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Regulation of STEAP1 gene and its clinical significance in human prostate cancer

Inês Margarida Amaral Santos Gomes

Tese para obtenção do Grau de Doutor em
Biomedicina
(3º Ciclo de Estudos)

Orientador: Prof. Doutor Cláudio Jorge Maia Baptista
Co-orientador: Prof.ª Doutora Cecília Reis Alves dos Santos

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"The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka" (I found it!) but "That's funny…".

Isaac Asimov

Agradecimentos

Depois de tantos altos e baixos, por entre momentos de sucesso e rasgados sorrisos e outros de tristeza e lágrimas, é com um grande sentimento de missão cumprida e grande felicidade que apresento este trabalho.

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Thesis Overview

This thesis is structured in eight main chapters. ***Chapter I*** presents a general introduction that includes a short description of prostate anatomy, physiology and pathology. The molecular pathways that are involved in prostate carcinogenesis are also summarized, stressing the role of androgens and estrogens. A brief description of prostate cancer biomarkers and the potential of STEAP1 as biomarker and immunotherapeutic target is underlined. A small part of this chapter was adapted from the original book chapter (see attachment I). ***Chapter II*** is intended to put forward an extensive review on STEAP1 structural features, its expression pattern both in normal and cancer tissues and cell lines. It is also summarized the role and regulation of STEAP1, as well as its physiologic roles, regulation and also STEAP1 implications in cancer. Additionally, the potential of STEAP1 as a biomarker and as an immunotherapeutic target are also stressed. This chapter is an adaption of the original paper 1 (see attachment II). In ***Chapter III***, the main goals established for the development of this thesis are described. The following chapters, ***Chapter IV***, ***Chapter V***, ***Chapter VI*** and ***Chapter VII*** present the results obtained during the course of the PhD, which were published or submitted as original research papers in scientific international journals. These are organized as follows:

Chapter IV refers to the original paper 2 and describes the role of androgens and estrogens on STEAP1 expression in LNCaP cells and rat prostate. STEAP1 expression was analyzed by qPCR, western blot and immunohistochemistry.

In ***Chapter V***, STEAP1 and STEAP1B expression are characterized in malignant and non-malignant prostate cell lines. In addition, STEAP1 stability and post-translational mechanisms regulating STEAP1 expression are analyzed (Original paper 3). Evaluation of mRNA and protein expression was performed using qPCR and western blot, and putative post-translational mechanisms were evaluated using *in silico* tools.

Chapter VI outlines STEAP1 role in cell proliferation and apoptosis in LNCaP cells. LNCaP cells were transfected with siRNA to induce the knockdown of STEAP1 gene. Cell proliferation and apoptosis were analyzed by MTS, flow cytometry and TUNEL assay. (Paper in Preparation)

Chapter VII describes STEAP1 expression and its clinical significance in prostate cancer. In order to evaluate whether STEAP1 expression is associated with histologic diagnosis or clinical-pathological data of patients, STEAP1 immunoreactivity was evaluated in several samples of human prostatic tissues using immunohistochemistry. (Original paper 4)

The last and final chapter, ***Chapter VIII*** summarizes the general conclusions of the work and future perspectives raised with the present thesis.

Resumo Alargado

A próstata é uma glândula acessória do sistema reprodutor masculino e desempenha um papel fulcral na fertilidade masculina. A morfogénese, desenvolvimento e homeostase celular desta glândula são altamente dependentes das hormonas esteróides sexuais, nomeadamente androgénios e estrogénios. De um modo geral, o cancro surge de um desequilíbrio entre a proliferação celular e apoptose, conduzindo a um crescimento descontrolado. O cancro da próstata é uma doença multifatorial, afetada por fatores de risco exógenos e endógenos, de entre os quais se destacam a história familiar e a idade. O cancro da próstata é o segundo tipo de cancro mais frequente na população masculina, e em conjunto com a hiperplasia benigna da próstata, é encontrado na maioria dos homens com idade superior a 40 anos. A etiologia do cancro da próstata é complexa, mas acredita-se que processos inflamatórios estejam por detrás do aparecimento de lesões pré-cancerosas. A atrofia inflamatória proliferativa e a neoplasia intraepitelial prostática (PIN) são frequentemente referidas como percursoras do carcinoma prostático localizado, que por sua vez evolui tornando-se invasivo e metastizando para tecidos mais longínquos. O cancro da próstata é muitas vezes descrito como uma doença bi-etápica, uma vez que é inicialmente responsivo aos androgénios (hormono-dependente), mas à medida que progride, torna-se não responsivo (hormono-independente). No final, o desenlace clínico é definido pelo potencial de crescimento, invasão e metástase do tumor. Disponíveis para a prática clínica, existem vários sistemas de classificação do grau e estadio tumorais que podem ser usados, dentro dos quais os mais frequentes são o sistema de Gleason e o sistema TNM.

As vias de sinalização envolvidas na carcinogénese da próstata são complexas. Neste trabalho destacam-se as vias de sinalização ativadas pelos androgénios e estrogénios. A 5α-dihidrotestosterona (DHT) apresenta-se como sendo o androgénio mais produzido, potente e o principal com ações fisiológicas na próstata. Também o 17β-estradiol (E_2) é produzido nesta glândula a partir da aromatização da testosterona, e também ele é o estrogénio mais potente. Tanto os androgénios como os estrogénios têm um papel central na diferenciação, proliferação e sobrevivência de células tumorais prostáticas, contribuindo assim para a origem e desenvolvimento do carcinoma da próstata.

A identificação de biomarcadores que permitissem um diagnóstico e tratamento mais precisos revolucionou a prática clínica. Atualmente, o PSA é o biomarcador por excelência no diagnóstico do cancro da próstata. No entanto, apesar de ser um bom indicador clínico, o PSA apresenta várias limitações. Como tal, é necessário que novos biomarcadores sejam identificados.

O STEAP1 foi inicialmente identificado como sendo sobre-expresso no cancro da próstata. No entanto, é também encontrado como sobre-expresso em vários outros tipos de tumores. Em tecidos normais, a expressão do STEAP1 é quase que totalmente restrita à próstata, onde está

principalmente localizado na membrana plasmática das células epiteliais, especialmente nas junções celulares. A regulação e funções biológicas do STEAP1 estão ainda pouco definidas, mas crê-se que actue como um canal iónico ou proteína transportadora, regulando a comunicação inter- e intra-celular. Finalmente, apesar de o seu significado clínico não ser ainda claro, as características apresentadas pelo STEAP1 fazem dele um potencial biomarcador e alvo imunoterapêutico.

Deste modo, foi primeiramente investigado se a expressão do STEAP1 é regulada pelas hormonas sexuais esteróides *in vitro* e *in vivo*. Os resultados mostraram que a expressão do STEAP1 é inibida tanto pelo DHT como pelo E_2 na linha celular maligna de cancro da próstata LNCaP, assim como na próstata de rato, sugerindo que o STEAP1 poderá desempenhar funções relevantes na progressão do cancro da próstata de responsivo para não-responsivo aos androgénios. Subsequentemente, foi avaliado o padrão de expressão de um gene relacionado com o STEAP1, o STEAP1B, e se a expressão do STEAP1 está sujeita a mecanismos de regulação pós-transcrição e pós-tradução. A análise *in silico* revelou que o STEAP1 e o STEAP1B1 partilham grande homologia estrutural, e que o STEAP1 é mais estável em células tumorais da próstata do que em células não tumorais. Ainda, foi possível demonstrar *in silico* que a expressão do STEAP1 poderá ser regulada por várias modificações pós-tradução. Seguidamente, centrou-se o foco nas funções do STEAP1. Utilizando um siRNA específico para reprimir a expressão do STEAP1 na linha celular LNCaP, analisou-se o papel do STEAP1 na proliferação celular e apoptose, assim como o efeito do DHT nas células LNCaP com baixos níveis de expressão do STEAP1. Utilizando as técnicas de MTS, citometria de fluxo e TUNEL, verificou-se que o silenciamento do gene do STEAP1 reduz a viabilidade e crescimento celular, e aumenta o número de células em apoptose. Por outro lado, a ação do DHT parece estar dependente dos níveis de expressão do STEAP1, uma vez que o DHT não foi capaz de reverter os efeitos provocados pela sub-expressão do STEAP1.

Muitos esforços têm sido dirigidos para clarificar a potencial utilização do STEAP1 como biomarcador e alvo imunoterapêutico. Neste trabalho investigou-se a associação entre os níveis de expressão do STEAP1 com dados clínicos e histológicos provenientes de doentes com cancro da próstata. Os resultados demonstraram que o STEAP1 é sobre-expresso em casos de PIN e cancro da próstata, e que a sua expressão está positivamente associada com a escala de Gleason.

Como principais conclusões deste trabalho, os resultados apresentados demonstram que a expressão do STEAP1 é regulada negativamente pela presença das principais hormonas sexuais esteróides presentes na próstata. Também a elevada estabilidade do STEAP1 em células tumorais da próstata em comparação com células normais leva a crer que os mecanismos de regulação pós-transcrição e pós-tradução são dependentes do estado clínico do tumor. Mais ainda, o silenciamento do gene do STEAP1 inibe a viabilidade e proliferação celulares das células LNCaP, ao mesmo tempo que induz a sua apoptose, levando a crer que STEAP1 poderá desempenhar um papel relevante no cancro da próstata, nomeadamente na iniciação do tumor e no aparecimento de células tumorais hormono-independentes. O uso do STEAP1 *per*

se poderá não ser suficiente para ser usado com biomarcador na prática clínica diária, mas poderá abrir caminho para novas estratégias de diagnóstico e tratamento do cancro da próstata.

Abstract

The prostate gland is an accessory gland of the male reproductive system and displays a critical role in male fertility. This gland is dependent of sex steroid hormones, namely androgens and estrogens, for the gland morphogenesis, development and cellular homeostasis. Prostate cancer (PCa) is a multifactorial disease, affected by both endogenous and exogenous risk factors, especially family history, age and sex hormones. PCa is the second most common type of cancer among men, and along with BPH, is found in the majority of men over 40 years old. Although PCa etiology is complex, inflammatory processes are thought to be behind the appearance of pre-cancerous lesions. At molecular level, PCa is often described as a two step disease. Initially it is responsive to androgens, but usually, the PCa becomes androgen-independent. In the end, the clinical outcome of PCa is defined by the potential of the tumor to grow, invade and metastasize. Regardless of all the knowledge gathered from prostate cancer pathophysiology and clinical management, identification of genes associated with the pathology, the role of sex steroid hormones in their regulation, and their potential to be used as biomarkers or immunotherapeutic targets is urgent.

STEAP1 was firstly identified as being overexpressed in prostate cancer. However, it can also be found overexpressed in several other types of tumors. STEAP1 expression in normal tissues is almost restricted to the prostate, where it is primarily localized in the plasma membrane of epithelial cells, especially at cell-cell junctions. STEAP1 regulation and biological function are yet uncertain, but it is believed to act as an ion channel or transporter protein, regulating inter- and intra-cellular communications. Finally, although its clinical significance is not clear, STEAP1 features stress its potential as biomarker and immunotherapeutic target.

This way, it was evaluated if STEAP1 expression is regulated by sex steroid hormones, using both *in vitro* and *in vivo* assays. It was found that STEAP1 is down-regulated by DHT and E_2 in LNCaP prostate cancer cells and in rat prostate, suggesting that STEAP1 may have a role on prostate cancer progression from androgen-dependent to androgen-independent. Subsequently, it was also evaluated the expression pattern of a STEAP1 related gene (STEAP1B) in cancer cell lines, and whether STEAP1 expression is subjected to post-transcriptional or post-translational regulatory mechanisms. *In silico* analysis revealed that STEAP1 and STEAP1B1 share high homology, and STEAP1 is more stable in prostate cancer cells rather than in non-malignant ones. Furthermore, it was demonstrated by *in silico* analysis that STEAP1 expression may be regulated by several post-translational modifications. In order to clarify the role of STEAP1 in prostate cancer, it was used a specific siRNA to decrease the levels of STEAP1 in LNCaP cells. Then, it was evaluated the role of STEAP1 in cell proliferation and apoptosis, as well as the effect of dihydrotestosterone (DHT) in LNCaP cells with low levels of STEAP1. Using MTS assay, flow cytometry and TUNEL assay, it was

evident that STEAP1 gene silencing appears to reduce cell viability and growth, and to increase apoptosis of LNCaP cells. On the other hand, the DHT action seems to be dependent of STEAP1 levels as it could not revert the effects induced by STEAP1 knockdown.

Much effort has been done to clarify the potential of STEAP1 as biomarker and immunotherapeutic target. Here, it was investigated the association between STEAP1 expression with histologic and clinical data of patients. It was demonstrated that STEAP1 is overexpressed in PCa and PIN lesions, and it is positively associated with Gleason score.

Taken together, the results here presented demonstrate that STEAP1 expression is negatively regulated by the two main sex steroid hormones presents on prostate. STEAP1 high stability in prostate tumor cells in comparison to normal ones lead us to believe that post-transcription and translational regulation mechanisms are dependent on the tumor stage. Moreover, STEAP1 gene silencing inhibits cell viability and proliferation, ate the same time that increases apoptosis in LNCaP cells, suggesting that STEAP1 may have an important role on PCa progression from androgen-dependent to hormone refractory. The use of STEAP1 *per se* may not be sufficient to be used as a biomarker in the daily clinical practice, but may open novel strategies for diagnosis and treatment of prostate cancer.

Keywords

Prostate cancer, STEAP1, expression, regulation, TMA's, clinical significance

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List of Abreviations

4-odione	Androstenedione
aa	Aminoacids
ACRATA	Apoptosis, cancer and redox associated transmembrane
ADC	Antibody-drug conjugates
ADIPOR1	Adiponectin receptor 1
AF	Activation Function
Akt	Protein kinase B
AP-1	Transcription factors activator protein 1
APC	Antigen-presenting cells
AR	Androgen receptor
ARE	Androgen responsive elements
ArKO	Aromatase deficiente mice
ATP	Adenosine triphosphate
Bcl-2	B cell lymphoma 2
BPH	Benign hyperplasia
BPSA	BPH-associated Prostatic specific antigen
C	Central domain
CAG	Polyglutamine
CBX7	Chromobox homolog 7
Chr	Chromosome
COX-2	Clycooxygenase-2
CTL	Cytotoxic T-lymphocyte
D	Hinge region
DBD	DNA-binding domain
DES	Diethylstilbesterol
DHEA	Dehydroepiandrosterone
DHT	5α- dihydrotestosterone
DNA	Deoxyribonucleic acid
E_1	Estrone
E_2	17β-estradiol
E_3	Estriol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERE	Estrogen-responsive elements
ERG-1	ETS related gene 1
ERK	Extracelular signal regulated kinase
ESR1	Estrogen receptor alfa encoding gene
ETV	ETS variant
FNO	$F_{420}H_2$:$NADP^+$ oxidoreductase
fPSA	Free Prostatic specific antigen
FRE	Ferric reductase
GGC	Polyglycine
GnRH	Gonadotropin-releasing hormone
GPR30	G-protein coupled receptor 30

List of Abreviations

GSTA1	Glutathione S-transferase alfa 1
GSTP1	Glutathione S-transferase-pi
HAT	Histone acetyl transferase
HER	Human epidermal growth factor receptor
HGPIN	High-grade prostatic intraepithelial neoplasia
HHD	HLA-A_0201 transgenic mice
HRPC	Hormone refractory prostate cancer
Hsp	Heat shock proteins
IGF-1	Insulin-like growth factor 1
IL-6	Interleukine 6
JNK	*c-jun* N-terminal kinase
KGF	Keratinocyte growth factor
KLK	Kallikrein
LBD	Ligand-binding domain
LEF	Lymphoid enhancer factor
LH	Luteinizing hormone
LUTS	Lower urinary tract symptoms
MAPK	Mitogen-activated protein kinase
MMAE	Monomethyl auristatin E
MMP	Matrix metalloproteinase
mPSCA	Murine prostate stem cell antigen
MSC	Mesenchymal stem cell
mSTEAP1	Murine six transmembrane epithelial antigen of the prostate 1
MVA	Modified vaccinia Ankara
NADPH	Nicotinamide adenine dinucleotide phosphate
NKX3.1	Neuromedin 3 homebox 1
NTD	N-terminal domain
PAP	Prostatic acid phosphatase
PCa	Prostate cancer
PCA3	Prostate cancer antigen 3
PI3K	Phosphatidylinositol-3 kinase
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PR	Progesterone receptor
PRC1	Polycomb repressive complex 1
PSA	Prostatic specific antigen
PTEN	Phosphate and tensine homolog
ROS	Reactive oxygen species
Sp-1	specificity protein 1
STEAP1	Six transmembrane epithelial antigen of the prostate 1
TAA	Tumor-associated antigens
TAF-1	Transcriptional activation function site 1
TAF-2	Transcriptional activation function site 2
TCF	T-cell factor
Tf	Transferrin
TfR1	Transferrin receptor 1
TGF	Transforming growth factor
Th	T helper

List of Abreviations

TMPRSS2	Type II transmembrane serine protease
TNF-α	Tumor necrosis factor alpha
TNM	Tumor Node and Metastasis
TRAMP-C	Transgenic adenocarcinoma mouse model derived cell line
TRPM2	Transient receptor potential cation channel M2
VEGF	Vascular endothelial growth factor
αERKO	Alpha estrogen receptor knockout
βERKO	Beta estrogen receptor knockout

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List of Scientific Publications

Papers related to this thesis

- Gomes IM, Santos CR, Gaspar C, Alvelos MI, Maia CJ, **Effect of down-regulation of STEAP1 by siRNA in cell cycle and apoptosis of human LNCaP prostate cells**, 2014 (Paper in Preparation)

- Gomes IM, Arinto P, Lopes C, Santos CR, Maia CJ, **STEAP1 is over-expressed in prostate cancer and prostatic intraepithelial neoplasia lesions, and it is positively associated with Gleason score**, Urologic Oncology Seminars and Original Investigations 2014; 32(1): 53.e23-53.e29 doi:10.1016/j.urolonc.2013.08.028

- Gomes IM, Santos CR, Maia CJ, **Expression of STEAP1 and STEAP1B in prostate cell lines, and the putative regulation of STEAP1 by post-transcriptional and post-translational mechanisms**, Genes & Cancer 2104; 5 (3-4): 142-151

- Gomes IM, Santos CR, Socorro S, Maia CJ, **Six transmembrane epithelial antigen of the prostate 1 is down-regulated by sex hormones in prostate cells**. Prostate 2012; 73(6):605-13. doi: 10.1002/pros.22601

- Gomes IM, Maia CJ, Santos CR, **STEAP proteins: From structure to applications in cancer therapy**. Mol Cancer Res 2012; 10(5):573-87 doi: 10.1158/1541-7786.MCR-11-0281

Book Chapters related to this thesis

- Gomes IM, Vaz CV, Rodrigues D, Rocha SM, Socorro S, Santos CR, Maia CJ. **Estrogens and prostate cancer: from biosynthesis to physiological effects**. Book Title: Estradiol synthesis: health effects and drug interactions. Nova Science Publishers, Inc, New York, USA. ISBN: 978-1-62808-962-2

Papers not related to this thesis

- Vaz CV, Maia CJ, Marques R, Gomes IM, Correia S, Alves M, Cavaco JE, Oliveira PF, Socorro S, **Regucalcin is an androgen-target in the rat prostate associated with modulation of apoptotic pathways**, Prostate 2013; doi: 10.1002/pros.22835

List of Scientific Communications

Poster communications related to this thesis

- Gomes IM, Santos CR, Alvelos MI, Gaspar C, Maia CJ. **Effect of down-regulation of STEAP1 by siRNA in cell cycle and apoptosis of human LNCaP prostate cells.** FEBS/EMBO Congress, 30 August- 4 September, Paris, France

- Gomes IM, Arinto P, Costa-Pinheiro P, Santos CR, Jerónimo C, Maia CJ. **Expression and regulation of STEAP1 and STEAP1B in prostate cell lines through mRNA and protein stability and epigenetic mechanisms.** 38th FEBS Congress- Mechanisms in Biology, 6-11 July 2013, Saint Petersburg, Russia

- Gomes IM, Arinto P, Santos CRA, Socorro S, Lopes C, Maia CJ. **Regulation of STEAP1 expression in prostate by sex steroid hormones.** 22nd Congress of European Association for Cancer Research - from Basic Research to Personalized Cancer Treatment, 7-10 July 2012, Barcelona, Spain

- Gomes IM, Santos CR, Socorro S, Lopes C, Maia CJ. **Regulation of STEAP1 expression in prostate by androgens.** XX Porto Cancer Meeting, Porto, Portugal. 28-29 April 2011

- Gomes IM, Santos CR, Socorro S, Lopes C, Maia CJ. **STEAP1 expression in prostate cancer and its regulation by androgens.** 16th International Charles Heidelberg Symposium on Cancer Research, Coimbra, Portugal. 26-28 September 2010

Poster communications not related to this thesis

- Marques R, Vaz CV, Peres CG, Gomes I, Santos CR, Maia, Socorro S. **Effect of androgens on the expression of Ca^{2+} -binding protein, regucalcin, and Ca^{2+} -channels in MCF-7 cells**. *22nd* Congress of European Association for Cancer Research - from Basic Research to Personalized Cancer Treatment, 7-10 July 2012, Barcelona, Spain

CHAPTER I

General Introduction

1. Prostate Anatomy and Physiology

The human prostate gland is part of the male reproductive system, having the shape and size of a walnut. This gland is situated frontal to the rectum and immediately below the bladder, surrounding its neck and the first part of the urethra [1, 2]. The prostate is partially enclosed by a capsule on the posterior and lateral sides, and the anterior fibromuscular stroma binds the anterior and apical surfaces [2]. The prostate is divided in four main zones. These are the peripheral zone, central zone, transition zone and anterior fibromuscular zone, as shown in Figure 1 [2, 3]. The peripheral zone comprises the bulk, approximately 70% of the prostate gland, and the central and transition zones the remaining 30% [2]. With aging, the transition zone becomes the most prominent prostate zone due to hypertrophy development [4].

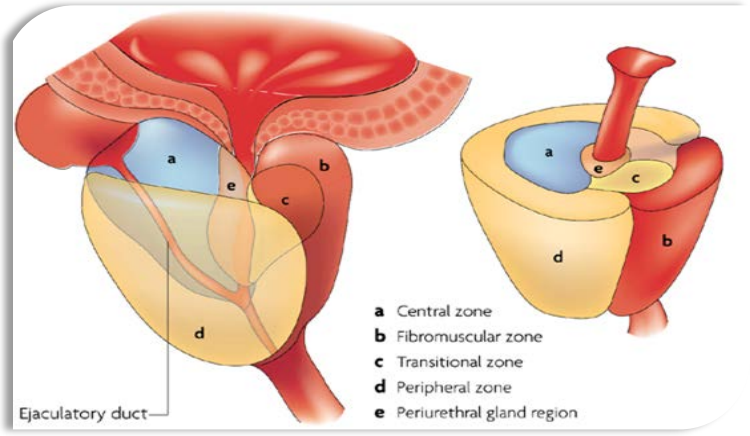

Figure 1- Representation of Prostate's anatomy. Adapted from [66].

