOX2-positive tumors revealed shorter disease-free survival duration, the results did not reach statistical significance and thus not consent the establishment of a definitive conclusion. Nevertheless, these data imply a probable SOX2 involvement in tumor recurrence and are in accordance in some previous studies. Russo and colleagues (2015) suggest a correlation between SOX2 expression in PC and biochemical recurrence-free survival (Russo et al., 2015). In ovarian cancer settings, SOX2 overexpression has been related to shorter disease-free survival durations (Zhang et al., 2012). Similar outcomes were obtained in hepatocellular carcinoma and esophageal squamous cancer

models, where SOX2 was able to convene larger disease aggressiveness, as males harboring SOX2-positive neoplams revealed significant shorter survival periods (Wang et al., 2009; Huang et al., 2011). Moreover, in sinonasal cancer SOX2 amplification was established to identify cancers which are more likely to relapse (Schröck et al., 2013). In contrast, Otsubo and colleagues (2008) reported that the expression of SOX2 is frequently downregulated in human gastric cancer and confers shorter survival periods and tumor suppressor features in gastric carcinogenesis (Otsubo et al., 2008).

In conclusion, we have consolidated SOX2 as a pivotal player in the progression of prostatic neoplams. Upon SOX2 knock-down mediated by siRNAs a decrease in the migratory capacities, as well as an increase in the number of cells undergoing apoptosis, was perceived. These results further support the premise that SOX2 may take part of an important transcriptional program responsible for ultimately driving malignancy. We also found that the protein expression of SOX2 in cancerous tissues is increased in comparison to the normal adjacent tissue. In addition, SOX2 closely associates with some clinical and pathological criteria, including risk of relapsing after therapy, further pointing to a potential participation in tumor recurrence mechanisms.

CONCLUSION

The present study showed that SOX2 plays an important role in the biological behavior of PC cell lines. SOX2 silencing mediated by siRNAs demonstrated that it acts during PC progression since we found a decrease in the migration capacity and also an increase in apoptosis of SOX2 knocked-down PC3 cells. SOX2 plays an important role in the biological behavior and subpopulation pattern of PC since it also affected the apoptosis resistance of PC3 cells, which showed increased expression of solely Annexin V or PI and Annexin V with concomitant early and late apoptosis, respectively.

Moreover, in what regards SOX2 expression pattern, basal-like cells overexpressed the protein in patient PC samples and significant associations with some important clinical criteria, including the risk of relapsing after therapy. These observations point to an important role of SOX2 in PC. Expression and activity of SOX2 is possibly involved in early tumor progression, invasion and relapse after therapy. These results indicate that the discovery of a SOX2 repressor could potentially be used for anti-cancer therapy.

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