At a closer look, the gland has an irregular shape due to the folds formed by the epithelium (Figure 2). This is arranged into pseudostratified columnar to cuboidal cells, and composed by two cellular layers, the luminal one with tall columnar secretory cells with basal nuclei that express androgen receptor (AR), cytokeratin 8 and 18 and prostatic specific antigen (PSA), and the basal layer, often incomplete and consisting of flattened basal cells with the ability to produce keratin, p63, cytokeratin 5 and 14 [2, 5-7]. Although the precise role of basal cells has long been discussed, several functions are attributed to the basal layer, namely as a reservoir of stem cells with capacity to differentiate into columnar secretory cells, or as displaying an active part in the molecular trafficking between luminal cells and extracellular space [8, 9]. Basal cells selectively express the estrogen receptor alpha (ERα), the progesterone receptor (PR) and other non-hormonal receptors such as human epidermal

growth factor receptor (HER)-1 or HER-2, enabling them to maintain cell growth and proliferation [7].

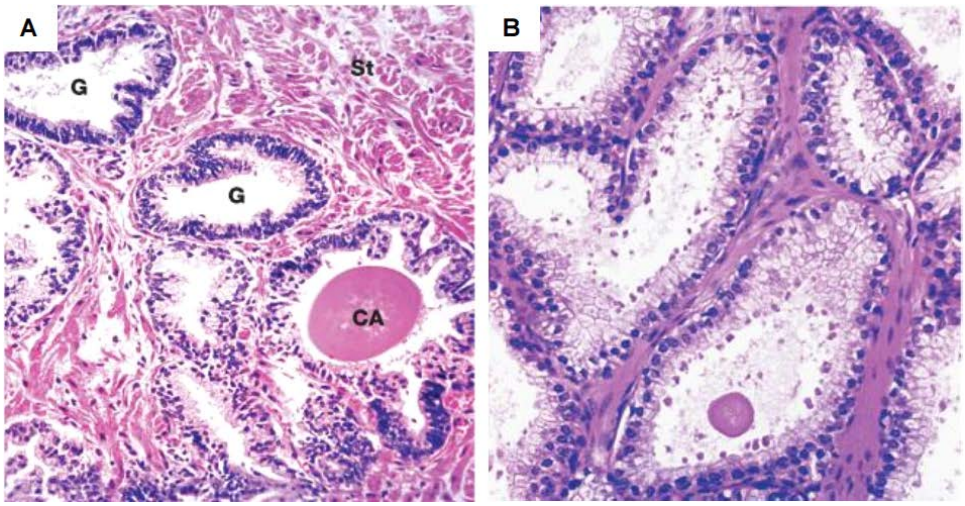

Figure 2- Human prostate histology. A – Human prostate H&E staining x30 magnification. B – Human prostate H&E staining x50 magnification. G- prostatic glands, CA- *corpora amylacea*, St- fibrous stroma. Adapted from [2].

A characteristic feature of aging is the appearance of *corpora amylacea*, a lamellated glycoprotein mass that later calcifies into prostatic concretions (Figure 2). The prostate stroma dwells on dense extracellular collagen matrix, fibroblasts, randomly arranged smooth muscle fibres, blood and lymphatic vessels, and nervous terminals [2, 10]. Prostate stroma is subdivided into two layers, the periacinar stroma that surrounds the epithelial ducts, and the interacinar stroma found between the adjacent periacinar sheaths [11]. Another type of cells found in the prostate are the neuroencodcrine cells, which are a rare type of cells located among epithelial cells and expressing chromogranin A [6, 7, 12].

Human prostate morphogenesis and growth takes place during fetal and puberty stages, while pathological modifications of this tissue begin around mid-forties [13]. Prostate growth period is characterized by increased rate of cell proliferation over cell death, whereas adult prostate displays comparable rates [14]. The initial epithelium differentiation is mediated by mesenchymal cells. The interaction between stroma and epithelial cells both in embryogenesis and adult differentiated prostate is imperative. The growth and development of the embryonic epithelium into differentiated prostate induced by androgens as well as the maintenance of the secretory epithelium in adult prostate is mediated by stroma cells [15, 16].

Physiologically, the maintenance of cellular homeostasis is not only attributed to androgens, but also to estrogens [17, 18]. The prostate gland secretes several factors, such as PSA, prostatic acid phosphatase (PAP), prostaglandins and citric acid, which play important roles in fertilization, sperm delivery and survival [19-23].

2. Prostate Pathology

In developed countries, prostate cancer (PCa) is the most frequently diagnosed malignancy in men [24, 25]. The etiology of this disease is complex and remains unclear. PCa is a gradual process that involves multiple genetic alterations in prostate cells, such as activation of oncogenes, inhibition of tumor suppressor genes and disturbance of stromal-epithelial balance [26, 27].

Prostate morphological zones display distinct architecture, histology and predisposition to disease development. Around 70 to 80% of the diagnosed prostatic adenocarcinomas emerge in the peripheral zone, while benign prostatic hyperplasia (BPH) commonly evolves in the transition zone [28].

The critical pathophysiological factor contributing to PCa development is the inhibition of apoptosis rather than enhanced cellular proliferation [29]. This event is characterized by a down-regulation of androgen responsive genes that inhibit proliferation, induce differentiation or mediate apoptosis [30-32]. There are three different stages involved in development of PCa. Initially, PCa progresses from precursor lesions, termed as prostatic intraepithelial neoplasia (PIN) and proliferative inflammatory atrophy (PIA), to carcinoma confined to the prostate, and finally, to metastatic and hormone-refractory carcinoma, a usually lethal prostate disease (Figure 5) [29].

2.1. Benign Prostatic Hyperplasia

BPH is one of the most prevalent benign neoplasm and chronic disease associated with male aging [33]. BPH was first described by McNeal, and is defined by an enlargement of the prostate transition zone due to an increase of epithelial and stromal cells and consequently causing the effacement of the central zone [34-36]. Although BPH is an heterogeneous process, it encompasses the formation of miscellaneous nodules that differ in terms of glandular tissue and fibromuscular stroma percentage (Figure 3) [37].

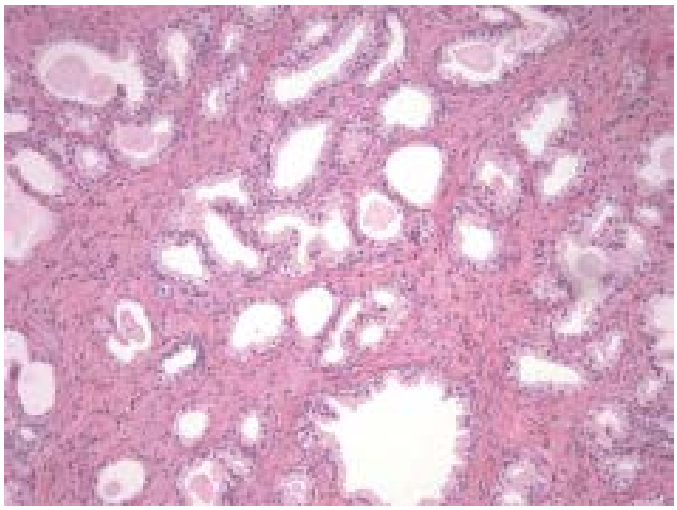

Figure 3- Benign prostatic hyperplasia, H&E staining. Microscopic image of a small gland pattern area of benign nodular hyperplasia, composed by small to medium-sized and irregular acini, with rounded lumens and surrounded by stroma. Adapted from [97].

The nodules evolve in the transition zone or in the periurethral region, but prostate enlargement occurs independently of their formation [38]. The bulk of early developed periurethral nodules are entirely composed of stromal cells. On the other hand, the majority of nodules in the transition zone are due to glandular tissue proliferation, an event that may be subjacent to loss of overall stroma. The initial development of BPH is accompanied by an increased number of nodules, but with a slow growth rate. Later in disease progression, the nodules significantly increase in size, specially the glandular ones [38].

BPH etiology is uncertain, and the observed cellular proliferation may be due to several factors. Although androgens are not known to cause BPH, its development does require their presence [39]. Androgens may act either in an autocrine or paracrine manner, increasing the transcription of several androgen-responsive genes involved in cellular proliferation, or in some conditions, in cell death. The imbalance between cellular proliferation and cell death in response to growth factors and androgens may also be linked to the appearance of BPH (Figure 4) [40].

BPH development is often correlated with the appearance of lower urinary tract symptoms (LUTS), and when left untreated, bladder dysfunction and hypertrophy appear and may evolve to acute urinary retention [40-43].

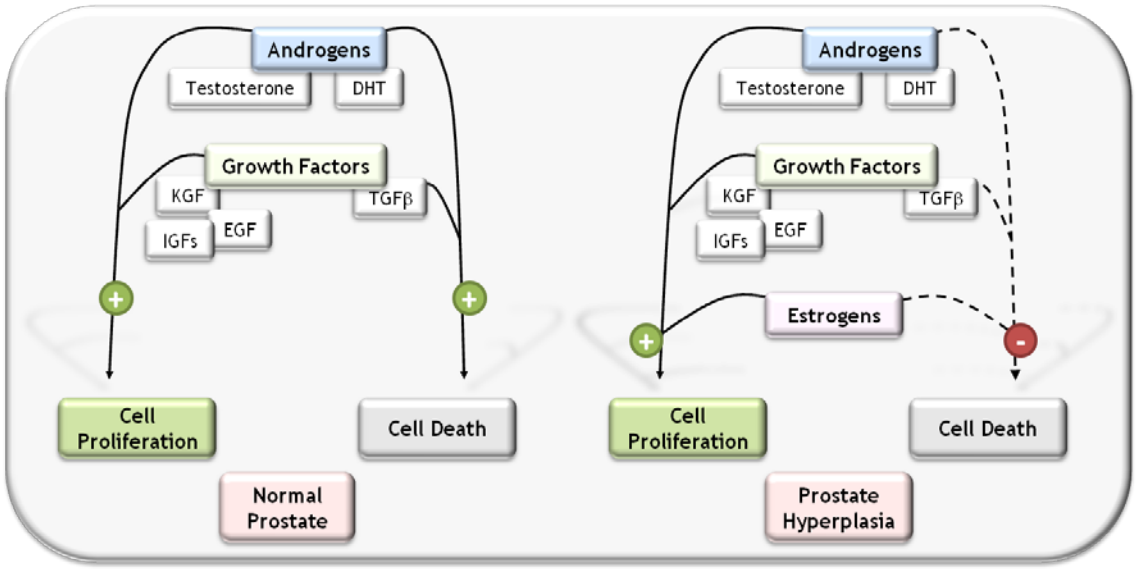

Figure 4- Global cellular homeostasis in normal prostate and BPH. As shown, androgens (testosterone and DHT) and growth factors (KGF, IGFs, EGF and TGFβ) can either have agonistic as well as antagonist effects on normal prostate, contributing to a rightful balance between cell proliferation and death. In BPH, this balance is disturbed and other hormones such estrogens may promote cell proliferation and inhibit cell death. DHT- 5α- dihydrotestosterone; KGF- Keratinocyte growth factor; IGF- Insulin-like growth factor; EGF- Epidermal growth factor; TGFβ- Transforming growth factor beta. Adapted from [40].

2.2. Prostate Cancer

PCa is one of the most common types of cancer diagnosed in the male population over 40 years old. As previously mentioned, the majority of PCa arise in the peripheral zone of the prostate. These tumors initially start as small foci of intraductal dysplasia, and may stay silenced for a long period of time, to eventually differentiate and progress into an invasive state [35]. The tumor foci leads to a disruption of prostate tissue and a decrease on glandular activity and fluid production [35, 44, 45]. Only a small portion of PCa arise in the transition zone, and the central zone is rarely associated with the disease [45-47]. Histologically, PCa is characterized by an obliteration of the basal cell layer with consequent basal derangement of basal lamina, decreased epithelial polarity and a scarce glandular acini connection [48].

PCa is thought to arise from pre-cancerous lesions originated as a consequence of inflammatory processes (Figure 5 and 6) [49-51].

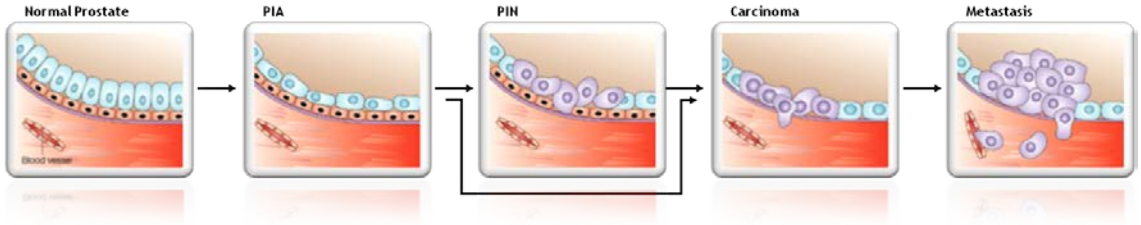

Figure 5- Schematic representation of prostate carcinogenesis. Prostate pathophysiology is a multistep process that comprises the appearance of pre-malignant lesions, proliferative inflammatory atrophy (PIA) and prostate intraepithelial neoplasia (PIN). This evolves into local carcinoma and locally invasive disease, and ultimately into metastasis with subsequent spread of tumor cells. Adapted from [396].

The stem cell population localized on the basal cell layer appears to have a preponderant role in pre-cancerous and PCa development. Occasionally, the population of adult basal stem cells may be restored, as in the case of prostatic tissue injury, and progresses into transit-amplifying/intermediate cells, which in turn can differentiate into luminal secretory epithelial cells or even neuroendocrine cells [49, 50, 52-54]. It has been proposed that a small number of these stem cells may represent the minority of epithelial cells that stand for PCa progenitor cells [49, 50, 52]. The continuous activation of signaling pathways by androgens, estrogens and other growth factors in progenitor epithelial cells may result in a heterogeneous population of progenitor cancer cells, provided with unlimited growth ability and possibility to trigger PIN-like lesions and PCa (Figure 6) [55].

Accordingly, the potential precursor of PCa is likely to be high-grade PIN (HGPIN) [56]. In general, PIN lesions are no more than an enhanced atypical cell number arising on the normal prostate gland architecture (Figure 6) [57]. According to the histomorphologic profile, PIN lesions were originally divided into three groups, namely Low-grade PIN1, grade 2 PIN and HGPIN (or grade 3 PIN) [58]. Later, PIN2 and 3 were fused and are mentioned as HGPIN [59]. Low-grade PIN architecture consists on crowding, stratified and irregular spacing epithelial cells, displaying anisonucleosis and rarely prominent and small nucleoli [58]. HGPIN lesion is characterized by architecturally benign prostatic acini and duct, lined by cytological atypical cells, with frequently prominent nucleoli, nuclear enlargement and crowding, increased cytoplasmatic density and multiple nucleolar size [58, 60]. HGPIN correlates with tumor stage, Gleason score and PCa risk [56, 61, 62]. Similar chromosomal alterations are found when comparing HGPIN with PCa, with loss of chromosome (chr) 8p, 10q and 13q and gain of chr 8q, 7 and Xq [63, 64]. In fact, the majority of the alterations that lead to PCa development appear to take place during benign epithelium to HGPIN transition [65].

PIA has also been pointed as a PCa precursor, possibly arising even before PIN-like lesions (Figure 6). PIA is thought to evolve in response to environmental factors that lead to cell damage and cause inflammation and oxidative stress. The resulting genetic modifications give rise to HGPIN lesions [66, 67]. PIA involvement on PCa etiology is somehow controversial. It is suggested that this type of lesion may only represent a primordial precursor, but is not related to PCa development [65]. Nonetheless, PIA displays molecular prints distinctive of

early neoplastic transformation, such as glutathione S-transferase-pi gene (GSTP1) hypermethylation, increased expression of glutathione S-transferase alpha 1 (GSTA1), cyclooxygenase-2 (COX-2) and B cell lymphoma 2 (Bcl-2), and reduced expression of NK3 homebox 1 (NKX3.1), early chromosomal abnormalities and shortening of telomere length [68-73]. In PIA lesions, discrete foci of proliferative glandular epithelium with the appearance of a simple atrophy or post atrophic hyperplasia in conjunction with inflammation can be found [74, 75]. PIA is characterized by the presence of two distinct cell layers. Among stromal and epithelial cells, a mononuclear and/or polymorphonuclear inflammatory cell layer can be found, and stromal atrophy with inconsistent fibrosis levels.

A number of different interactions between PCa cells and the stromal cell layer stimulate the stromal compartment originating a reactive microenvironment, where AR has a preponderant role promoting PCa cells growth and proliferation by activating distinct signaling pathways mentioned further ahead [76].

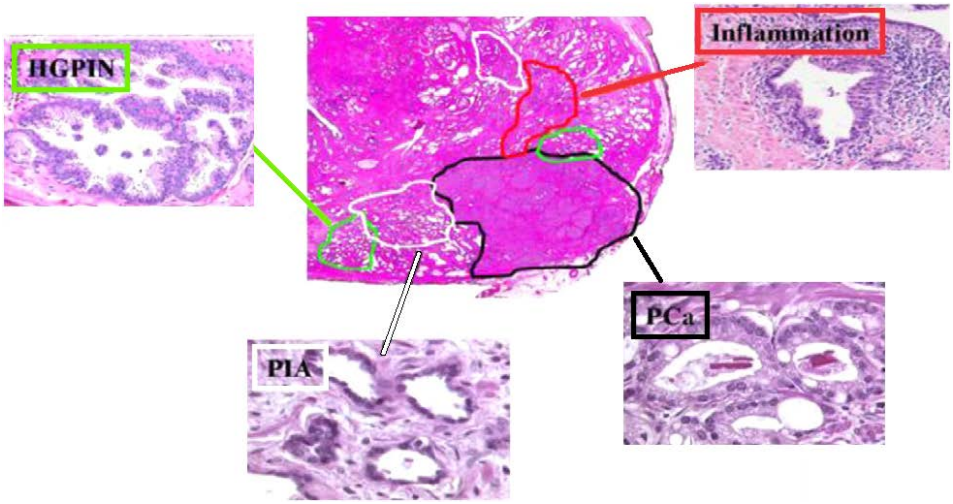

Figure 6- The distribution of inflammation, PIA, HGPIN and PCa in the human prostate H&E staining tissue sample, indicated, respectively, by red, white, green and black lines and arrows. PIA- proliferative inflammatory atrophy; HGPIN- High grade prostate intraepithelial neoplasia; PCa- Prostate cancer. Adapted from [397].

Hormone refractory prostate cancer (HRPC) is triggered after ablation therapies fail and tumor development is once again established. HRPC is considered a multifactorial and heterogeneous disease, and includes the activation of several signaling pathways in tumor cells. These induce a continuous AR-signaling because of an upregulation of AR expression, enhanced ligand-dependent activation by 5α-dihydrotestosterone (DHT) and testosterone and a broadened AR activation by non-androgenic and non-steroidogenic ligands, such as estrogens, progestins or growth factors. Furthermore, bypassing the AR signaling, cell growth and survival is also maintained by AR-independent mechanisms such as Bcl-2 activation, differentiation of neuroendocrine cells, ERα and PR signaling cascades, and others, and finally

by a continuous reposition of tumor cells through cancer stem cell regeneration [77-85]. Interestingly, much of the mechanisms culminating in HRPC are also essential for the growth and survival of the normal prostatic epithelium when exposed to adverse environments such limiting levels of androgens. The proper maintenance of the prostatic epithelium lies on basal cells and their particular resistance to androgen deprivation and invasive therapeutic approaches, much due to their androgen-independent phenotype and the harboring of a stem cells reservoir, as previously mentioned [7]. HRPC cells share great similarity to normal basal cells behavior, suggesting that HRPC cells mimic normal basal and stem cells in order to attain multidrug resistance, greatly improving their survival and proliferation ability [86].

The existing pseudocapsule surrounding the periphery and isolating it from the central zone may pose itself as a barrier for cancer cells to spread into the transition zone. When in the transition zone, tumors tend to be restricted to the prostate and have a better prognosis when comparing to peripheral tumors [47, 87]. Upon penetration of the capsule, PCa cells may spread and invade peri-prostatic tissues, firstly to the pelvic lymph nodes reaching as far as the distant lymph nodes, bones, brain, liver and lungs [88-91].

In the end, the clinical outcome associated with PCa is determined by the tumor growth, invasion, angiogenic and metastatic potential. PCa cells often metastasize to the bone tissue [92, 93]. The mechanism behind the high incidence of bone metastasis is poorly understood, but it may possibly be due to the tumor cell microenvironment and to the characteristics of the bone-specific matrix that facilitates the formation of metastasis [94].

Among the several grading systems available for tumor grading, the Gleason score system is the best known. This system is based on the tumor architecture pattern, glandular pattern, differentiation degree and interaction with stroma. Initially, the Gleason system comprised nine different patterns that were later compiled into five (Figure 7) [95, 96]. Briefly, Gleason patterns 1, 2 and 3 represent tumors with a normal resemblance and patterns 4 and 5 represent those with an increasing anaplastic appearance [95]. Gleason patterns 1,2, 3A and 3B indicate glands that are small in size, with separated acinis of various sizes. A large glandular pattern, with large simple acini, presence of papillary and cribiform structures and possible existence of central necrosis involving round duct-like structures, is attributed to Gleason 3A, C and 5A. Gleason patters 4A and 4B represent fused glandular adenocarcinomas. Finally, Gleason pattern 5B is attributed to solid adenocarcinomas, consisting on sheets, cords and single infiltrating cells [97]. A common finding among prostatic adenocarcinomas is the presence of heterogenic patterns within the tumor [98]. This way, the two most common patterns are selected and their sum accounts for a final Gleason score of the tumor. The Gleason system has been modified to better fit the modern needs, and nowadays the scale is restricted to patterns 3 to 5. Gleason patterns 1 and 2 are now considered as normal variants of prostate architecture [99].

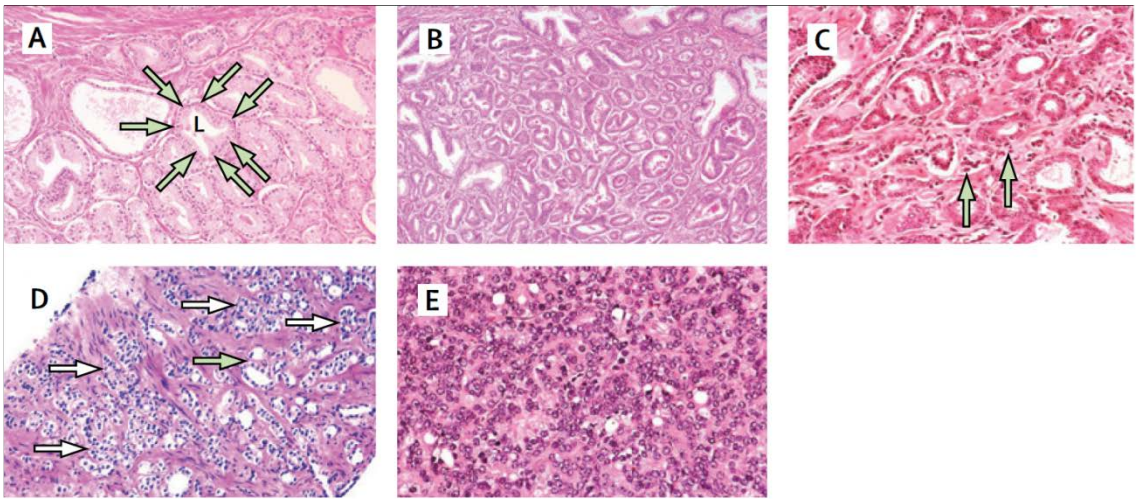

Figure 7- Gleason grading pattern system. A- Gleason pattern 1. Arrows indicate an individual acinus, and the lumen (L). 40x magnification. B- Gleason pattern 2, 40x magnification. C- Gleason pattern 3. Arrows indicate a small cell infiltration into the surrounding stroma. 100x magnification. D- Gleason pattern 4. Signs of increased stromal invasion. White arrows indicate areas of gland fusion and poorly defined lumens and green arrows indicate an area of Gleason pattern 3. 40x magnification. E- Gleason pattern 5. Presence of solid sheets of cells with no glandular structure and poorly differentiated cells. 100x magnification. Adapted from [398].

Another system has also been introduced to classify the tumor stage and that could accurately correlate with the pathological stage and disease prognostic, the Tumor Node and Metastasis (TNM) system [100]. The TNM classification is based on the extent of the primary tumor (T stage), the absence or presence of migration to nearby lymph nodes (N stage) and the absence or presence of metastasis to distant organs (M stage) (Table 1) [101].

Table 1- TNM system for staging of prostate cancer. Adapted from [399].

Stage	Sub-stage	Definition
T1		*Clinically unapparent tumor, not detected by digital rectal examination nor visible by imaging*
	T1a	Incidental histological finding; ≤5% of tissue resected during TURP
	T1b	Incidental histological finding; >5% of tissue resected during TURP
	T1c	Tumor identified by needle biopsy
T2		*Confined within the prostate*
	T2a	Tumor involves half of the lobe or less
	T2b	Tumor involves more than one half of one lobe but not both lobes
	T2c	Tumor involves both lobes
T3		*Tumor extends through the prostate capsule but has not spread to other organs*
	T3a	Extracapsular extension (unilateral or bilateral)
	T3b	Tumor invades seminal vesicle(s)
T4		*Tumor is fixed or invades adjacent structures other than seminal vesicles*
	T4a	Tumor invades bladder neck and/or external sphincter and/or rectum

Stage	Sub-stage	Definition
	T4b	Tumor invades levator muscles and/or is fixed to pelvic wall
Node		**Regional lymph nodes**
	NX	Regional lymph nodes can not be assessed
	N0	No regional lymph nodes metastasis
	N1	Regional lymph node metastasis
Metastasis		**Systemic spread**
	MX	Distant metastasis can not be assessed
	M0	No distant metastasis
	M1a	Non-regional lymph node(s)
	M1b	Bone(s)
	M1c	Metastasis at other site(s)

2.2.1. Epidemiology

PCa is the most commonly diagnosed cancer and the second most common cause of cancer related death in men in the Western world. In general, PCa in mainly found in the elderly men, turning it into an issue in developed countries rather than developing ones [102]. In 2012, it was estimated that almost 240000 men would be diagnosed with PCa, and around 29.000 would die due to the disease [103].

In the United States of America, PCa alone will account for 27% (233.000) of all newly diagnosed cancers in men, and 29.480 estimated deaths for the year 2014. Possibly due to early diagnosis of PCa through PSA testing, the incidence rates of PCa appear to be declining since 2000 [25].

In the European continent, PCa is the most common non-skin solid tumor with an incidence of 214 cases per 1.000 individuals [104]. Across different countries, the incidence rate of PCa widely differs [24, 102]. An optimist trend appears to be setting, as from 2009 to 2013, all cancer age-standardized mortality rates are predicted to decrease by 6% (140,1 per 100.000 men). In the European Union only, the predicted number of deaths as a consequence of cancer is thought to affect 1.314.296 individuals, 737.747 of which are men, and 10,5 per 100.000 will die from PCa [105].

In Portugal, around 4000 new cases of PCa were diagnosed in 2002, and 2000 deaths were expected [106, 107]. According to the National Statistical Institute of Portugal, in 2008 PCa was the second most incident type of cancer, representing 12,3% of the total malignant tumors recorded [108]. During 2012, malignant tumors were the origin of 23,9% of all deaths recorded in Portugal, corresponding to a mortality rate of 245 deaths per 100.000 habitants, of which 1,7% were due to PCa, accounting for 1.814 deaths [109].

2.2.2. Risk Factors

The risk factors for a high prevalence of PCa can be classified as endogenous (age, family history, ethnicity, hormones and oxidative stress) or exogenous (dietary factors, physical inactivity, obesity, environmental factors, occupation, smoking). Of all these factors, family history and age are considered the strongest risk factors for PCa [110-112]. Epidemiologic data demonstrate that family history is often linked to PCa risk. The predisposition for PCa increases in individuals who have a genetic linkage to an affected relative, being even higher according to the number of relatives with PCa [113- 117]. Nevertheless, due to the fact that men with family history of PCa are being screened more thoroughly, it is more likely to obtain positive findings [118]. Increasing age leads invariably to a physiological decline, diminishing the ability to fight stress, damage and disease. It affects neuroendocrine and immunological responses, causes cellular senescence, and increases oxidative stress, predisposition for DNA damage and somatic mutations [119, 120]. Some studies demonstrated that increasing age decreases the vitamin D levels, p53 activity, expression of genes with anti-oxidant action and androgens levels. On the other hand, it increases inflammatory mechanisms and reactive oxygen species (ROS) levels. All together, these events turn prostate cells more susceptible to malignant transformation [121]. Ethnicity is also one of the risk factors behind PCa development. PCa is more incident among African-American males, and those in conjunction with Hispanic men are usually diagnosed at younger ages when compared to white males [122-124]. The mortality rate is rising worldwide, especially in Asian countries rather than in western countries, most likely because of earlier diagnosis [125]. The disparities between PCa risk associated with race are thought to be due to multiple factors, such as environmental exposure, diet and genetic background. Furthermore, the hormonal levels found between different races may also account for the distinct PCa risk incidence, although this is controversial [126-129]. The presence of specific nucleotide repeats of polyglutamine (CAG) and polyglycine (GGC) within AR gene has also been implicated in PCa development, and vary in number and length according to ethnicity [130, 131]. In fact, epidemiologic studies suggest that the high risk of PCa found in African-American men may be related to an higher frequency of shorter and fewer CAG and GGC repeats when compared to other populations [130, 132].

Deregulation of hormones metabolism, particularly that of androgens, may also be in the genesis of PCa [133, 134]. Analysis of several prospective studies are inconsistent and no clear association between circulating and intraprostatic levels of androgens and PCa has been established [135]. The role of estrogens is also unclear and the controversial data recorded demonstrate that no association between estrogen levels and PCa risk has yet been established [126, 136, 137]. To better understand the reason why androgens and estrogens are involved in PCa, the roles of both are described further ahead.

Consumption of food enriched in antioxidants has proven itself on preventing PCa progression. Inclusion of vitamins C, D and E, soy, lycopene and selenium on diet has a protective action over PCa etiology and incidence [138-142]. Although controversial, low fat intake has also been related with a lower tumor growth rate [143-146].

Both BPH and PCa epidemiologic studies suggest that their incidence and prevalence are linked to chronic prostatic inflammation [147, 148]. The persistence of inflammation arising from an infection or tissue injury origins an unbalanced repair versus inflammatory response [66, 149, 150]. This tends to increase cell proliferation and an increasing predisposition for malignant transformation due to cellular and DNA damage caused by a stable production of reactive species, cytokines, chemokines and growth factors [51, 151, 152].

3. Molecular Pathways of Carcinogenesis: The role of sex steroid hormones in Prostate Cancer

Considering the several cellular and genetic alterations in PCa, the molecular pathways associated with carcinogenesis are poorly understood. The relationship between hormones and the pathogenesis of PCa has been extensively studied. PCa is generally considered a paradigm of androgen-dependent tumor. However, the role of estrogens appears to be equally important in both normal and malignant prostate. Recent epidemiologic and experimental data have clearly pinpointed the key roles of estrogens in PCa development and progression [153, 154].

The signaling pathways responsible for PCa carcinogenesis do not only include AR or ER genomic signaling. A number of other nongenomic pathways are involved on prostate pathogenesis and eventually crosstalk in between each other.

The cell surface receptor family of integrins has been thought to take part on cancer progression by modulating apoptosis, cell adhesion, cellular growth, gene expression and migration [155-158]. PCa cells display different extracellular matrix from the normal cells, suggesting that integrins display an important role in PCa progression and metastasis [159-161]. Integrins act alongside other cell adhesion molecules, such as cadherins, which are crucial for activation of epithelial-mesenchymal transition and PCa metastasis [162]. The loss of expression of the tumor suppressor gene E-cadherin correlates with higher tumor grades, bone metastasis and an unfavorable diagnosis [163-166]. In contrast, the increased expression of N-cadherin correlates with advanced PCa and metastasis [167, 168].

Prostate carcinogenesis is also regulated by the activation of a number of other signaling pathways, some of which interacting with AR signaling. These include growth factor receptor signaling, mitogen-activated protein kinase (MAPK) signaling, cytokine signaling and

Wnt signaling. The insulin-like growth factor 1 (IGF-1) overexpression has been correlated with higher PCa risk, and potentially modulates AR signaling by interfering with its phosphorylation, translocation to the nucleus or by enhancing AR cofactors action [169-171]. The epidermal growth factor receptor (EGFR), and its ligand epidermal growth factor (EGF) are also associated with high Gleason scores and promotion of AR co-activators, respectively, thereby facilitating prostate tumor progression and metastasis [172-174]. EGF also stimulate the HER receptor tyrosine kinases 1-4, members of the EGFR family, highly expressed on PCa cells, leading to activation of downstream pathways involving MAPK and phosphatidylinositol-3 kinase (PI3K)/ protein kinase B (Akt). Upon heregulin binding, HER2 and HER3 stimulate AR reporter genes transactivation [175]. Transforming growth factor alpha (TGFα) and EGFR act in a paracrine way influencing prostate growth and function [176]. Transforming growth factor beta (TGF-β) appears to have a dual effect, acting as a tumor suppressor and proliferation enhancer of PCa [177]. The phosphate and tensine homolog (PTEN) has also a vital role suppressing PCa initiation and progression, inhibiting AR and PI3K/Akt signaling [178, 179]. In PCa cells, PTEN is usually deleted, thus driving PCa progression [180]. The MAPK signaling cascade is a merging point of several pathways regulating prostate carcinogenesis. The primary endpoints of this cascade are the extracellular-signal regulated kinase (ERK), a protein involved on proliferation and usually activated by mitogens, *c-jun* N-terminal kinase (JNK) and p38 MAPK, both activated upon cellular stress and responsible for regulating the activity of nuclear transcription factors and proteins [181]. In PCa, ERK has either anti-apoptotic or proliferative activity, increasing PSA, and as p38, it correlates with increased cell proliferation and PCa initiation [182-185]. In opposite, PCa cells with activated JNK have increased apoptosis [186, 187].

PCa signaling pathways also include cytokine signaling, which are essential on the control of inflammation, cellular organization, apoptosis and cell survival. The tumor necrosis factor alpha (TNFα) is known to trigger the extrinsic apoptotic pathway, but contrarily to what happens in androgen-responsive PCa cells, normal and androgen-insensitive PCa cells are more resistant to apoptosis and growth arrest by TNFα suggesting that TNFα signaling differs according to AR expression in normal and PCa cells [188-190]. Another cytokine, interleukine-6 (IL-6) is prone to regulate inflammation, immune responses and tumor growth [191]. AR activity is also partially regulated by IL-6 by multiple pathways that include MAPKs and PI3K/Akt, and usually lead to AR expression and transcriptional activity enhancement, although mTor, a downstream target of Akt, suppress it [192-197]. Wnt signaling has also been implicated in PCa [198]. Binding of a Wnt ligand to the cell surface receptor frizzled family leads to nuclear translocation of β-catenin, where it complexes with lymphoid enhancer factor (LEF) and T-cell factor (TCF) activating several target genes transcription, such as c-myc and cyclin D1 [199, 200]. PCa is also characterized by an existing crosstalk between Wnt and AR signaling pathways. Both β-catenin and LEF-1/TCF complex induce AR upregulation and AR-regulated gene transcription [199]. β-catenin expression also correlates

with tumor grade and stage, leading to believe that altering Wnt pathway contributes to PCa progression into an androgen independent phase [201-206].

The Hedgehog pathway is directly implicated on embryonic development of the prostate tissue and the Hedgehog proteins expression in the human prostate decreases throughout prostate development until the adult stage, promoting cell growth and epithelial differentiation [207-212]. The downstream effects of this pathway include induction of proliferation and apoptosis repression, facilitating invasion and metastasis of tumor cells [213, 214]. Several studies have pointed out that the reactivation of this embryonic signaling pathway in PCa cells correlates with higher Gleason score [210, 215, 216].

Despite the high complexity of signaling crosstalk in PCa, it is clear that the signaling pathways triggered by AR and ER play an important role in PCa progression. Therefore, the comprehension of molecular mechanisms underlying the action sex steroid hormones is crucial to better understand the molecular mechanisms underlying PCa and to implement new therapeutic strategies.

3.1. Androgens and Androgen Receptor

The major circulating androgen, testosterone, is produced in the testis under hormonal control of the luteinizing hormone (LH) and gonadotropin-releasing hormone (GnRH). In several tissues, testosterone can be enzymatically converted into DHT by 5α-reductase [217]. Other androgens, such dehydroepiandrosterone (DHEA) and androstenedione (4-odione) are produced in small amounts in the adrenal glands [218]. DHT is the most potent androgen and is the main androgen on the prostate. As shown in Figure 8, both testosterone and DHT bind to AR. However, DHT is more potent and displays a higher affinity towards the receptor [219]. This way, the presence of DHT and a functional 5α-reductase enzyme is crucial for a complete prostate morphogenesis, as this organ is androgen-dependent [220, 221].

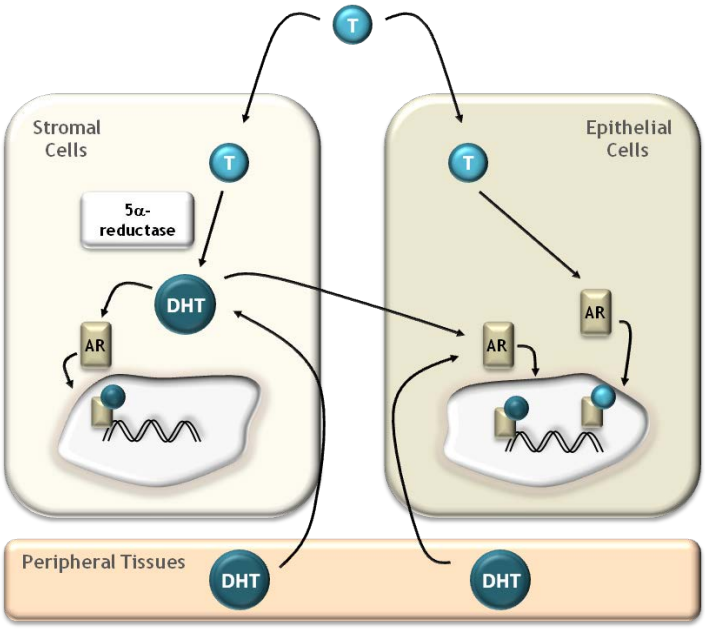

Figure 8- Testosterone (T) is captured by prostatic epithelial and stromal cells, and can either bind to the androgen receptor or be converted into 5α- dihydrotestosterone (DHT) in the stromal cells. DHT can act in an autocrine manner inside the stromal cell or diffuse to the proximal epithelial cells acting in a paracrine manner. DHT produced by peripheral tissues can also diffuse into the prostate. Adapted from [40].

Subsequent to prostate development, androgens continue to promote survival of the secretory epithelium, the primary cell type thought to be in the origin of prostate adenocarcinoma [222]. Cellular homeostasis is modulated by several growth factors secreted by epithelial and stromal cells. The reduction of prostatic DHT levels leads to a decreased protein synthesis, inactivation of androgen-dependent genes required for cell proliferation, and activation of others involved in apoptosis [223, 224]. Furthermore, androgen regulated growth factors and their receptors that mediate autocrine and paracrine androgenic signaling pathways are also affected. Consequently, castration promotes a massive loss among prostate secretory epithelial cells which undergo apoptosis [225]. The basal epithelia and stromal cells remain unaffected disclosing that prostatic stroma still retains the capacity to respond to androgens even though androgens are not required for its survival. In addition to apoptosis of secretory epithelial cells, castration also results in apoptosis and degeneration of prostatic capillaries and constriction of larger blood vessels, which precedes the appearance of epithelial apoptosis and contributes to a decrease in vascular function [2, 226]. The outcome of supra-physiological levels of serum androgen in humans is an increased cell proliferation in the prostate transitional zone [227].

Androgens have a pivotal role in PCa cell proliferation and survival, but in some conditions, androgens may also take part on inhibition of cell proliferation or promotion of apoptosis. Overall, the effects underpinning androgens actions are varied, and a crosstalk between downstream signaling elements may occur [228].

The main mechanism of action of androgens is through binding to AR (Figure 9), which belongs to the superfamily of nuclear receptors and acts as a transcription factor [229]. The AR gene, spans around 180Kb and is composed by a total of 8 exons and is located on the chr X (Xq11-12) [230]. The mature protein is composed by 919 aminoacids (aa), has an estimated molecular weight of 110KDa and comprises four distinct domains known as N-terminal domain (NTD), DNA-binding domain (DBD), hinge region and ligand-binding domain (LBD). The NTD is the largest domain and harbors CAG and GGC repeats, as well as the transcriptional activation function site 1 (TAF-1), making this an important co-regulator at the interface region [231-233]. The DBD contains two zinc fingers indispensable for recognition of response elements, such androgen responsive elements (AREs). The hinge region contains the nuclear localization signal responsible for the translocation of AR to the nucleus. Finally, the LBD is responsible for mediating ligand binding affinity and contains a second transcription activation function site (TAF-2) which is ligand-dependent [234].

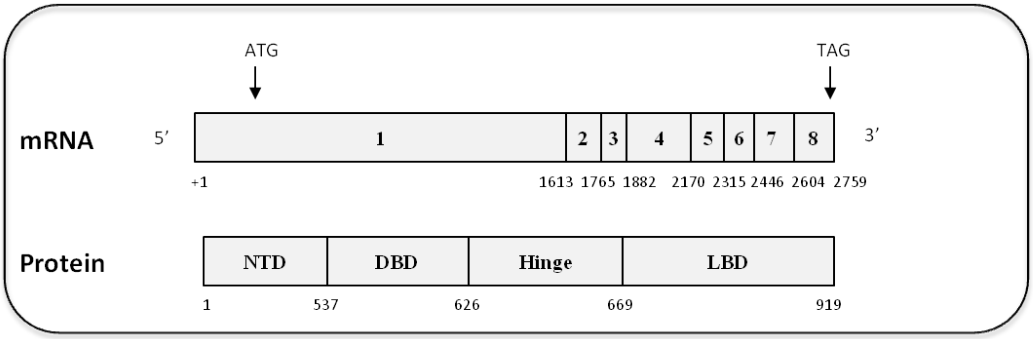

Figure 9- Structural organization of the androgen receptor (AR) mRNA and protein. Shading boxes indicate coding regions. Indicated below mRNA and protein structures are, respectively, exons size (bp) and aminoacid number. Adapted from [236].

Prior to ligand binding, AR is held inactive through association with inhibitory heat shock proteins (Hsp) and is precluded from DNA binding. Ligand binding releases Hsp, allowing AR translocation to the nucleus where it binds DNA as a homodimer on AREs within the regulatory regions of target genes (Figure 10) [235-237]. At this point, the intramolecular interaction between LBD and its NTD is crucial for an optimal AR activity and chromatin binding [238]. During the process, several factors of the transcriptional machinery such as chromatin remodeling complexes (SWI/SNF), AR coactivators, which either possess themselves histone acetyl transferase (HAT) activity or recruit proteins with HAT activity to the AR-regulated promoters, and RNA polymerase II are recruited in order to facilitate transcriptional initiation and AR-dependent gene expression [236, 239-241]. AR and chromatin interaction is extremely dynamic and transient, taking only a few seconds to bind and be displaced from the chromatin [242].

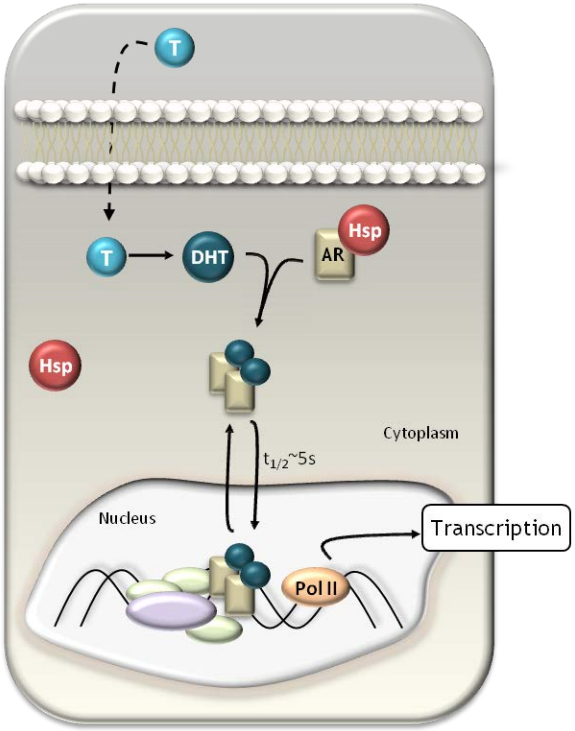

Figure 10- Androgen Receptor (AR) genomic signaling pathway. AR is held inactive wild bound to Hsp. Upon ligand binding, Hsp is released and hormone/receptor homodimers translocate to the nucleus, where they bind DNA on AREs. AR transcriptional activity is regulated by chromatin remodeling complexes (purple), coactivators (light green), and RNA Polymerase II (PolII). AR shuttles between the chromatin-bound and free cytoplasmic state with a $t_{1/2}$ around 5s. T- Testosterone; DHT- 5α-dihydrotestosterone; Hsp- Heat shock proteins; AREs- androgen responsive elements. Adapted from [230].

In the normal adult prostate tissue, AR is expressed in all types of luminal cells, and to some extent in epithelial basal, intermediate cell types and in the stroma [243]. In the epithelial cells, AR is responsible for the production of proteins, which are later secreted. In the stromal cells, AR is not only implicated on promoting cell proliferation but also cell survival [244]. On the other hand, AR can also act as a pro-apopotic factor [245]. During normal prostate development and homeostasis, AR present in stromal cells promotes the androgen-induced release of paracrine growth factors that modulate epithelial cell proliferation, survival and differentiation. A tight balance between luminal cells survival and basal cells proliferation is maintained by AR, for it acts as a growth inducer on the firsts and a suppressor on the seconds [246-248].

In primary PCa, AR retains the same role as in normal prostate, like inducing PSA synthesis and modulating lipid metabolism. However, it also promotes other events that promote epithelial cell growth, as induction of the type II transmembrane serine protease (TMPRSS2):ETS fusion [249, 250]. The TMPRSS2 is an androgen-regulated gene overexpressed in PCa, and frequently suffers chromosomal rearrangement that leads to fusion between its promoter and the 3' end of ETS family members, ETS related gene 1 (ERG-1), ETS variant (ETV)1 or ETV4 [251, 252]. The TRMPRSS2-EST fusion is considered the most common chromosomal PCa rearrangement and drives ETS oncogenes overexpression, previously

identified as the most expressed proto-oncogenes present on malignant epithelial prostate cells [253-255].

AR is heterogeneously expressed within prostate tumors, being found in primary, HRPC and metastasis notwithstanding tumor stage or grade and with no apparent correlation with diagnosis or duration of hormonal treatment, but there appears to exist a relationship between AR levels and subsequent disease progression [256-259]. Although there are many conflicting results, it has been noticed that increased AR heterogeneity and lower AR positivity on periepithelial stroma correlate with higher grades and poorer prognosis [258, 260]. This heterogeneity suggests that the increased AR expression may not be behind PCa initiation or that HRPC does not evolve from an AR-negative foci [77]. The role of AR in PCa progression and development of HRPC has been attributed to several factors, such as AR gene amplification, activating mutations and aberrant co-activators expression. These alterations lead to an increased AR expression, activation of AR by non-androgenic ligands, broadened ligand specificity and sensitivity and increased AR transactivation, which ultimately contribute to tumor cell growth in low androgen environment [261-269]. Furthermore, recent data indicate that an increased expression of constitutively active AR splice variants follows castration and are associated with poor prognostic and a rapid recurrence of PCa [270-273].

Identification of prostate-specific androgen or AR regulated genes is of extreme importance, especially those that demonstrate the potential to be used as PCa biomarkers or therapeutic targets.

3.2. Estrogens and Estrogen Receptors

Biosynthesis of estrogens precursors is achieved through the high levels of circulating androgens, such as testosterone, androstenedione and DHEA [274-277]. Natural estrogens can be divided into three basic and biologically active subtypes: estrone (E_1), 17β-estradiol (E_2) and estriol (E_3), of which E_2 is the most potent one [278]. Many studies suggest that E_2 may be produced locally within the prostate via aromatization of testosterone, a process catalyzed within the prostate stroma by aromatase [17, 279, 280]. Evidence has arisen that local E_2 metabolism may contribute to PCa and play an important role in tumor progression because some metabolites have been linked to free radical generation and direct genomic damage [281].

It is well characterized the association between androgens and the development of PCa, but recently, several studies have shown that aromatase expression is altered in PCa tissues, giving further support to a role of estrogens in this disease. Abnormal levels of E_2 and/or estrone and, especially, some of their hydroxylated derivates have also been implicated in tumor onset and progression [282, 283]. In non-malignant prostate tissues, aromatase expression is confined to the stromal cells, while it is expressed throughout microdissected epithelial tumor cells and PCa cell lines [279].

The importance of estrogens in the normal growth, differentiation and development of prostate as well as in the uncontrolled growth and transformation has been well established in PCa [284, 285]. Experimental animal models support the view that estrogens, alone or in combination with androgens are potent inducers of cell growth and differentiation in PCa [286, 287]. The administration of estrogens at pharmacological doses, alone or with androgens, results in the development of benign lesions known squamous metaplasia, and pre-cancerous lesions similar to PIN and HGPIN in prostates of various mammalian species, including humans [288-291]. In addition, estrogens lead to progression of human PCa and metastasis at distant organs [292]. *In vitro* studies also refer that both androgen and estrogens affect proliferation of cultured PCa cells. More specifically, physiological concentrations of estrogens stimulate LNCaP cell proliferation, and may decrease in a dose-dependent manner the growth of PC3 cells [293, 294]. The effect of estrogens in controlling cell growth appears to be mainly receptor-mediated [294].

ERα and ERβ belong to the nuclear hormone receptor superfamily, and are encoded by distinct genes found in distinct chromosomes and both display several mRNA splicing variants [295].

ERs exhibit a modular structure and are composed by six functional domains. These are the NTD or A/B domain, the DBD or Central domain (C), the hinge region (D), the LBD or E domain and the unique C-terminal F domain with unknown function (Figure 11) [296-298]. The low homology, less than 24%, present on the NTD domain of ERα and ERβ may account for the ER subtype–specific functions [192]. The AF1 situated on the NTD domain is ligand-dependent and displays promoter- and cell-specific activity [296, 298]. Unlike NTD, the DBD domain is highly conserved and is responsible for specific DNA binding, such estrogen responsive elements (EREs) sequences, and receptor dimerization [299, 300]. This domain is also provided by two zinc-finger motifs that confer its functionality [296]. The hinge region contains a nuclear localization signal important for nuclear translocation of ER. It is also poorly conserved between ERα and ERβ [301]. The LDB domain is responsible for ligand binding and receptor dimerization and contains an hormone-dependent AF-2 [296]. Finally, the F domain is unique to the ERs family and is thought to modulate the receptor stability and co-activator recruitment, as well as modulate the activation of AF-2 [302, 303].

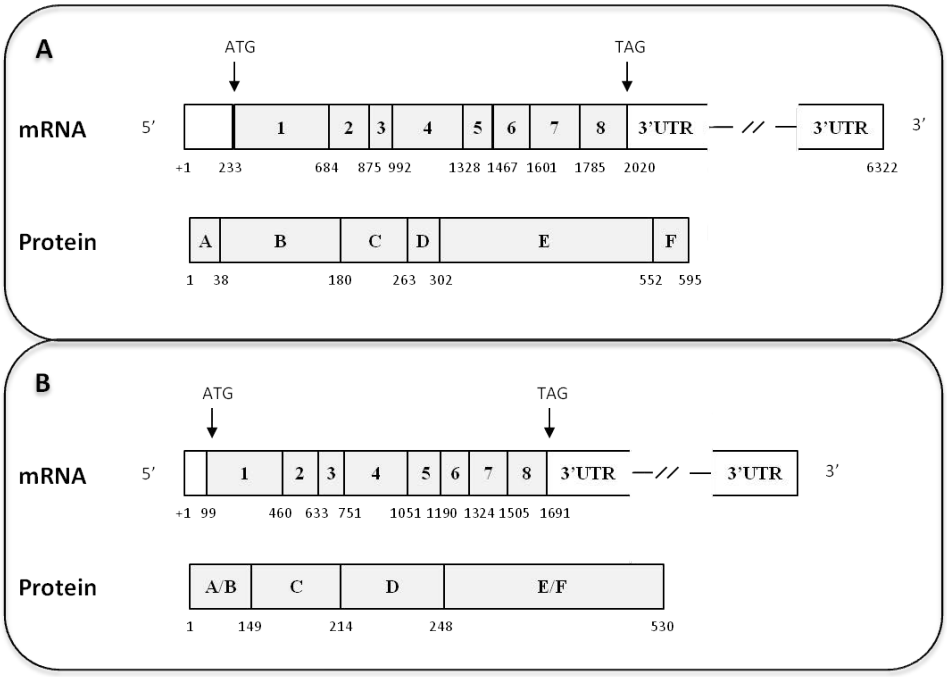

Figure 11- Structural organization of ERα (A) and ERβ (B) mRNA and protein. Shading boxes indicate coding regions. Indicated below mRNA and protein structures are, respectively, exons size (bp) and aminoacid number. ERα- Estrogen Receptor α; ERβ- Estrogen Receptor β. Adapted from [295].

The ERα encoding gene, ESR1, is localized on the Chr 6q25.1 and spans over 300kb and contains eight exons comprising a translated region of 140kb [304]. Six alternative promoters, A to F, can be found regulating ERα expression, thereby originating several splice variants, most with diminished transcriptional activity [305, 306]. The mature protein contains 595 aa and 66KDa [307].

ERβ gene is located on Chr 14q22-24, has 8 exons and a translated region spanning about 40kb [308]. Two upstream promoters, promoter OK and promoter ON, can also be found [309, 310]. Several transcripts have been identified and display transcriptional activity [311]. The mature protein comprises 530 aa with a predicted molecular weight of 60KDa [312].

More recently, the role of estrogens on prostate pathophysiology has been recognized, thus the signaling pathways involving estrogens and the ERs have been investigated. The mechanisms underlying the action of estrogens in PCa development involve several growth factor dependent signaling pathways, that promote aberrant cell growth, e.g., TGFα/ EGF receptor, TGFβ, insulin-like growth factor 1 (IGF-1) and vascular endothelial growth factor (VEGF), ER, prolactin receptor and MAPK activation, the majority of which are explained below in more detail [313-318]. Furthermore, estrogens appear to have a stimulatory effect in the Src/Raf-1/Erks signal transducing pathway in LNCaP cells, allowing PCa cell survival [319].

Evasion of apoptosis is another mechanism of estrogens action, in which estrogens promote the overexpression of anti-apoptotic mediators, such as metallothionein and transient receptor potential cation channel M2 (TRPM2) /clusterin [320, 321]. In addition,

changes in gene-expression profiles related to cell proliferation, DNA damage, activation of proto-oncogenes and transforming factors are well established in *in vivo* studies [322].

The effect of estrogens in controlling cell growth appears to be mainly receptor-mediated [294]. ERα and ERβ seem to exert opposite effects in prostate cell proliferation (Figure 12). ERα stimulation has been described to result in hyperplasia, inflammation and dysplasia of prostate, and ERα inhibition prevents PCa progression [323-325]. On the other hand, ERβ exerts an anti-proliferative effect in the prostate, directly or through ERα inhibition, and a loss of ERβ expression has been related with PCa progression [326]. *In vivo* studies using the beta estrogen receptor knockout (βERKO) mice showed that these animals develop prostatic hyperplasia with aging, which is not observed in wild-type or alpha estrogen receptor knockout (αERKO) mice [327]. In addition, treatment of neonatal rats with diethylstilbesterol (DES) leads to prostatic hyperplasia and dysplasia, which probably results from ERα upregulation and ERβ downregulation [328]. *In vitro* studies are well correlated with these data, as in prostate cells, ERβ caused a marked reduction of proliferation [329]. This mechanism involves, besides the ER activation, the recruitment of specific co-regulators, the interaction of the complex with EREs on the DNA and subsequently the activation of target gene transcription [330]. Furthermore, ERα and ERβ can interact with other DNA binding sites, such as those containing the transcription factors activator protein 1 (AP-1) and specificity protein 1 (Sp-1), which may regulate gene transcription in opposite directions [330].

The estrogen-ER complex regulates a set of genes involved in cell proliferation and cell cycle progression. It has been described that estrogens may exert their mitogenic effects regulating cyclin D1 expression, which increased expression causes aberrant G1 to S transition and lead to cell cycle progression [331]. In LNCaP cells, which are androgen dependent, ERβ expression increases from the G1 to the S phase and decreases before entering G2/M phase, and activates transcription via ERE during the S phase in a ligand-dependent manner. In addition, ERβ overexpression leads to an arrest in early G1 with a decrease in c-jun phosphorylation and cyclin D1 expression [332]. Others refer that estrogens induce the expression of Cyclin D1 via ERβ by increasing c-fos and c-jun expression in PC3 cells. In PCa tissues, cyclin D1 is overexpressed and it is positively correlated with ERβ expression, while ERα was almost undetectable [333]. Pravettoni et al. showed that E_2 or a ERβ selective agonist, diarylpropionitrile, decrease DU145 cell proliferation, which is accompanied by an increase of p21, a nuclear protein and a key point in control of cell cycle progression due to its effect inhibiting cyclin-dependent kinases [109]. Also, another study using a flavonoid, shows that genistein arrests the cell cycle at the G2/M phases and also suppresses cyclin B and p21, and p21 is induced via both p53-dependent and p53-independent pathways [334].

ERα and ERβ can also regulate cell proliferation through the activation of specific kinase activity, regulating EGFR and the activation of PI3K and MAPK signaling pathway, or induction of calcium influx [335-337]. ERβ inhibits cell growth and invasiveness of PCa cells,

and strongly stimulates apoptosis regulating the expression of Bax, Poly(ADP-ribose) polymerase and caspase-3 [329]. In addition, ERβ induces apoptosis via caspase-8 in prostatic stromal, luminal and castrate resistant basal epithelial cells of aromatase deficient (ArKO) mice, in Gleason score 7 xenografted tissues and in PC3 and DU145 PCa cell lines [338]. The upregulation of AIB1 expression, an ER co-regulator associated with cell proliferation stimuli and cell motility and invasion, was significantly correlated with lymph node metastasis of PCa. AIB1 directly regulates the transcription of matrix metalloproteinase (MMP)-2 and MMP-13 [339]. Interestingly, E_2 treatment leads to MMP2 production through stimulation of TGFβ1 expression in prostatic stromal cells which in turn promotes PCa cell invasion [340]. Although the effects of estrogens are known to be mainly mediated by ERα and ERβ, other alternative mediators may play important actions in PCa progression. ERβ2 and ERβ5 isoforms are reported to promote metastasis and their expression was associated with shorter metastasis in patients with PCa [341]. Recently, G-protein-coupled receptor-30 (GPR30) was referred to have estrogens-binding affinity, regulating cell growth. Activation of GPR30 by its specific ligand G-1, inhibits PCa cell growth by activating Erk1/2 and also c-jun and c-fos, which in turn upregulates p21 that mediates G2-M arrest, suggesting GPR30 as a novel mediator of estrogenic actions in the prostate [342].

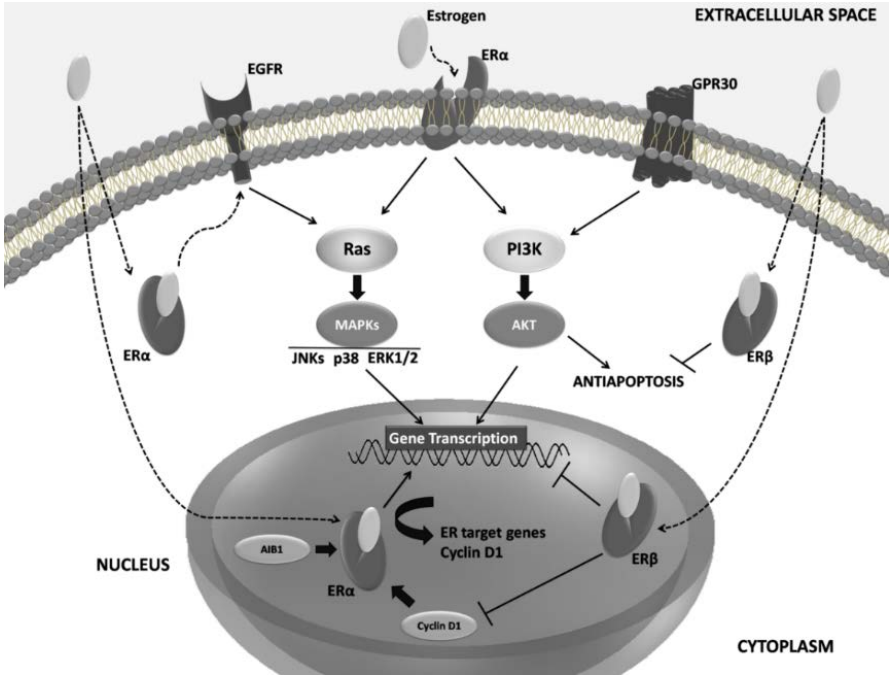

Figure 12. Schematic diagram of estrogen signalling in prostate cancer. Binding of Estrogen to the Estrogen Receptor α (ERα) promotes the translocation of this complex to the nucleus and the transcription of ER target genes along with a set of independent genes, including Cyclin D1. Estrogen-ERα complex also activates Ras-MAPK and PI3K signalling pathways, which in turn triggers cell proliferation and anti-apoptotic routes. Estrogens may stimulate the transcription of anti-apoptotic genes, via PI3K-AKT pathway, upon activation of G protein-coupled receptor 30 (GPR30). In addition, Cyclin D1 and AIB1 potentiate the transcription of ER-target genes. Contrarily, the binding of estrogen to ERß seems to inhibit cell growth and anti-apoptotic events. Adapted from [400].

The miscellaneous effects of estrogens in prostate may be explained by different expression patterns of ERs in different cellular compartments of the prostate tissue. The classical ER, ERα, has been detected almost exclusively on the stroma and in subsets of basal cells [343-348]. Both in normal, HGPIN and prostatic adenocarcinoma tissues, ERα maintain this restricted cellular expression. Interestingly, some studies show a positive correlation between ERα expression and high grade tumors (Gleason 4 and 5) as well as recurrent and metastatic tumors, leading to the assumption that the increase of receptor expression accompanies the development of PCa [349]. However, this is slightly controversial because although ERα is expressed in BPH and PCa cells, no clear or significant differences were found between the two pathologies, or even between hormone-responsive and refractory prostate carcinomas [350]. Epigenetic modifications have been pointed as one of the mechanisms involved in the regulation of ERα expression. It has been showed that the transcriptional activity of ERα gene is under control of methylation patterns, which is responsible to decrease ERα expression in higher grades of PCa [351].

ERβ expression, on the other hand, seems to be restricted to the luminal and basal epithelial cells, with lower or no expression in stromal cells [308, 346, 352-354]. However, *in vitro* studies using normal and malignant prostate epithelial cell lines, show that this receptor is highly expressed [355]. In human prostate tissue specimens, ERβ expression pattern alters significantly across PCa progression, suggesting that it may have an important role on prostate carcinogenesis and metastasis. Diminished levels of ERβ have been associated with the appearance of HGPIN, and this receptor expression is progressively lost on primary PCa stages. Nonetheless, ERβ expression appears to be retained on tumors with higher rates of recurrence and regained in metastasis [346, 353, 356, 357]. The regulation of ERβ expression appears to be through epigenetic mechanisms, specifically by hypermethylation CpG islands in the ERβ promoter leading to gene silencing [355, 358]. This event seems to be in accordance with the proposed role of ERβ as a putative tumor suppressor gene [329].

4. Biomarkers of Prostate Cancer

The identification of biomarkers, molecules whose detection and expression evaluation allow a better disease diagnosis and management, revolutionized the standard clinical approach. These molecules can be of several kinds, from proteins to metabolites, RNA transcripts, DNA or even epigenetic modifications of DNA sequences, and can be detected from the patient's tissue samples or body fluids. In cancer management, biomarkers are expected to have the potential to address disease disposition, screening, diagnostic, prognostic, prediction, monitoring and pharmacogenetics [359]. The ideal biomarker would be

safely and easily measured, display high sensibility and sensitivity, high positive and negative outcome values.

During many decades, the standard PCa treatments were limited to the use of cytotoxic agents, radiation, surgical removal of prostate and chemical castration. Nowadays, these approaches are becoming disadvantageous for the patient, and new strategies for targeting PCa are urgently needed [360-362].

The first biomarker ever used in PCa was PAP, as it was elevated on the serum of metastatic PCa patients [363]. With the appearance of PSA, PAP was rapidly replaced [364]. PSA is commonly used for disease prediction, detection and monitoring after hormonal therapy, once elevated serum PSA levels are an alarming signal in PCa [22]. PSA, also referred to as KLK3, is a member of the kallikrein (KLK) family secreted by prostate epithelial cells and displaying proteolytic activity [365]. Since it was first introduced, PSA based PCa widespread screening led to a more timely PCa diagnosis. On the other hand, more males with indolent PCa were being diagnosed and subjected to biopsies, turning PCa an overdiagnosed and overtreated type of cancer [366]. Moreover, PSA has several limitations as a biomarker, mainly due to its poor specificity. For example, increased PSA levels can be detected in response to noncancerous events, such as BPH. On the other hand, low levels of PSA can be detected in some patients with PCa [22, 365, 367].

Total PSA accounts for a combination of several distinct molecular PSA forms that can be divided into free PSA (fPSA) and complexed PSA (cPSA), mainly bound to the protease inhibitor α1-antichymotrypsin [368]. fPSA can be found in four subfractions: pro-PSA, intact free PSA, nicked PSA and BPH-associated PSA (BPSA) [369]. Both fPSA and cPSA have been considered adjuvant for total PSA tests. It has been noticed that fPSA levels decrease in PCa, allowing a more accurate distinction between BPH and PCa bearing individuals, while pro-PSA appears to increase in PCa [370-372]. Furthermore, a combined measurement of pro-PSA and fPSA has also been associated with higher PCa risk and aggressiveness [373, 374]. BPSA is expressed in men with BPH, and elevated serum BPSA levels appear to be correlated with pathologic nodular prostate hyperplasia, making it a reasonable marker for BPH [375, 376].

Although PSA is a valuable indicator for clinical practice, new PCa biomarkers are needed for disease detection, monitoring and recurrence. Emerging PCa biomarkers include prostate cancer antigen 3 (PCA3), TMPRSS2-ERG gene fusions, germline risk loci, circulating tumor cells, exosomes and some isoforms of the six transmembrane epithelial antigen of the prostate (STEAP) family [377-388].

In fact, STEAP1 was chosen for further studies because it meets the criteria for a good immunotherapeutic target: strong expression in multiple cancer types and restricted expression in normal tissues, cell-surface localization and no homology to any other known proteins [389, 390]. The identification of tumor-associated antigens (TAA) and their correspondent epitopes add up to an interesting research area for the development of anti-tumor vaccines with wide applications [391]. Because of its characteristics, STEAP1 has been considered as a potential TAA for the development of T cell–based immunotherapy [392-394].

Recently, it has been reported the presence of STEAP-related RNA derived from tumor cells in the serum of tumor patients [395]. Even though much is already known about STEAP1, there is still a gap to fulfill concerning its clinical meaning which is not fully understood.

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CHAPTER II

STEAP1 proteins: From structure to applications in cancer therapy

Adapted from the original paper 1

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1. STEAP proteins

The STEAP family of proteins includes 4 members, named 6-transmembrane epithelial antigen of prostate 1 to 4 (STEAP1–STEAP4). They all have in common a 6-transmembrane domain, a COOH-terminal domain with significant homology to the yeast ferric reductase (FRE) family of b-type cytochrome metalloreductases, and an *N*-terminal with homology to the archaeal and bacterial $F_{420}H_2$:$NADP^+$ oxidoreductase (FNO)–binding proteins [1]. STEAP proteins uptake iron and copper because of 2 conserved histidine residues predicted to bind at least an intramembranar heme group [2, 3]. The heme-binding 6-transmembrane domain is also present in the Nox and YedZ family, in which only 2 histidine residues are present and received the designation of apoptosis, cancer, and redox associated transmembrane (ACRATA) [4]. In mammals, STEAP proteins contain an exclusive FNO-like domain, enabling them to use intracellular flavin adenine dinucleotide– or flavin mononucleotide–derivate flavins as electron donors for iron and copper reduction [1, 3]. Other features common to most STEAP proteins are the presence of the YXXØ consensus sequence (in which Ø is a large hydrophobic amino acid) that is responsible for targeting transmembrane proteins to lysosomes and endosomes, and the Rossman fold (GXGXXG/A motif), a feature of proteins with oxidoreductase and dehydrogenase functions [3, 5]. The first role attributed to this family of proteins was their contribution to metal homeostasis by reducing iron and copper, thereby allowing their uptake. The only exception is STEAP1, which does not reduce metals, possibly owing to the absence of the FNO-like domain and the Rossman fold. Nevertheless, the partial colocalization of STEAP1 with transferrin (Tf), transferrin receptor 1 (TfR1), and endosomes specialized in iron uptake suggest that STEAP1 may also have a role in iron metabolism [1].

2. STEAP1: State of the Art

2.1. Structural Features of STEAP1 gene and protein

STEAP1 was the first member of the STEAP family to be identified. The STEAP1 gene (Table 1) is located on chr 7q21.13 and comprises 10.4 kb, encompassing 5 exons and 4 introns. Transcription of the STEAP1 gene gives rise to 2 different mRNA transcripts of 1.4 kb and 4.0 kb. However, only the 1.4 kb transcript is processed into the mature protein, which contains 339 amino acids with a predicted molecular weight of 36 kDa [6, 7].The 4.0 kb transcript contains a large intron of 2.399 bp, and it is not translated into a mature protein. The protein contains 6-transmembrane domains with the COOH- and *N*-terminals located in the cytosol, and 3 extracellular and 2 intracellular loops [6].

Recently, there has been found a STEAP1 related gene, STEAP1B. Although in the same chr, STEAP1B gene is located on the short arm of chr7 (7p15.3), is likely to originate two different transcripts and shares a high homology to STEAP1. The only transcript described has a coding region of 1.2kb and is predicted to originate a protein of 245 aa, which lacks the NADPH-oxidoreductase domain and the heme binding domain [8]. However, no expression or functional studies have yet been performed. Further *in silico* analysis was performed by our research group [9], and is described in Chapter V.

Table 1. Characterization of STEAP1 and STEAP1B genes, mRNA transcripts and proteins. Adapted from [54].

	Chromosome location	Gene size (Kb)	Exon/intron	mRNA transcripts (Kb)	Amino acids	MW (KDa)	References
STEAP 1	7q21.13	10,4	5/4	1,4 4,0	339	36	[6, 7]
STEAP1B	7p15.3	80,8	5/4	1,3 1,2	342 245	39 28	[8,9]

2.2. Tissue Expression and Cellular Localization

STEAP1 is overexpressed in several types of human cancer tissues and cell lines, namely prostate, bladder, colon, pancreas, ovary, testis, breast, cervix, and Ewing sarcoma (Tables 2–3; [6, 10]). Among normal tissues, the prostate gland is where STEAP1 expression is more abundant. Other nontumoral human tissues, such as ureter, fallopian tubes, uterus, pituitary, pancreas, stomach, colon, and breast show diffuse and low-intensity staining [6, 10]. In the prostate, STEAP1 is primarily expressed in the plasma membrane of the epithelial cells, particularly at cell–cell junctions [6].

Table 2. Expression of STEAP1 mRNA and proteins in normal and cancer tissues. Adapted from [54].

Normal Tissues	STEAP 1		References	Cancer Tissues	STEAP 1		References
	mRNA	Protein			mRNA	Protein	
Brain	+	-	[1,6]	Lungs		++	[11]
Pituitary gland		+	[6]	Colon		+	[6]
Fetal liver	++		[1]	Kidney		++	[44]
Liver	++	-	[1,6]	Bladder		++	[6]
Heart	+		[1]	Breast		++	[10]
Lungs	+	-	[1, 6, 11]	Prostate		+++	[6]
Thymus	+	-	[1,6]	Prostate Lymph node metastasis		+++	[11]
Lymph node		-	[6]	Prostate bone metastasis		+++	[11]
Bone marrow	+	-	[1,6, 33]				
Thyroid gland		-	[6]				

Pancreas	+	+	[1,6]
Spleen	-	-	[6]
Adrenal gland		-	[6]
Stomach	+	+	[1,6]
Duodenum	+		[1]
Colon	+	+	[1,6]
Kidney	++	++	[1,44]
Bladder	+	++	[6, 11,44]
Breast	++	++	[10]
Placenta	+	-	[1,6]
Ovary		-	[6]
Fallopian tubes		+	[6]
Uterus		+	[6]
Prostate	+++	+++	[1, 6,11]
Testis		-	[6]
Ureter		+	[6]
Skeletal muscle	+	-	[1,6]
Skin		-	[6]

+ low levels, ++ medium levels, +++ high levels, - not detectable

Table 3- Expression of STEAP1 mRNA and proteins in cell lines. Adapted from [54].

Cell Lines		STEAP 1 mRNA	STEAP 1 Protein	References	Cell Lines		STEAP 1 mRNA	STEAP 1 Protein	References
Melanoma	Mel 526	+		[41]	Lungs	TTC 1105	-		[41]
	Mel 624	+							
	Mel 888		+	[44]	Breast	MCF 7*	+		[6, 55, 57]
Myeloid Leukemia	KCL-22	+		[6]		MCF 7-LCC1			-11
Lymphoma	Jurkat		-	[44]		MDA-MB-435*	+		[6, 56]
Pancreas	BxPC-3	+	-			CAMA-1*	-		[6, 58]
	HPAC	++	-	[6]		DU4475	-		[6]
	Capan-1	++	-			SKBr3*		-	[33, 38]
Colon	Colo 205	++	+		Ovary	OV-1063	++	++	[6]
	CaCo-2	+	+	[6]		PA-1	+	+	
	LoVo	++	+			SW 626	++	+	[6,41]
	T84	++				CAOV-3	+		[6]
Kidney	Caki-1		+		Cervix	HeLa	+	-	[6]
	SW839		+	[44]		A431	+		[6]
	ACHN		+		Prostate	LNCaP*	+++	+++	
	SMKTP3		-			PC3#	+	+	[6, 59]
Bladder	UM-UC-3	++	++	[6, 44]		DU145#	+	+	
	5637	+++	++		Testis	NTERA-2	+		
	EJ-1		+	[33]		NCCIT	-		[6]
	T24		+			TERA-1	+		
	TCCSUP	+				TERA-2	+		
	HT1197	-		[6]	Ewing's sarcoma	RD-ES	++	+	[6]
	SCABER	+				A 673	++		[41]
	J82	-				TC 32	++		

*AR+, #AR-
+ low levels, ++ medium levels, +++ high levels, - not detectable

2.3. Physiologic Roles, Regulation and Implications in Cancer

Because of its localization on the cell membrane and its predicted secondary structure as a 6-transmembrane protein, it is believed that STEAP1 acts as an ion channel or transporter protein in tight junctions, gap junctions, or in cell adhesion, taking part in intercellular communication. As STEAP1 is overexpressed in cancer, it has been suggested that STEAP1 may facilitate cancer cell proliferation and invasion, perhaps through modulation of concentration of ions such as Na^+, K^+, and Ca^{2+} and small molecules [11-13]. Higher levels of voltage-gated Na^+ channels confer a highly invasive phenotype to PCa cells and the presence of these channels seems to be linked with the loss of androgen receptor expression and function and the progression to androgen-independent cell stages [13-16]. In addition, modulation of Ca^{2+} and K^+ levels seems to be very important for the progression of prostate tumors toward androgen-insensitive stages, by conferring an apoptotic resistant cellular phenotype [17-20]. Therefore, the relationship between STEAP1 and ion channels should be addressed in the future. STEAP1 also plays an important role in intercellular communication. Blocking STEAP1 with specific monoclonal antibodies in LNCaP cells increases cell death, suggesting that STEAP1 may promote proliferation of cancer cells or prevent apoptosis [11]. In accordance, Yamamoto and colleagues also demonstrated the importance of STEAP1 in tumor growth *in vivo*, and also that STEAP1 expression is also important for cell-cell interaction between tumor cells and adjacent tumor stromal cells *in vitro* [21]. The pathways underlying these effects are still unknown and need to be studied. On the other hand, STEAP1 seems to facilitate cell growth by raising the intracellular level of ROS, showing that STEAP1 acts both on inter- and intracellular pathways (Fig. 1; [22]). Furthermore, in correlation to Pan et al., recent data also demonstrated that STEAP1 is associated with invasiveness and oxidative stress phenotype of Ewing tumors, as STEAP1 promotes tumor cells proliferation, anchorage-independent colony formation and invasion *in vitro*, and induces growth and metastasis of tumors xenografts *in vivo*. It is unknown the mechanisms by which STEAP1 overexpression induces ROS levels elevation and its precise function in cancer processes, but it's predicted that the pro-invasive MMP1 and the pro-proliferative adiponectin receptor 1 (ADIPOR1) are downstream mediators of STEAP1 [23].

Until recently, 17β-Estradiol seemed to be the only known regulator of STEAP1 expression. This hormone downregulates STEAP1 expression both in rat mammary gland and in MCF-7 breast cancer cells [10]. Currently, the Polycomb protein member chromobox homolog 7 (CBX7) of the polycomb repressive complex 1 (PRC1) is also known to regulate STEAP1 expression by directly interacting with its promoter in human thyroid cancer cells and rat normal thyroid cells [24]. CBX7 is usually under-expressed in several types of cancers, including thyroid where its expression ends up being almost inexistent [25- 28]. Further analysis demonstrated yet that STEAP1 is upregulated in human papillary thyroid

carcinomas and lung carcinomas, suggesting that loss of CBX7 expression may contribute to the development of malignant phenotypes [24].

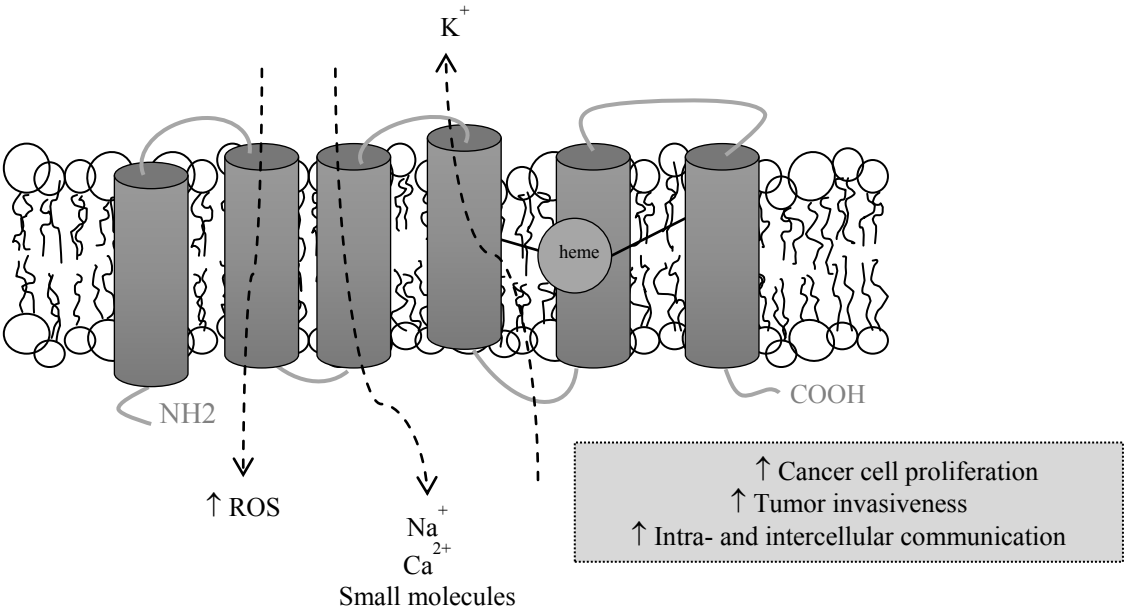

Figure 1- Schematic representation of STEAP1 protein structure, cellular localization and physiologic functions. Similar on the structure, presenting a six transmembrane structure, intracellular C- and N- terminal and intramembrane heme group, STEAP1 lacks the innate metalloreductase activity conferred by the presence of FNO-like domain. STEAP1 actively increases intra- and intercellular communication through the modulation of Na^+, Ca^{2+} and K^+ concentration, as well as the concentration of small molecules. It stimulates cancer cell proliferation and tumor invasiveness capacity. Adapted from [54].

2.4. STEAP1 as a Disease Biomarker

Initial studies on STEAP1 expression during the development of PCa did not find any significant alterations along the different cancer stages [6]. However, a later analysis of PCa cases, benign prostate hyperplasia, and nonprostatic malignancies disclosed a negative correlation between STEAP1 expression and histologic grading of PCa cells [29]. In addition, STEAP1 protein expression is higher in primary colon and bladder cancer when compared with colon and bladder cell lines derived from metastatic cancers [6]. Not a while ago, the enhanced outcome of Ewing sarcoma patient's was associated with STEAP1 high expression at the tumor's cell membrane. These were corroborated by the fact that STEAP1 silencing conferred cell resistance to chemotherapeutic agents, leading to believe that STEAP1 sensitizes cells to these drugs, perhaps by elevating ROS levels [23, 30]. Additionally, STEAP1 was found yet again overexpressed in several types of human cancers, being positively correlated with poor overall survival in colorectal cancer, diffuse large B cell lymphoma, acute myeloid leukemia and multiple myeloma [31]. Finally, the latest data indicate that

STEAP1 elevated expression in PCa human tissues correlates with high Gleason scores, seminal vesicle invasion, biochemical recurrence and metastatis, all features of poor prognostic outcomes. STEAP1 expression multivariate analysis also revealed that STEAP1 is an independent biomarker for biochemical recurrence [32]. So, more studies are required to clarify the association of STEAP1 expression with histologic grading in different cancer types, to understand the clinical significance of STEAP1 and its importance in cancer progression, particularly in PCa initiation, development, and metastasis.

Recently, STEAP1 mRNA has been identified by real-time PCR in serum of patients with cancer [33]. This highly sensitive and specific method allowed the distinction between 50 patients bearing pancreatic, bladder, breast, prostate, colon-rectal, lung, or stomach tumors and healthy subjects. External factors like age, histologic type, and clinical stage of cancer and therapy were evaluated, but none of them had a direct influence on STEAP mRNA levels. Therefore, the idea that STEAP1 may be a useful marker for several types of cancer, as well as its potential for cancer diagnosis, was reinforced [33-35]. Although STEAP1 has been almost exclusively associated with PCa, it is also differentially expressed in murine and human mesenchymal stem cells (MSCs), and in human bone marrow cells [36]. As MSCs can differentiate into several types of cells that compose bone marrow and, due to the cell-surface localization of STEAP1, this epithelial antigen could also be a useful marker for isolation and purification of MSCs, distinguishing between normal and abnormal populations of bone marrow cells. In addition, its possible involvement in the regulation of the differentiation process of the MSCs should be further investigated [37].

2.5. STEAP1 protein as Immunotherapeutic target

STEAP1 has been considered to be a good target for T cell–based immunotherapy, with applications in prostate, colon, pancreas, bladder, Ewing sarcoma, breast, testicular, ovarian, and melanoma cancers, as it has the required features of TAA, specifically, cell-surface localization, high expression levels in several types of tumors, especially in the prostate, and absence of expression in vital organs [6, 38]. Good immunotherapy techniques require the increase of expression or cross-presentation of self-peptides to naïve T cells. Therefore, the ultimate purpose of tumor immunotherapy is the production of an effective vaccine containing epitopes that elicit both $CD8^+$- and $CD4^+$ –T-cell immune responses, leading to tumor regression. This vaccine should be administrated to patients with cancer without using invasive techniques [39, 40]. The identification of STEAP1 epitopes has been directed toward prostate, renal, and bladder cancers with some success. The first STEAP1 epitopes used to trigger an antitumor immune response were $STEAP_{292}$ (MIAVFLPIV), a naturally processed peptide, and its modified version, $STEAP_{292.2L}$ (MLAVFLPIV). Their selection was based on their strong binding to HLA-A_0201 molecules and ability to elicit a sustained cytotoxic T-lymphocyte (CTL) response. In fact, $STEAP_{292}$, and especially $STEAP_{292.2L}$, induced

naïve $CD8^+$ T cells into CTL capable of recognizing peptide-loaded cells with high specificity. Moreover, CTL induced by $STEAP_{292.2L}$ peptide not only recognized peptide-loaded cells, but also tumor cells from prostate, colon, bladder, Ewing sarcoma, melanoma, and embryonic rhabdomyosarcoma that expressed STEAP1 [41]. Two additional nonameric STEAP1 epitopes ($STEAP_{86-94}$ and $STEAP_{262-270}$) were found to be HLA-A*0201–restricted epitopes. Both can be found in human and mouse, but with slightly different constitutions; human $STEAP_{86-94}$ differs at position 9 from the mouse peptide (FLYTLLRE**V**→FLYTLLRE**I**) and human $STEAP_{262-270}$ differs at position 6 from the mouse peptide (LLLGT**I**HAL→LLLGT**V**HAL). The latter has been initially identified as a TAA of STEAP3 [42, 43]. Despite the differences mentioned, both $STEAP_{86-94}$ and $STEAP_{262-270}$ peptides are immunogenic in vivo, in HLA-A_0201 transgenic mice (HHD), and in vitro, in peptide-specific human $CD8^+$ T cells from healthy donors. Furthermore, $STEAP_{86-94}$ and $STEAP_{262-270}$ from human and mouse $CD8^+$ T cells were able to recognize STEAP1 expressed in human tumor cells in an HLA-A*0201–restricted manner. Also, specific CTL-expressing $STEAP_{86-94}$ were amplified ex vivo, from the peripheral blood of 3 out of 5 patients with non-small cell lung carcinoma and 2 out of 3 patients with PCa, reinforcing the protective role of STEAP1. However, the contribution of tumor cells expressing STEAP1 to the observed immunologic response generated by the naturally processed STEAP1, as well as the quantitative and qualitative discrimination of the T cells expressing $STEAP_{86-94}$ and $STEAP_{262-270}$ remain to be identified [42]. As a TAA, STEAP1 was also thought to be able to trigger an immune response to eliminate a tumor by specifically eliciting $CD4^+$ helper T cells. Consequently, 2 specific synthetic STEAP1 peptides, $STEAP_{102-116}$ (HQQYFYKIPILVINK) and $STEAP_{192-206}$ (LLNWAYQQVQQNKED), which strongly bind to different classes of HLA-DR, can also be presented by those different classes to $CD4^+$ helper T cells [40, 44]. These naturally processed epitopes are both presented to $CD4^+$ helper T cells by tumor cells such as PC3 prostate cells, m697 melanoma cells, and Epstein Barr virus–transformed lymphoblastoid cell lines, but only $STEAP_{102-116}$ is presented to $CD4^+$ helper T cells by antigen-presenting cells (APC; [40]). The reactive $CD4^+$ helper T cells present in the different types of cancer may play a crucial role in the immune response that follows their activation; these cells would initiate the immune response sending the appropriate signals to APCs and CTL, resulting in CTL expansion, maturation, costimulation, and generation of memory CTL populations [45]. In fact, both of these peptides were identified as being processed endogenously, through direct presentation of STEAP1 peptides by HLA-DR molecules, and exogenously, by APCs that process STEAP1 peptides derived from cell lysates, as seen in vitro with renal and bladder cancer [44]. A new STEAP1 synthetic peptide was found to be effective in lung cancer, complementing previous observations. Resorting to a computer based algorithm system, $STEAP_{281-296}$ was selected as having the higher potential as a promiscuous HLA-class II peptide epitope able to elicit a $CD4^+$ T cell response. And indeed, $STEAP_{281-296}$ was able to efficiently activate $CD4^+$ T cells, in a HLA-DR restricted manner, obtained from the peripheral blood of healthy individuals to generate reactive clones. The presentation of this epitope was seen to occur both endogenously, by direct recognition of lung cancer cell lines expressing STEAP1

and HLA-DR, and exogenously, through direct recognition of naturally processed STEAP1 antigen in lung cancer tumor presented by dendritic cells. Furthermore, $STEAP_{281-296}$ induced a T cell immune response in lung cancer patients, possibly underlying the presence of STEAP reactive T cell precursors in these patients [46].

Immunotherapeutic strategies based on the presence of memory T cells may present a potent tool for tumor antigen effective target. In an attempt to identify the presence of circulating T cells reactive to STEAP1 in Ewing sarcoma patients, Altvater and colleagues found *ex vivo* that the frequency of STEAP1-reactive memory T cells is extremely low in the peripheral blood of healthy donors. This frequency is held low even in newly detected and relapsed Ewing sarcoma patients, leading to believe that spontaneous T cell reactivity against STEAP1 is rare. STEAP1-specific CTLs were only efficiently generated *ex vivo* under professional presentation with autologous dendritic cells and under optimized cytokine conditions, but even then they failed to interact with STEAP1 expressing Ewing sarcoma cells, making theme unfit for therapy [47].

Application of therapeutic vaccination, using STEAP1 as a target, that efficiently attenuates or even stops cancer progression is still in its early steps. No commercial formulations are available, particularly in PCa, as the efficacy of the developed vaccines relies on the immunosuppressive state of the patients included in clinical trials and their tumor microenvironment, which prevents triggering of an immune response. Two studies that applied a mouse STEAP1 DNA prime/Venezuelan equine encephalitis virus-like replicon particles boost vaccine showed the efficacy of this therapeutic strategy against PCa [39, 48]. The capacity of this vaccine to trigger an immune response could be seen by the increasing number of $CD8^+$ and $CD4^+$ T cells and by the production of cytokines such as TNF-α, IFN-α, IL-2, and IL-12 following vaccination of C57BL/6 mice injected with a cell line derived from the transgenic adenocarcinoma mouse model, TRAMP-C2 [39]. Protection against PCa dramatically increases when vaccination occurs in mice mimicking earlier stages of cancer. Immunosuppressive mechanisms that lead to a reduction of T helper 1 (Th1) and T helper 2 (Th2) function, reduction of proinflammatory cytokines, and increased expression of immunosuppressive factors activated during PCa progression tend to interfere with the efficacy of the vaccine [48]. Avoiding the establishment of an immunosuppressive tumor microenvironment seems, therefore, to be the key to the success of therapeutic vaccination in later cancer stages.

Other viral vectors have been used to deliver TAAs, such the highly attenuated strains of modified vaccinia Ankara (MVA) which has been widely used in clinical trials [49]. The prophylactic efficacy a STEAP1 DNA prime/MVA boost revealed promising results on PCa prevention in mice. Upon primary immunization with murine STEAP1 (mSTEAP1) DNA vector and posterior boost with MVA recombinants, C57BL/6 mice with TRAMP-C1 cell line infiltrates add a marked reduction on tumor growth, proving mSTEAP1 DNA prime/MVA boost vaccine anti-tumor activity. A combination of mSTEAP1 with murine prostate stem cell antigen (mPSCA) demonstrated to have a even higher anti-tumor potential, and when applied in

TRAMP mice it decreased tumor burden, reduced the severity prostatic lesions and the incidence of high-grade PCa, diminished the proliferation index and led to an increase of T cells infiltrated on the tumor, all without adversely affecting vital organs. This vaccination strategy promising results on mice immunization may be therefore a good candidate to test in humans with PCa.

It has been known the potential of antibody-drug conjugates (ADCs) as a therapeutic approach, once it combines the specificity of the monoclonal antibody and the cytotoxic character of the chemotherapeutic drug to which the antibody is conjugated [50, 51]. Resorting to ADC technique, a second human monoclonal STEAP1 antibody tagged with monomethyl auristatin E (MMAE) has been constructed [52]. MMAE is a synthetic drug with potent antimycotic and anticancer activity, inhibiting tubulin polymerization [53]. MMAE was linked to the STEAP1 antibody through a thioeter bond between the linker maleimide moiety and a cysteine thiol that usually forms the interchain disulfide bond at the hinge region of the antibody. The impact of MMAE conjugation of the ADC generated STEAP1 antibody resulted on appreciable, although slight, impact on pharmacokinetics and tissue distribution with high clearance and a general trend to an increased hepatic uptake and diminished levels on other highly vascularized organs [52].

Thus, all together, these observations offer new possibilities for novel immunotherapeutic strategies, not only directed toward prevention but also for the treatment of patients with cancer.

3. References

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CHAPTER III

Aims of the Thesis

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Considering the summarized revision found in Chapters I and II, the pivotal role of sex steroid hormones on PCa pathophysiology is well established, as well as the increasing need to identify and perceive the role of novel immunotherapeutic targets, as well as their potential as disease biomarkers. A growing body of evidences put forward STEAP1 as a key player in PCa development, diagnosis and treatment. Nonetheless, it is urgent to clarify its expression pattern, regulation and physiological role to further understand how it correlates with PCa. The main aims of the present research work were to:

1. Determine the effect of sex steroid hormones in STEAP1 expression;
2. Compare STEAP1 stability between malignant and non-malignant prostate cells;
3. Understand the role of STEAP1 in PCa cell proliferation and apoptosis;
4. Explore the putative role of STEAP1 as a biomarker for PCa.

These goals are addressed in the following Chapters IV to VII, in the form of scientific publications.

CHAPTER IV

Six transmembrane epithelial antigen of the prostate 1 is down-regulated by sex hormones in prostate cells

Original Paper 2

Gomes IM, Santos CR, Socorro S, Maia CJ, Six transmembrane epithelial antigen of the prostate 1 is down-regulated by sex hormones in prostate cells. Prostate 2012; 73(6):605-13. doi: 10.1002/pros.2260

The Prostate 73:605–613 (2013)

Six Transmembrane Epithelial Antigen of the Prostate I Is Down-Regulated by Sex Hormones in Prostate Cells

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BACKGROUND. STEAP1 is over-expressed in several types of tumors, especially prostate cancer, where it is localized in the plasma membrane of epithelial cells, at cell–cell junctions. Its role in prostate carcinogenesis and its regulation in prostate cells remain unknown. Therefore, we propose to study the effect of sex hormones in the regulation of STEAP1 expression in prostate cells in vitro and in vivo.

METHODS. LNCaP prostate cells were incubated with fetal bovine serum (FBS), charcoal-stripped FBS (CS-FBS), 5α-dihydrotestosterone (DHT), and 17β-estradiol (E_2) for different periods of stimulation. In addition, adult male Wistar rats were castrated and treated with DHT and E_2. The levels of STEAP1 in response to treatments were analyzed by real-time PCR, Western blot, and immunohistochemistry.

RESULTS. The treatment of LNCaP cells with DHT or E_2 induces a down-regulation of STEAP1 expression, while incubation with CS-FBS has the opposite effect. Experiments using inhibitors of androgen and estrogen receptor (AR and ER) showed that down-regulation of STEAP1 is AR-dependent, but ER-independent. However, the mediation of six transmembrane epithelial antigen of the prostate 1 (STEAP1) expression by AR seems to be dependent of de novo protein synthesis. In vivo studies showed that castrated rats express higher levels of STEAP1 protein when compared to intact rats, an effect reversed by DHT or E_2 replacement.

CONCLUSIONS. STEAP1 is down-regulated by DHT and E_2 in LNCaP cells and in rat prostate. *Prostate 73: 605–613, 2013.* © 2012 Wiley Periodicals, Inc.

KEY WORDS: **STEAP1; cancer; 5α-dihydrotestosterone; 17β-estradiol; rat; LNCaP**

INTRODUCTION

Prostate cancer is one of the most prevalent types of cancer and one of the principal causes of mortality among men [1]. Prostate carcinogenesis is mainly characterized by two stages, androgen-responsive and androgen-insensitive with poor prognosis [2]. Despite the overall advances in prostate cancer management, there are still few molecular tools for correct prostate cancer diagnosis and treatment, stressing the importance of the identification of novel biomarkers and immunologic therapeutic targets. Six transmembrane epithelial antigen of the prostate 1 (STEAP1) is over-expressed in prostate cancer and in the spontaneous transgenic mouse model of prostate cancer (TRAMP), with restricted expression in normal tissues, but a wide expression in several types of cancers, including breast, cervix, pancreas, bladder, colon, ovary, testis and Ewing sarcoma [3–5]. STEAP1 is primarily located in the plasma membrane of epithelial cells, particularly at cell–cell junctions, but it can also be seen dispersed on the cytoplasm [3,4]. Although STEAP1 biological functions are yet to be totally ascertained, it is believed that it acts as an ion channel or transporter protein possibly in tight junctions, gap junctions and in cell adhesion [3,4]. STEAP1 seems to regulate intercellular communication and

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the rate of cell proliferation, but it may also regulate cell fate and cancer cells invasiveness [6–8]. Besides intercellular communication, STEAP1 also appears to be involved in intracellular pathways, acting as an active intervenient on cell growth by raising reactive oxygen species (ROS) [9]. Furthermore, the expression of STEAP1 on Ewing sarcoma has been correlated with increasing ROS levels, which in turn regulate a number of genes and signaling pathways responsive to ROS [10]. STEAP1 is differentially expressed in prostate cancer cell lines according to their metastatic potential, being more expressed in those cells that mimic earlier stages of the disease [3]. Although it is clear that STEAP1 expression is definitely linked to prostate cancer, the regulation of STEAP1 expression in this tissue is still a question to be address in more detail. So far, it was demonstrated that zoledronic acid, which is used to inhibit bone resorption in patients with cancer, decreases STEAP1 mRNA expression in prostate cancer cells [11]. Recently, our research group also demonstrated that STEAP1 is down-regulated by 17β-estradiol (E_2) in a human breast cancer cell line (MCF-7) and in rat mammary gland [4]. Estrogens also have an active role in invasion and metastasis of prostate cancer cells [12,13]. In addition, cell proliferation and apoptosis and several genes involved in the onset and development of prostate cancer are regulated by androgens [14,15]. Therefore, the aim of this study was to determine the effects of androgens and estrogens, specifically 5α-dihydrotestosterone (DHT) and E_2, on STEAP1 expression in in vitro and in vivo.

MATERIALS AND METHODS

Animals and Cell Lines

The human prostate cancer adenocarcinoma cell line (LNCaP) was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK).

A total of 64 adult male Wistar rats (250–300 g; approximately 3 months old) were maintained in an animal room at constant temperature, humidity, a 12 hr light–dark cycle, and with food and water available ad libitum, for 1 week before use. Research was conducted according with the National and European Union guidelines (Directive-2010/63/EU).

Cell Culture and HormoneTreatment

LNCaP cells were maintained in RPMI 1640 medium phenol red (Gibco, Invitrogen, Paisley, Scotland) supplemented with 10% of fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany) and 1% penicillin/streptomycin (Invitrogen, New York, NY) and maintained at 37°C with 5% CO_2. In all experiments, cells were grown up to 60% confluence and 24 hr before stimulation the cells medium was replaced with phenol red-free RPMI 1640 medium containing 10% charcoal-stripped fetal bovine serum (CS-FBS) devoid of steroid hormones (Gibco, Invitrogen). The effect of the presence of steroid hormones in general and DHT (Fluka, Sigma, Steinheim, Germany) or E_2 (Sigma, Saint Louis) in particular was studied as follows. Cells were cultured on a 10% CS-FBS or 10% FBS supplemented medium during 4, 8, 12, 24, or 48 hr. For the same period of time, LNCaP cells were also exposed to two different concentrations of DHT or E_2 (0 and 10 nM). Next, a dose-dependence assay was performed using 0, 0.1, 0.5, 1, 10, or 100 nM DHT or E_2 for 24 hr. Finally, the effect of 24 hr incubations with 10 nM DHT, 10 nM E_2, 1 μM Flutamide (Sigma), 1 mg/ml Cycloheximide (Sigma), 100 nM ICI 182,780 (Tocris Cooksob, Bristol, UK), combination of DHT or E_2 with each inhibitor, or 10 nM E_2 conjugated to BSA (E_2:BSA) was assessed. Inhibitors were added to cell cultures 30 min before hormone stimulation. All assays were carried out in hexaplicate.

Animal Treatment

Rats were divided into four distinct groups, each containing 16 animals. Animals from two groups were orchidectomised (ORCHX) under anesthesia (Clorketam 1000, Vétoquinol, Lure, France). Five days after surgery, they were given daily intraperitoneal injections of DHT (500 μg/kg day) (ORCHX + DHT) or physiologic serum/ethanol 30% alone (ORCHX + vehicle) for 5 days. The remaining groups were constituted by intact rats that were injected daily with vehicle alone (control) or with E_2 (250 μg/kg day) (intact + E_2). The doses of DHT and E_2, and duration of treatment were chosen on the basis of other similar studies to evaluate gene regulation by sex hormones [16–18]. After treatment, animals were sacrificed under anaesthesia and prostrates removed. The organs were then frozen in liquid nitrogen and stored at −80°C or fixed in PFA 4% for immunohistochemistry. In each group of 16 animals, 8 whole prostates were used for protein extraction and 8 prostates were used for fixation in PFA 4%.

Total RNA Extraction and cDNA Synthesis

Total RNA from the prostate cell line was extracted using TRI reagent (Ambion, UK) according to the manufacturer's instructions. The quantity and quality of total RNA was assessed by espectrophotometry at 260 and 280 ηm (Pharmacia Biotech, Ultrospec 3000), and agarose gel electrophoresis, respectively. One micro-gram of total RNAs was used for cDNA synthesis

applying a First Strand cDNA Synthesis Kit (Fermentas), according to the manufacturer's instructions.

Total Protein Extraction

LNCaP cells and prostate homogenized tissues were lysed in an appropriate volume of radioimmunoprecipitation assay buffer (RIPA) (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris, 1 mM EDTA, 1% Protease cocktail and 10% PMSF) and total proteins (supernatant) were recovered after a 12,000 rpm centrifugation for 20 min at 4°C. Quantification of total protein extracts was assessed using the *Bradford* method (Bio-Rad Laboratories, CA).

Real-Time PCR

Real-time PCR was carried out to compare the mRNA levels of STEAP1 in LNCaP cells subjected to DHT and E_2 treatments. Specific primers to STEAP1 located in different exons were used (Table I). STEAP1 expression was normalized with the internal controls: human GAPDH (hGAPDH) and human beta-2-microglobulin (hβ2M) (Table I). The efficiency of real-time PCR was determined for all designated primers with serial dilutions (1; 1:10; 1:100; 1:1,000) of the cDNA from LNCaP cells. Real-time PCR reactions were carried out using the Maxima^{TM} SYBR Green/Fluorescein qPCR Master Mix (Fermentas) and primers for each gene. To evaluate the specificity of the amplified PCR fragments, melting curves were obtained by a series of 10 sec cycles leading to an increase of temperature from 55 to 95°C (0.05°C/s). STEAP1 expression was assessed using the mathematical model proposed by Pfaffl ($2^{-(\Delta\Delta Ct)}$) [19].

Western Blot

Total proteins were resolved in a 12% SDS–PAGE gel and electrotransferred to a PVDF membrane (GE Healthcare, Buckinghamshire, UK) at 750 mA for 40 min. Membranes were blocked for 1 hr in a 3% casein (Sigma) solution and then probed overnight with a rabbit polyclonal antibody against human STEAP1 (H105) diluted 1:300 (Santa Cruz Biotechnology, Santa Cruz), as used by other groups [20,21]. The membranes were then incubated with an alkaline phosphatase conjugated goat polyclonal antibody against rabbit IgG diluted 1:20,000 (GE Healthcare). Finally, STEAP1 immunoreactivity was visualized after membrane exposure to the ECF substrate (GE Healthcare) on the Molecular Imager FX (Biorad, Hercules). The same membranes probed with anti-STEAP1 were incubated with mouse anti α-tubulin antibody diluted 1:5,000 (Sigma) and then with goat anti-mouse IgG phosphatase alkaline conjugated secondary antibody diluted 1:20,000 (Abcam, Massachusetts, MA).

The expression levels of STEAP1 and α-tubulin proteins were quantified by densitometry using Quantity One software (Biorad, Hercules). The signal of STEAP1 was normalized to the corresponding α-tubulin signal.

Immunohistochemistry

STEAP1 was detected by immunohistochemistry in paraffin-embedded rat prostate tissue sections. These were deparaffinized with xylene and rehydrated using ethanol solutions with increased water content. Endogenous peroxidases were inactivated by a 3% hydrogen peroxide solution and unspecific protein bounds were eliminated through 30 min incubation with 5% normal goat serum (Santa Cruz Biotechnology). Incubation with rabbit primary antibody against human STEAP1 (H105) diluted 1:50 (Santa Cruz Biotechnology) for 1 hr 30 min was followed by 1 hr incubation with a biotinylated goat anti-rabbit IgG 1:20 (Sigma), both at room temperature. After Avidin-conjugated rabbit peroxidase (Sigma) exposure for 30 min and incubation with DAB (Dako, Denmark), immunoreactivity of STEAP1 was detected. Finally, sections were counterstained in Mayer's hematoxylin (Richard-Allan Scientific, MI), dehydrated, cleared and mounted with Entellan® neu mounting media

TABLE I. Sequences and Resulting Amplicon Sizes of the Specific Primers and Cycling Conditions Used in Real-Time PCR for Amplification of Human STEAP1, GAPDH and b2M

Oligo name	Sequence (5′–3′)	Amplicon size (bp)	Annealing temperature (°C)
hSTEAP_619	GGC GAT CCT ACA GAT ACA AGT TGC	128	60
hSTEAP_747	CCA ATC CCA CAA TTC CCA GAG AC		
hGAPDH_74	CGC CCG CAG CCG ACA CAT C	75	
hGAPDH_149	CGC CCA ATA CGA CCA AAT CCG		
hβ2M_347	ATG AGT ATG CCT GCC GTG TG	92	
hβ2M_439	CAA ACC TCC ATG ATG CTG CTT AC		

(Merck, Darmstad, Germany). Negative controls were performed by omission of the primary antibody.

Statistical Analysis

Data from all experiments are shown as mean ± SEM of n = 6 for in vitro and n = 8 for in vivo studies. The statistical significance of the differences in STEAP1 expression between different experimental groups was assessed by one-way ANOVA followed by the Bonferroni test. Significant differences were considered when $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***) compared to control values.

RESULTS

Sex Steroid Hormone Withdrawal Up-Regulates STEAP1 Expression in LNCaP Cells

In order to determine if the sex steroid hormones in the culture medium influence STEAP1 expression, LNCaP cells were cultured with 10% FBS (control) or 10% CS-FBS for 12, 24, 48, 72, and 96 hr. mRNA and protein levels were analyzed by real-time PCR and Western blot, respectively. STEAP1 expression was detected in both groups but with significant differences. It was clear that both mRNA (Fig. 1A) and protein (Fig. 1B) expression levels were up-regulated when LNCaP cells were cultured with CS-FBS in comparison with controls ($P < 0.05$). These increased levels were maintained throughout the time-course assay, but no differences were found between the levels of expression at the distinct times of incubation with CS-FBS.

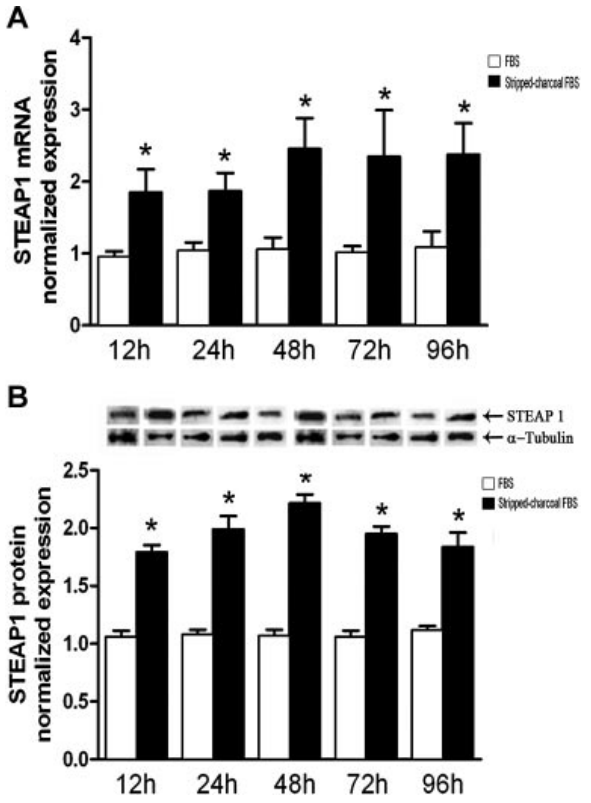

Fig. 1. Time-course effect of CS-FBS on STEAP1 mRNA and protein expression in LNCaP cells determined by real-time PCR (**A**) and Western blot (**B**). STEAP1 mRNA expression was normalized with hGAPDH and hβ_2M expression and protein expression was normalized with α-tubulin. Error bars indicate mean ± SEM of n = 6. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (one-way ANOVA followed Bonferroni test) compared with control values.

DHT and E2 Down-Regulate STEAP1 Expression in LNCaP Cells

To assess the effects of androgens and estrogens in STEAP1 expression in LNCaP cells, time-course and dose-dependent assays with DHT or E_2 treatment were carried out. In order to validate our experiments, prostate specific antigen (PSA) gene was used as positive control. The results obtained (Supplementary Fig. 1) showed that PSA is up-regulated in response to DHT or E_2, as described by others in LNCaP cells [22,23]. Treatment with 10 nM DHT resulted in a down-regulation of STEAP1 mRNA (up to 40% of control values, Fig. 2A) and protein (up to 50% of control values, Fig. 2B) after 8 hr of stimulation, suggesting that the effect of DHT is time-dependent. The decreased levels of STEAP1 mRNA and protein expression due to E_2 treatment were also time-dependent, being visible only after 24 hr decreasing even further thereafter. The levels of STEAP1 mRNA and protein decreased up to 60% and 40% compared to controls after 48 hr of incubation, respectively (Fig. 2). Subsequently, the effect of different DHT (0, 0.1, 0.5, 1, 10, and 100 nM) and E_2 (0, 1, 10, and 100 nM) concentrations was assessed after 24 hr of stimulation, a period of time chosen based on the time-course assay. Dose–response analysis of both hormones showed that 1, 10, and 100 nM decreases the levels of STEAP1 mRNA (Fig. 3A). Regarding the STEAP1 protein (Fig. 3B), the results showed that down-regulation is visible with 0.5, 1, 10, and 100 nM DHT, but not with 0.1 nM DHT. The down-regulation of STEAP1 protein by E_2 is visible with 10 and 100 nM, but not with 1 nM E_2.

DHT and E_2-mediated decrease in STEAP1 mRNA and protein levels could result from diminished transcription, RNA destabilization or a combination of these events. To analyze whether STEAP1 regulation is mediated through the activation of the androgen receptor (AR) or estrogen receptor (ER), both were

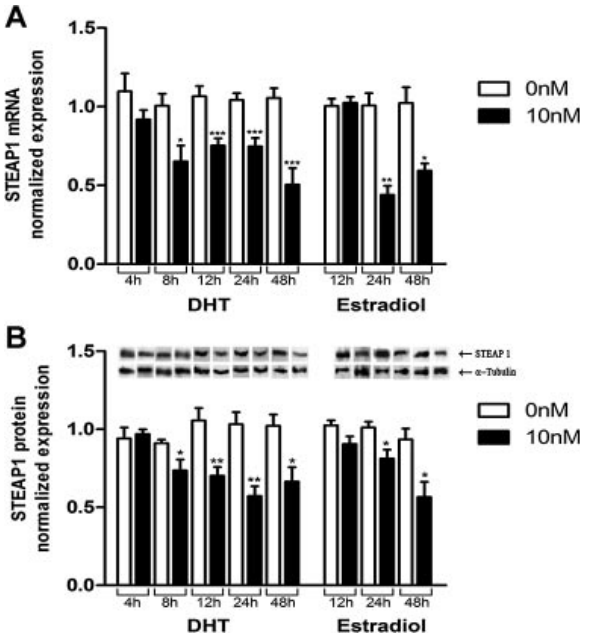

Fig. 2. Time-course effect of DHT on STEAPI mRNA and protein expression in LNCaP cells was determined by real-time PCR (**A**) and Western blot (**B**). STEAPI mRNA expression was normalized with hGAPDH and hβ_2M expression and protein expression was normalized with α-tubulin. Error bars indicate mean ± SEM of n = 6. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (one-way ANOVA followed Bonferroni test) compared with control values.

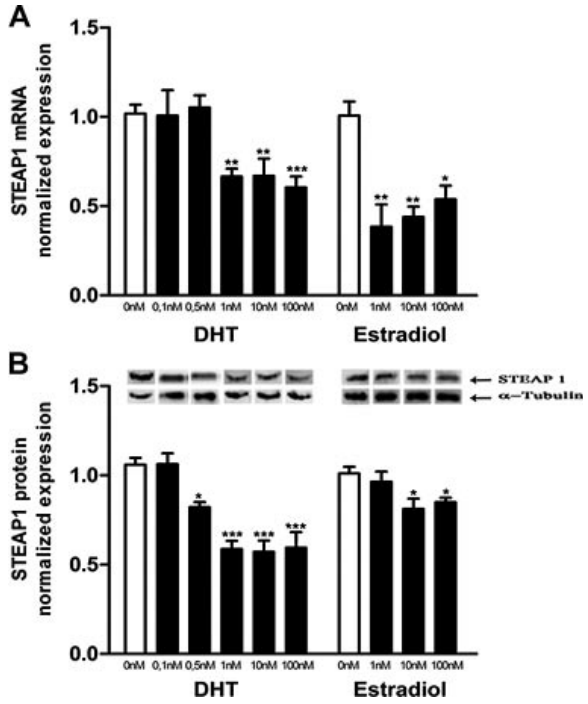

Fig. 3. The dose-dependence effect was analyzed by real-time PCR (**A**) and Western blot (**B**). STEAPI mRNA expression was normalized with hGAPDH and hβ_2M expression and protein expression was normalized with α-tubulin. Error bars indicate mean ± SEM of n = 6. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (one-way ANOVA followed Bonferroni test) compared with control values.

blocked with specific antagonists, Flutamide (AR antagonist) and ICI 182,780 (ER antagonist), respectively. The inhibitory effect of DHT and E_2 on STEAP1 mRNA expression was once more observed after 24 hr of stimuli (Fig. 4). The effect of DHT in STEAP1 expression was abrogated in the presence of Flutamide (Fig. 4). To determine if newly synthesized proteins are required for the down-regulation of STEAP1 expression, the effect of DHT was compared in the presence of the protein synthesis inhibitor, cycloheximide (Chx). The results showed that the effect of DHT on mRNA expression was withdrawn by Chx-treatment (Fig. 4). The down-regulation of STEAP1 in response to E_2-treatment was not blocked in the presence of ICI 182,780, and E_2–BSA treatment reproduced the same effect of E_2 alone (Fig. 4). Flutamide, Chx, and ICI 182,780 alone did not modulate STEAP1 expression when compared to controls.

Effect of Castration and DHT/E_2-Replacement in STEAPI Levels of Rat Prostate

The levels of STEAP1 protein were determined in response to DHT and E_2 in rat prostate. Western blot analysis showed that castration alone increased STEAP1 protein levels approximately twofold when compared to control (intact rats). Administration of DHT (500 μg/kg day) nearly restored STEAP1 expression to the levels of controls. Regarding the treatment of intact rats with E_2 (250 μg/kg day), no immunoreactive bands were detected (Fig. 5).

To evaluate the cellular localization of STEAP1 protein and intensity of staining in rat prostate, eight tissue sections of each experimental group were analyzed immunohistochemically. As shown in Figure 6, STEAP1 protein was essentially visualized in the columnar epithelial cells (brown staining), particularly in the cell junctions and at the luminal side of the membrane. Castration resulted in a size decrease of epithelial cells, which was restored by DHT treatment. In control animals, STEAP1 staining was low in four tissue sections and negative in the remaining ones. In all castrated rats, STEAP1 staining intensity ranged from low (2 out of 4) to high (2 out of 4). Treatment of castrated rats with DHT restored STEAP1 expression levels, resulting on a staining intensity comparable to that observed in intact rats, that is five tissue sections were negative and the remaining were

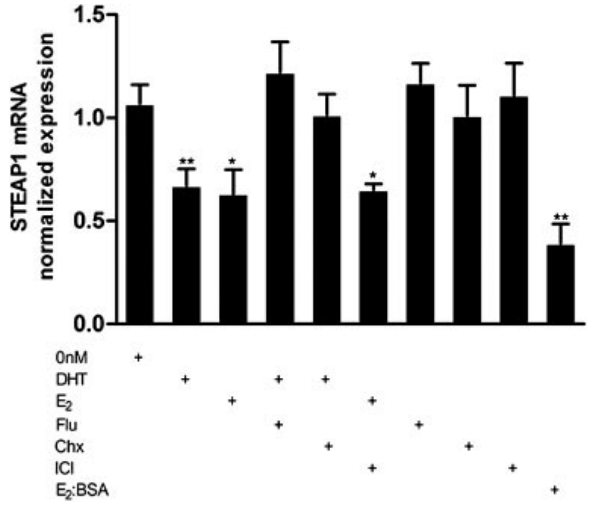

Fig. 4. STEAPI mRNA expressions in response to DHT (10 nM), E_2 (10 nM), flutamide (1 μM), cyclohexamide (1 μg/μl), ICI 182,780 (100 nM), and E_2–BSA (10 nM) treatment was evaluated in LNCaP cells at 24 hr of stimulation by real-time PCR. STEAPI mRNA expression was normalized with hGAPDH and hβ2M expression. Error bars indicate mean ± SEM of n = 6. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (**One-way ANOVA followed Bonferroni test) compared to control.

low. Regarding intact animals treated with E_2, no staining was observed in the corresponding tissue sections.

DISCUSSION

STEAP1 is a protein with six transmembrane domains over-expressed in several types of human cancer, namely prostate and breast adenocarcinoma

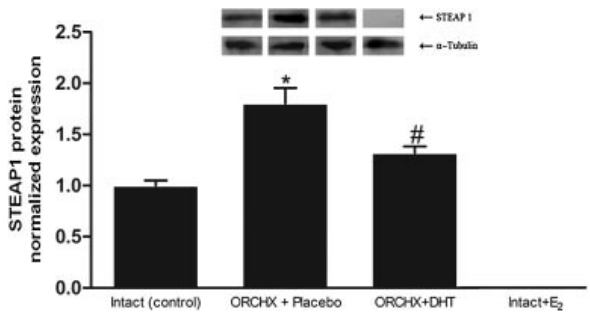

Fig. 5. STEAPI protein expression in rat prostate. Castrated rats received i.p. injections of vehicle (ORCHX + vehicle) or DHT (ORCHX + DHT, 500 μg/kg day) for 5 days. Intact animals were administrated with E_2 (250 μg/kg day) or vehicle (control). STEAPI protein expression was analyzed by Western blot and normalized with α-tubulin. Data are presented as the mean ± SEM of n = 8. $^{*,\#}P < 0.05$ (one-way ANOVA followed Bonferroni test) compared with intact and ORCHX + Placebo values, respectively.

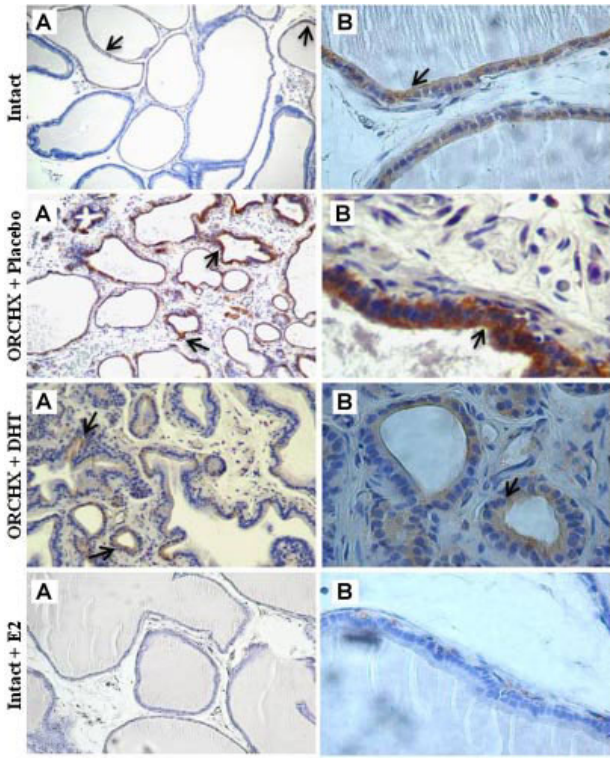

Fig. 6. Representative images of immunohistochemical staining for STEAPI protein in tissue sections from whole prostate of intact animals, castrated animals treated with vehicle (ORCHX + Pla- Placebo) or DHT (ORCHX + DHT) for 5 days, and intact animals treated with E_2 for 5 days (Intact + E_2). Negative control (without primary antibody) was done in tissue sections of all groups (data not shown). Images were amplified 100× (**A**), and 400× (**B**). Arrows indicate stained structures.

[3,4]. The factors that control STEAP1 expression in prostate cells are still unknown, but sex steroid hormones are potential candidates. Therefore, we have evaluated whether factors present in serum, which could be removed by charcoal-treatment, are involved in STEAP1 gene regulation. Several studies have used CS-FBS to selectively remove hormones, namely androgens, estrogens, progesterone, insulin, T3 and T4, without nonspecific loss of other serum components [24,25]. Hence, LNCaP prostate cancer cells were treated with FBS serum (used as control) and CS-FBS. An increase in STEAP1 mRNA or protein levels was noticed after exposing cells to CS-FBS, suggesting that the loss of some factors present in serum induce an up-regulation of STEAP1. This means that FBS serum may contains signaling factors that may down-regulate STEAP1 expression. As androgens and estrogens play an important role in the onset and progression of prostate cancer [24,25], studies to evaluate the effect of DHT and E_2 in STEAP1 regulation in

prostate were conducted. Both hormones, DHT and E_2, markedly decreased the expression of STEAP1 in LNCaP cells. The effect of DHT in down-regulation of STEAP1 seems to be time-dependent, with the effect of DHT and E_2 being visible after 8 and 24 hr of stimulation, respectively. Next, the dose of DHT and E_2 in the regulation of STEAP1 levels was evaluated. It was observed that 1 nM was the minimum dose of DHT necessary to observe down-regulation of STEAP1 mRNA expression at least for 24 hr incubation. However, the expression of STEAP1 protein requires 0.5 nM of DHT to decrease over the same time period. Regarding the effect of E_2 in the regulation of STEAP1 expression, dose-dependence was observed after 24 hr of stimulation. However, the effect of 1 nM E_2 in the down-regulation of STEAP1 is detected at the mRNA level, but not at protein level. Together with the effect of DHT, our results show that lower doses of both hormones may trigger different effects in the expression of STEAP1 mRNA and protein. Although other studies are required, these results suggest that these steroid hormones may influence the stability or degrading rates of mRNA or protein, as described by other groups [26,27]. It is well known that DHT and E_2 produce most of their cellular effects through their cognate nuclear receptors, AR and ER, respectively. After binding to the hormone, AR or ER form homodimers that interact directly with AR or ER elements or indirectly with other transcription factors that bind DNA in the regulatory region of the target gene promoters [28–30]. AR or ER activation result in alteration in the rate of protein synthesis and in the generation of a cellular response. Our results show that down-regulation of STEAP1 by DHT is inhibited by the AR antagonist, flutamide, which promotes the assemblage of a transcriptionally inactive AR [31]. In addition, as down-regulation of STEAP1 by DHT-treatment is abolished in the presence of cycloheximide and no statistical differences are detected between cycloheximide alone and controls, de novo protein synthesis may be required to produce this effect. This suggests that AR should not act directly at the promoter region of the STEAP1 gene, but rather on another transcription factor. To support this idea, we have analyzed the STEAP1 promoter region using a bioinformatic program (www.gene-regulation.com) and no androgen responsive elements (ARE) were detected. Contrarily, the presence of ER antagonist, ICI 182,780, did not counteract the E_2 inhibitory effect, suggesting that the down-regulation of STEAP1 by E_2 does not involve the nuclear ER signaling pathway. In fact, the E_2:BSA treatment also down-regulates the expression of STEAP1. Taking into account that E_2–BSA does not cross the cell membrane; this result suggests that the effect of E_2 may be mediated through a membrane-receptor. This observation is in accordance with what has been previously described by our research group in MCF-7 breast cancer cells [4]. Although not fully understood, it has been suggested that ERs can translocate to the plasma membrane. It is believed that the mechanism behind this process is accompanied by post-transcriptional lipid modifications, such as palmitoylation, and by several scaffold proteins that include caveolins, striatin, Shc and others [32]. Although STEAP1 levels in normal cells appear to be almost inexistent, we detected STEAP1 mRNA and protein in rat prostate. STEAP1 mRNA was amplified by PCR using specific primers to rat STEAP1, and the identity of the PCR product was confirmed by sequencing (data not shown). Therefore, the effect of DHT and E_2 in STEAP1 protein levels was also evaluated in rat prostate. The obtained results indicate that castrated rats present higher levels of STEAP1 protein and that this effect was abrogated by DHT replacement, suggesting that DHT down-regulates STEAP1 protein expression. Although the detection of STEAP1 expression has not been achieved in some tissue sections of rat prostate, similar observations were also obtained by Immunohistochemical analysis, in which prostates from castrated rat show higher STEAP1 immunoreactivity when compared to intact or DHT treated animals. In intact animals treated with E_2, we have not detected STEAP1 expression. Taking into account that STEAP1 expression is very low in normal prostate of intact animals and that E_2 may down-regulate STEAP1 expression, it is not surprising that we could not detect expression of STEAP1 in the prostate of these animals. STEAP1 protein was localized mainly in the columnar epithelial cells, particularly at the apical membrane of cell–cell junctions, as previously described [3].

At the present time, it is difficult to speculate about the pathophysiological significance of STEAP1 down-regulation. Some studies indicate that STEAP1 may be involved in intercellular communication facilitating cell growth of prostate cells [3]. On the other hand, it is well known that androgens and estrogens are involved in maintenance of cell proliferation and apoptosis. Taking into account that the effect of steroid hormones should not be directly mediated by its cognate receptors, it is possible that cells decrease the levels of STEAP1 in order to overcome the proliferative effects of steroid hormones. Therefore, it would be interesting to evaluate how does STEAP1 levels influence the proliferation induced by androgens and estrogens in prostate cells.

Several studies have suggested that STEAP1 can be used as a biomarker. The STEAP1 mRNA was detected in serum of patients with different tumors,

including prostate cancer [33]. In addition, other studies have suggested that STEAP1 levels may predict the clinical outcome in Ewing's sarcomas [34]. Bearing in mind that STEAP1 is down-regulated by androgens and that STEAP1 may facilitate cell proliferation, it is expected that AR-refractory tumors with higher levels of STEAP1 may be more aggressive than tumors with lower levels of STEAP1. To address this question, studies to evaluate the clinical significance of STEAP1 in human prostate cancer, particularly to assess if there are any correlations between STEAP1 levels and AR-status, Gleason score, metastasis, or survival outcome, are required.

CONCLUSION

STEAP1 is down-regulated by both DHT and E_2 in LNCaP prostate cancer cells and in rat prostate, suggesting that STEAP1 may influence prostate cancer progression in an androgen- and estrogen-dependent manner. Further studies are required to evaluate the biological role of this down-regulation in response to sex steroid hormones.

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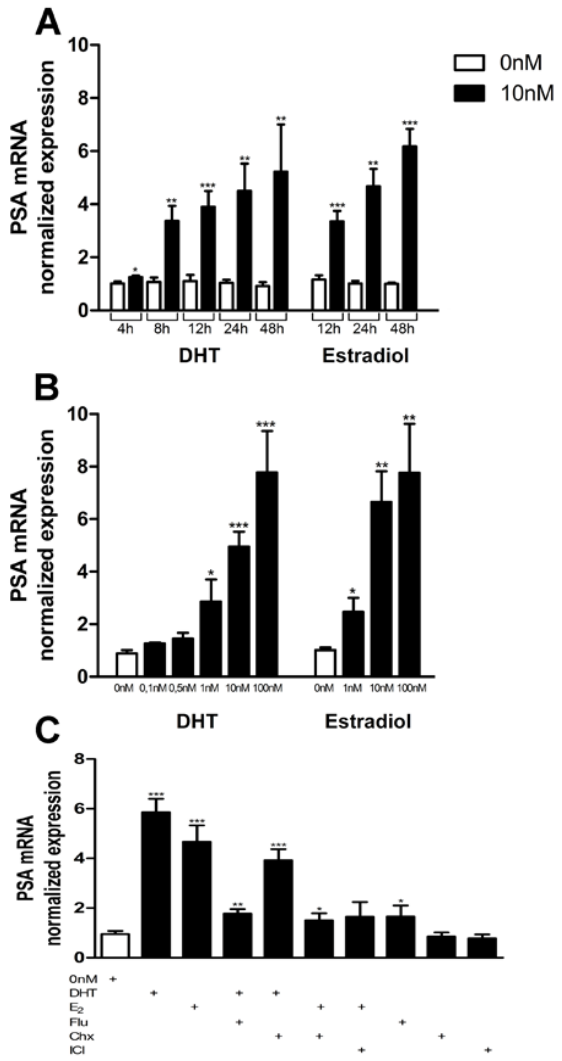
Supplemental Figure



Supp Fig 1. Effect of DHT in PSA mRNA expression was determined in LNCaP cells by real-time PCR. A- Time-course; B- Dose-dependence; C- Effect of flutamide (1µM), cycloheximide (1µg/µl), and ICI 182,780 (100nM) on DHT and E_2 regulation of STEAP1 mRNA expression at 24h of stimulation. STEAP1 mRNA expression was normalized with hGAPDH and β2M expression. Error bars indicate mean ±SEM of n=6. *p<0.05; **p<0.01; ***p<0.001 (One way Anova followed Bonferroni test) compared to control.

CHAPTER V

Expression of STEAP1 and STEAP1B in prostate cell lines, and the putative regulation of STEAP1 by post-transcriptional and post-translational mechanisms

Original paper 3

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Expression of STEAP1 and STEAP1B in prostate cell lines, and the putative regulation of STEAP1 by post-transcriptional and post-translational mechanisms

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ABSTRACT

STEAP1 gene is overexpressed in several kinds of tumors, particularly in prostate cancer. Besides STEAP1, there is another related gene, STEAP1B, which may encode two different transcripts. Although several studies have been pointing STEAP1 as a putative immunotherapeutic target and biomarker, the mechanisms underlying its regulation are not fully understood. *In silico* analysis allowed us to show that STEAP1 and STEAP1B share high homology, but with slight differences at structural level. Experiments with prostate cells showed that STEAP1B2 is overexpressed in cancer cells. Regarding STEAP1 regulation, it is demonstrated that the stability of mRNA and protein is higher in LNCaP than in PNT1A cells. Of note, serum triggered opposite effects in LNCaP and PNT1A in relation to STEAP1 stability, e.g., increasing it in PNT1A and decreasing in LNCaP. These results suggest that STEAP1 may be regulated by post-transcriptional and post-translational modifications (PTM), which may differ between non-neoplastic and neoplastic cells. These PTM are supported through *in silico* analysis, where several modifications such as N-glycosylation, N-Glycation, Phosphorylation and O-linked β-N-acetylglucosamine, may occur in STEAP1 protein. In conclusion, these data indicate that STEAP1B2 is overexpressed in neoplastic cells, and PTM may be involved in regulation of STEAP1 expression in prostate cells.

INTRODUCTION

The six transmembrane epithelial antigen of the prostate 1 (STEAP1) is overexpressed in prostate cancer, and other malignant tumors with slightly less intensity [1-4]. Regarding normal tissues, STEAP1 expression is almost restricted to prostatic cells preferentially located on the plasma membrane of epithelial cells, particularly on cell-cell junctions, and to a lesser extend dispersed on the cytoplasm [2, 3, 5]. Although its precise cellular function is still not fully understood, STEAP1 appears to function as a transporter or ion channel, taking part on inter- and intracellular communication, possibly regulating cell proliferation and invasiveness [1-3, 6]. Several studies have been pointed out the STEAP1 as a potential immunotherapeutic target as well as a biomarker, emphasizing its clinical relevance [7-11]. Besides STEAP1 gene, another related gene, STEAP1B, is encoded by the human genome. This gene is located at a different arm of the same chromosome as STEAP1, and may originate two different transcripts, STEAP1B1 and STEAP1B2. However, its expression in prostate cells is unstudied. Recently, our research group has demonstrated that STEAP1 is regulated by androgens and estrogens on the LNCaP cell line [5]. However, the mechanisms underlying STEAP1 over-expression on prostate cancer remain to be elucidated. It is well known that the regulation of gene expression could occur not only at transcription level but also at post-transcriptional and post-translational levels. In fact, events like cell-cycle progression, signal transduction and apoptosis, which are closely associated with oncogenesis, are influenced by mRNA stability as well as the rate of protein degradation [12-17]. It has been reported that tumors may activate endogenous mechanisms to increase mRNA stability of genes encoding oncogenes, cytokines, growth factors, and enzymes, leading to an

enhanced protein over-expression, consequently raising cell growth and inflammatory processes known to be involved in the onset and progression of carcinogenesis [18-23]. Post-translational modifications (PTM) are intrinsically involved on regulating protein function, and therefore, are crucial for a variety of cellular processes, such as transcription, replication, cell cycle, apoptosis and cell signaling [24, 25]. Overall, we aimed to conduct an extensive *in silico* analysis of STEAP1 and STEAP1B, and to evaluate STEAP1 and STEAP1B expression in human prostate cell lines. In addition, the putative post-transcriptional and PTM modifications are evaluated through STEAP1 mRNA and protein stability, supplemented by a post-translational *in silico* analysis.

RESULTS

STEAP1 and STEAP1B gene share high homology and are differentially expressed in human prostate cell lines

A detailed *in silico* analysis allowed to compare the genomic organization of STEAP1 and STEAP1B genes. STEAP1 gene is found close to the telomeric region on chromosome 7q21.13, encoding a transcript with 1.3 Kb, which originates a 339 aa mature protein (39.72KDa), with six predicted transmembranar regions, connected by three extracellular and two intracellular loops, and both COOH and NH_2 intracellular terminal. The transmembrane domains are thought to be located between 73-95, 117-139, 164-182, 218-240, 252-274, 289-311 of the aa sequence (www.ncbi.nlm.nih.gov/protein/NP_036581.1; http://www.cbs.dtu.dk/services/TMHMM/) . STEAP1B gene is localized on chromosome 7p15.3 and may originate two different transcripts, namely STEAP1B1 and STEAP1B2 (Figure 1). In comparison to the STEAP1 gene, STEAP1B1 has an additional exon, and a very large intron 4, with 53809bp on STEAP1B1 and 72728bp on STEAP1B2. STEAP1B1 is the longer transcript spanning approximately 1.3 Kb, and may encode the longer isoform with 342 aa (39.547KDa), containing four potential transmembranar regions between aa 117-139, 163-182, 218-240 and 250-267, two intracellular and two extracellular loops, and COOH and NH_2 intracellular terminal regions (www.ncbi.nlm.nih.gov/protein/NP_001157932.1; http://www.cbs.dtu.dk/services/TMHMM/). STEAP1B2 transcript has approximately 1.2Kb, and may encode a protein with 245 aa (28.684 KDa) with three potential transmembranar regions situated between aa 98-120, 144-163 and 199-221, one intracellular and one extracellular loops, an intracellular NH_2 termini and an extracellular COOH region (www.ncbi.nlm.nih.gov/protein/NP_997225.1; http://www.cbs.dtu.dk/services/TMHMM/) (Figure 1). STEAP1B2 uses an alternate in-frame splice site in the 5' coding region and an alternate 3' exon with a distinct 3' coding region and 3' UTR, compared to variant 1. The resulting isoform lacks an internal segment near the N-terminus and has a shorter and distinct C-terminus when compared to isoform 1 (http://www.ncbi.nlm.nih.gov/gene?term=STEAP1B). . This detailed analysis reveals that STEAP1 and STEAP1B1 isoforms share 89% and STEAP1 and STEAP1B2 91% of homology (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

STEAP1, STEAP1B1 and STEAP1B2 mRNA are differentially expressed in prostate cells lines. On the non-neoplastic prostate cells, PNT1A and PNT2, STEAP1, STEAP1B1 and STEAP1B2 mRNAs have little to no expression. On the other hand, on the malignant prostate cells, LNCaP and PC3, STEAP1 and STEAP1B2 are highly expressed, particularly STEAP1 (Figure 2A). STEAP1B1 mRNA is mainly expressed on PNT2 and PC3 cells, and under-expressed on LNCaP cells. The expression of STEAP1 protein was evaluated by western blot analysis (Figure 2B). Two immunoreactive bands could be identified, one of 30 KDa on PNT1A and other of 36 KDa on LNCaP cells. As seen in Figure 2B, STEAP1 protein is highly expressed on LNCaP cells, followed by PNT1A, PC3 and PNT2 with no expression.

Stability of STEAP1 mRNA and protein in human prostate cell lines

To evaluate the hypothesis that post-transcriptional and PTM mechanisms could be involved in the differential expression of STEAP1 between non-neoplastic and neoplastic cells, PNT1A and LNCaP cells were chosen to determine STEAP1 mRNA and protein stability through qPCR and western blot analysis, respectively. Cells were primarily cultured on CM, and STEAP1 mRNA stability was established by assessing the relative decay rates of STEAP1 after Act D treatment at 0, 1, 2, 4, 8 and 12h (Figure 3A). STEAP1 protein stability was determined after inhibiting protein synthesis with Chx for 0, 1, 2, 4, 6, 8 and 16h (Figure 3B). On PNT1A cells, the approximate half-life of STEAP1 mRNA levels is 4h, whereas on LNCaP is about 10h. Comparing the decaying mRNA levels of both cell lines, it is noticed that STEAP1 mRNA is more stable on LNCaP cells than on PNT1A cells. Regarding to STEAP1 protein expression after exposure to Chx, the approximate half-life of STEAP1 protein on PNT1A cells is about 6h, whereas the levels on LNCaP are still high even after 16h of treatment with Chx. In accordance to the mRNA data, it is clear that STEAP1 protein is also more stable on LNCaP than on PNT1A cells. However, no relationship seems to occur between the decay rates of mRNA and protein, namely on LNCaP cells.

It is well known that FBS (serum) contains hormones

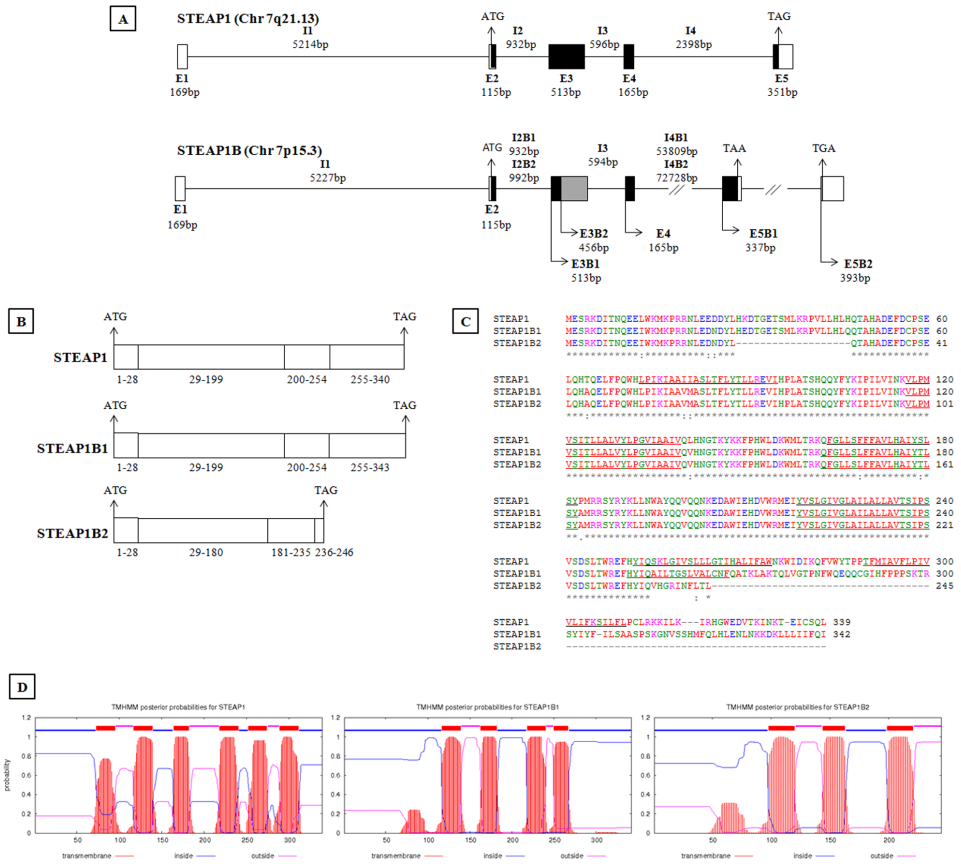

Figure 1: In silico analysis of human STEAP1 and STEAP1B gene. Genomic organization (A) and transcripts (B) resulting from STEAP1 and STEAP1B gene. Exons (E), Introns (I) and their molecular sizes in bp (base pairs) are indicated. The sequence ATG and TAG/ TAA corresponds to initiation and STOP codons, respectively. White boxes indicate non-coding exons, and black or grey boxes represent regions of coding exons depending on transcript encoded by STEAP1B gene. C- Alignment of amino acids sequences of STEAP1 and putative STEAP1B isoforms. The underlined amino acids sequences correspond to predicted transmembrane regions. * indicate identical amino acids among STEAP1s proteins; ":" indicate different amino acids but with similar physical-chemistry properties. D- Prediction of transmembrane helices in STEAP1, STEAP1B1 and STEAP1B2 proteins. All sequences were retrieved from http://genome.ucsc.edu/ and the alignment was carried out using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The prediction of transmembrane helices was performed resorting to Center for Biological Sequence analysis (http://www.cbs.dtu.dk/services/TMHMM/).

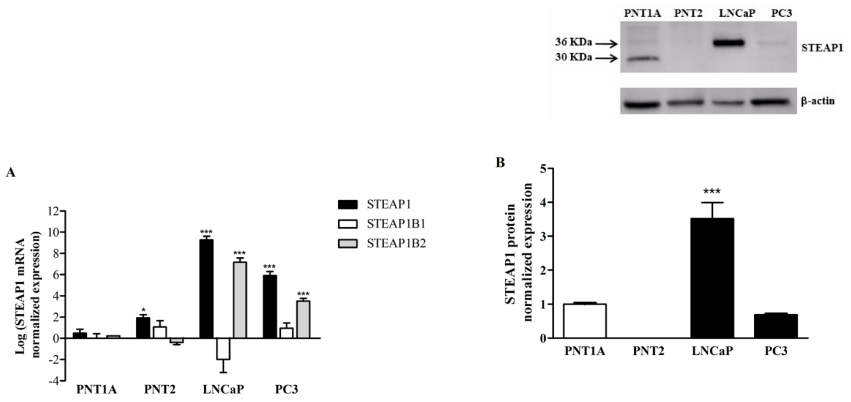

Figure 2: Differential expression of STEAP1, STEAP1B1 and STEAP1B2 on prostate cell lines. A. mRNA expression of STEAP1 and its isoforms were determined by qPCR. B. STEAP1 protein expression was determined by Western blot. mRNA and protein expression was normalized with hGAPDH and β-actin, respectively. Error bars indicate mean ± SEM of n=6. *p<0.05, ***p<0.001 (One-way ANOVA followed by Bonferroni test) compared with PNT1A expression.

and growth factors important to cell homeostasis. Therefore, the effect of serum on STEAP1 mRNA and protein stability was investigated. In the presence of FBS, STEAP1 mRNA expression increased 2 fold after 8h of stimuli on PNT1A cells (Figure 4A, upper left), whereas on LNCaP cells STEAP1 mRNA expression decreased almost 2 fold after 12h of stimuli with FBS (Figure 4B, lower left). The effect of FBS in STEAP1 protein levels at these time points was in accordance to STEAP1 mRNA, i.e., increased at 8h on PNT1A (Figure 4A right upper) and decrease after 12h on LNCaP cells (Figure 4B, lower right). These results show a clear opposite response to hormones and growth factors, which are present in FBS, in non-neoplastic and neoplastic prostate cell lines.

In silico analysis of post-translational modifications in STEAP1 protein

In an attempt to uncover the causes behind the above mentioned differences on STEAP1 protein stability, a closer insight into PTM was achieved through an extensive *in silico* analysis. Alterations such as N-glycosylation, glycation, phosphorylation sites and O-β-GlcNAc anchor sites on STEAP1 aa sequences were investigated. As seen on Figure 5A, two N-X-S/T consensus sequences [32] were found on STEAP1 sequence, but a single potential N-glycosylation site was identified at position 143, corresponding to the asparagine aa. Although another asparagine was signaled, the potential score was below the threshold line. Potential glycation sites occur in several lysines (9/24) of STEAP1 protein. These potentially glycated aa are located at positions 5, 15, 17, 30, 108, 148, 149, 156 and 162 (Figure 5B). It is well known that three distinct aa (serine, threonine and tyrosine) have the ability to be phosphorylated. According to the results retrieved from NetPhos 2.0 Server, four serines (positions 3, 187, 240 and 244), two threonines (positions 160 and 246) and four tyrosines (positions 27, 147, 219 and 252) are potentially phosphorylated on STEAP1 (Figure 5C). Several kinase enzymes with the potential to

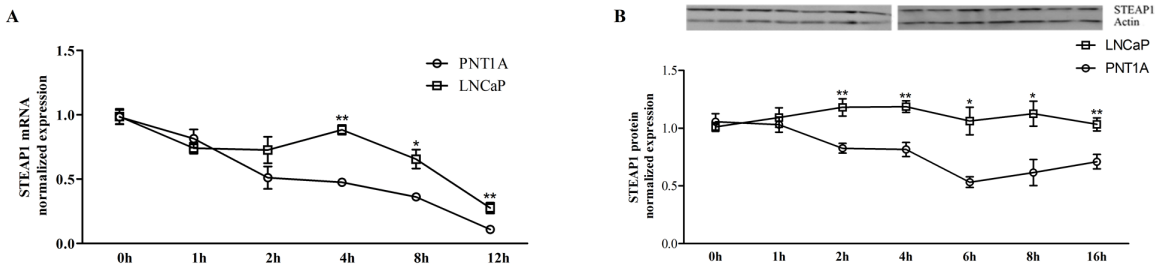

Figure 3: STEAP1 mRNA and protein stability in human prostate cell lines. Comparison between PNT1A and LNCaP STEAP1 mRNA (A) and protein (B) stability in complete culture medium. mRNA and protein expressions were determined by qPCR and Western blot, respectively. mRNA and protein expression was normalized with hGAPDH and β-actin, respectively. Error bars indicate mean ± SEM of n=6. *p<0.05, p**<0.01 (t-test) comparing PNT1A and LNCaP STEAP1 expression at each indicated time.

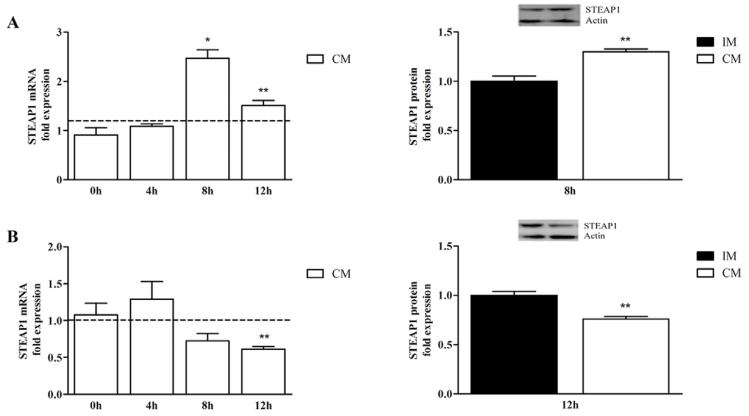

Figure 4: Effect of serum on PNT1A (A- upper left and right) and LNCaP (B- lower left and right) STEAP1 mRNA and protein stability, after treatment with Act D and Chx, respectively. mRNA and protein fold variation relatively to incomplete medium (IM) were determined by qPCR and Western blot, respectively. mRNA and protein expression was normalized with hGAPDH and with β-actin, respectively. Error bars indicate mean ± SEM of n=6. *p<0.05, **p<0.01 (t- test) comparing (IM) and complete medium (CM) STEAP1 expression at each indicated time.

Table 1: Sequences, amplicons sizes and annealing temperatures for the different STEAP1 mRNA and the internal controls used for quantitative Real-Time PCR.

Oligo name	Sequence (5´-3´)	Amplicon Size (bp)	Anneling temperature
hSTEAP1_619fw	GGC GAT CCT ACA GAT ACA AGT TGC	128	60ºC
hSTEAP1_747rv	CCA ATC CCA CAA TTC CCA GAG AC		
hSTEAP1B1fw	CTGGAAGCCTGGTAGCTTTG	162	
hSTEAP1B1rv	GGCTGGCTGCTGATAAAATG		
hSTEAP1B2fw	CGATTATTTGCAAACAGCCC	173	
hSTEAP1B2rv	GGGAAGTTGCTAAAGGGTGA		
hGAPDH_74fw	CGC CCG CAG CCG ACA CAT C	75	
hGAPDH_149rv	CGC CCA ATA CAA TCC G		

phosphorylate different aa on STEAP1 sequence were also identified. As seen on Table 2, the threonine at position 160 presents the highest score to be phosphorylated by protein kinase C. GlcNAcylation usually occurs in parallel with phosphorylation, and the two are frequently mutually exclusive [33, 34]. In fact, the potential O-β-GlcNAc anchor sites can be found on threonines 236 and 333, as well as on serines 237 and 242 of the STEAP1 aa sequence (Figure 5D).

DISCUSSION

STEAP1 is mainly expressed in normal and malignant prostate and the pursuit for understanding how its expression is regulated and modulated by internal and external factors is fundamental. Recently, Grunewald and colleagues described the existence of a STEAP1 homolog gene, named STEAP1B [35]. STEAP1 and STEAP1B genes are located on chromosome 7, but on different arms, i.e., STEAP1 gene is located at the long arm (7q21.13) and STEAP1B on the short arm (7p15.3). Considering the high homology between STEAP1 and STEAP1B genes, it is possible that gene duplication may have occurred during genome evolution. STEAP1B gene gives rise to two transcripts, STEAP1B1 and STEAP1B2, which share several similarities to STEAP1, but may encode two different mature proteins of 332 aa and 245 aa, respectively. Although the expression of STEAP1B at protein level still needs to be verified, these putative proteins also display transmembrane regions like STEAP1, but in a smaller number, suggesting that different roles could be played by these isoforms in comparison to STEAP1 protein. There are some common features present on the structure of STEAP family of proteins, such as the presence of an N-terminal with homology to the archaeal and bacterial $F_{420}H_2$:$NADP^+$ oxidoreductase (FNO)–binding proteins and human NADPH-oxidoreductases, two conserved histidines that bind an intramembranar heme group known as the ACRATA domain, the presence of the Rossman fold within the N-terminal and the strikingly similarity between the C-terminal domain to the yeast FRE cytochrome b metalloreductase domain, indicating their possible role as oxidoreductases [36-39]. Both STEAP1B isoforms, lack the FNO-like domain, the Rossman fold and the NADPH-oxidoreductase domain, as STEAP1 [35, 36]. In addition, STEAP1B2 appears to be

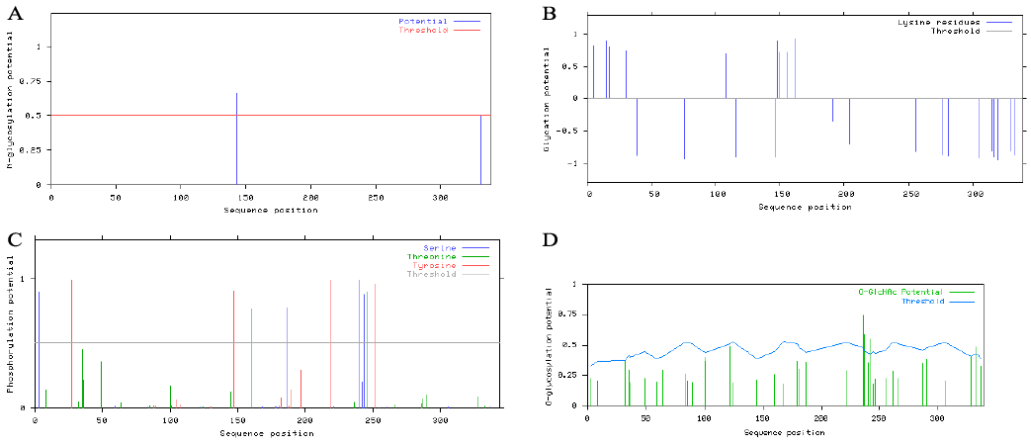

Figure 5: Prediction of N-glycosylation, glycation, phosphorylation and O-β-GlcNAc sites of STEAP1 using: A. NetNGlyc 1.0, B- NetGlycate 1.0, C. NetPhos 2.0 and D- YinOyang 1.2, respectively.

Table 2: Putative Kinases enzymes involved on STEAP1 phosphorylation. Prediction was performed using NetPhosK 1.0 server.

Site	Kinase	Score
S-3	PKC	0,67
T-8	CKII	0,57
Y-27	EGFR	0,58
Y-27	INSR	0,55
T-64	DNAPK	0,59
T-64	ATM	0,60
S-83	DNAPK	0,55
S-83	PKA	0,74
T-85	PKC	0,64
T-100	PKC	0,67
S-122	PKA	0,67
T-145	PKC	0,59
T-160	PKC	0,91
S-179	PKC	0,59
S-181	PKC	0,68
S-187	PKC	0,87
S-187	PKA	0,58
Y-197	EGFR	0,60
S-221	PKA	0,55
T-246	CKII	0,53
T-246	PKC	0,86
S-255	PKC	0,78
S-255	Cdc2	0,56
S-261	PKA	0,68
T-287	Cdk5	0,52
S-306	PKA	0,63
T-333	PKC	0,63
S-337	DNAPK	0,58
S-337	ATM	0,64

also missing the heme-binding sites, making it unfit to act as a ferric oxidoreductase [35].

STEAP1 mRNA overexpression is well described on LNCaP and PC3 cells [2]. We demonstrated that STEAP1B1 and STEAP1B2 mRNAs are also expressed on prostate cell lines. Alike in STEAP1, STEAP1B2 mRNA is also overexpressed in neoplastic cells when compared to non-neoplastic cells, suggesting that also STEAP1B2 may be dysregulated in cancer and demonstrating its potential application as a biomarker. STEAP1B1 mRNA can only be found in diminished levels on PNT2 and PC3 cells, and little or no expression on the LNCaP and PNT1A cells. These results seem to suggest that STEAP1B1 mRNA is not differentially expressed between normal and prostate cancer. However, it is needed to carry out studies using human prostate cancer cases to evaluate the expression profile of STEAP1B1 and STEAP1B2, as well as to clarify their clinical significance.

A strong immunoreactive band of approximately 36 KDa was detected on LNCaP cells, which must correspond to STEAP1 protein, as described by others [1, 2, 6]. Interestingly, an immunoreactive band of 30 KDa was obtained on PNT1A cells. The different molecular weight of STEAP1 between LNCaP and PNT1A may result from different PTM on protein, as suggested by our results and discussed below. It is also possible that the immunoreactive band of 30 KDa may correspond to STEAP1B2, because this is the predicted molecular weight of STEAP1B2 isoform. Nonetheless, further analysis using specific antibodies against STEAP1B1 and STEAP1B2 are required to determine their expression.

Numerous studies have described that STEAP1 is overexpressed in several kinds of tumors, but the mechanisms underlying its overexpression remain to be clarified. With this work we also attempted to unveil if STEAP1 expression would be regulated by post-transcriptional and PTM. Considering that both mRNA and protein stability display an important role at cellular level, in regulation of gene induction/expression, proliferation, cell signaling and apoptosis [12-16, 40], the STEAP1 stability in non-neoplastic and neoplastic prostate cell lines was evaluated. mRNA half-life and turnover are dependent on development stage or environmental factors, such cytokines, hormones, among others, decreasing its rate of transcription [40, 41]. The regulation of mRNA stability is intrinsically associated to the regulation of protein production [40]. The most stable mRNA and proteins are usually associated with vital processes such as translation, metabolic pathways and respiratory machinery [42]. According to the collected data, STEAP1 mRNA and protein are less stable in the PNT1A cells in comparison to the highly stable STEAP1 mRNA and protein on LNCaP cells. The enhancement of STEAP1 stability in LNCaP cells suggests that post-transcriptional and PTM may differ between non-neoplastic and neoplastic cells, contributing for STEAP1 overexpression in cancer cells. The evaluation of the effect of serum on STEAP1 stability shows that serum has opposite effects on STEAP1 stability, increasing it on PNT1A and decreasing on LNCaP cells. Recently, our research group demonstrated that treatment with serum or DHT down-regulates STEAP1 expression through androgen receptor (AR) [5]. Considering that DHT increases AR mRNA stability and consequently the levels of AR protein, it is liable to speculate that DHT present on serum may contribute to decreased STEAP1 stability [43]. However, other factors may be involved in stability of STEAP1.

The opposite effect of serum in STEAP1 stability on PNT1A cells reinforce that the mechanisms involved in regulation of STEAP1 may differ between non-neoplastic and neoplastic cells. Furthermore, the increased stability of both mRNA and protein on LNCaP cells is in accordance that stable mRNAs allow a wider translational timeframe for genes that are expressed at high levels [44].

There are several factors that could control mRNA

and protein turnover on cells, and in an attempt to determine possible PTM's in STEAP1 protein, *in silico* analysis was carried out. Four different types of PTM's were found to be likely to occur in STEAP1 protein, namely N-glycosylation, glycation, phosphorylation and O-β-GlcNAcylation. STEAP1 protein is therefore potentially subjected to multisite modification, a phenomenon that modulates protein function by "loss-of-function" and/or "gain-of-function" mechanisms as a result of interaction between the different modifications [25]. It is well documented that these types of modifications tend to confer higher stability to proteins and are often implicated on the etiology and pathogenesis of several diseases, including cancer [25, 45-50]. Furthermore, certain PTM's may even be used as diagnostic targets and are known to enhance tumor cell proliferation and invasion, as in prostate cancer [51-53]. Further analysis is required to ascertain if these particular PTM's are occurring on cells, and if they occur, it is crucial to evaluate the role of these modifications in carcinogenesis.

In conclusion, STEAP1B transcripts have similar structural features to STEAP1, but may encode proteins with less transmembrane domains. STEAP1B2 transcript is also overexpressed on neoplastic prostate, making it worth to evaluate its potential as cancer biomarker. For the first time, we demonstrated that STEAP1 expression and regulation could also be under the control of several PTM. Clarifying the regulation of STEAP1 as well as the expression and function of STEAP1B on cells may open novel strategies for diagnosis and treatment of prostate cancer.

MATERIAL AND METHODS

Cell culture and treatment

Human prostate cell lines (PNT1A, PNT2, LNCaP and PC3) were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). All cell lines were grown in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 10% FBS (Biochrom AG, Berlin, Germany) and 1% penicillin/streptomycin (Invitrogen, NY, USA), in a humidified chamber at 37ºC and a 5% CO_2 atmosphere. Determination of STEAP1, STEAP1B1 and STEAP1B2 expression was determined through the collection of cells from three different passages. Evaluation of mRNA and protein stability was achieved with treatment with inhibitors of transcription [actinomycin D (Act D)], and translation [cycloheximide (Chx)], respectively. Two different approaches were carried out in order to evaluate STEAP1 mRNA and protein stability in cells: a) LNCaP and PNT1A cells were grown up to 60% confluence in Complete Medium [(CM) RPMI 1640, 10% FBS and1% penicillin/streptomycin]. This medium was then replaced by CM supplemented with Act D 1μg/mL, or Chx 1μg/mL. Cells were harvested after 0h, 1h, 2h, 4h, 8h and 12h to assess mRNA stability, and after 0h, 1h, 2h, 4h, 8h and 16h for protein stability; b) LNCaP and PNT1A cells were grown up to 60% in Incomplete Medium [(IM) without serum]. This medium was posteriorly supplanted by CM or IM supplemented with Act D 1μg/mL, or Chx 1μg/mL. Cells were harvested after 0h, 4h, 8h and 12h for mRNA stability, and after 8h and 12h for protein stability in PNT1A and LNCaP cells, respectively.

Total RNA extraction and cDNA synthesis

Total RNA from human prostate cell lines was obtained using TRI reagent (Ambion, UK) according to the manufacturer's instructions. Total RNA integrity and quantification were assessed by agarose gel electrophoresis and measuring 260 and 280ηm absorvances on a nanospectrometer (Pharmacia Biotech, Ultraspec 3000). cDNA synthesis was carried out using NZY First-Strand cDNA Synthesis Kit (Nzytech, Lisboa, Portugal) according to protocol.

Total Protein Extraction

PNT1A, PNT2, LNCaP and PC3 cells were lysed on an appropriated volume of RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris, 1 mM EDTA) supplemented with 1% protease cocktail and 10% PMSF. The total protein extract (supernatant) was obtained after centrifugation the cell lysate for 20min at 12000 rpm and 4ºC. Quantification of the total protein bulk was measured using the Bradford method (Biorad, CA, USA).

Quantitative Real-Time PCR analysis

Quantitative real-time PCR (qPCR) was used to determine the expression of STEAP1, STEAP1B1 and STEAP1B2 in prostate cell lines and to establish PNT1A and LNCaP half-life of STEAP1 mRNA. qPCR reactions were performed on IQ5 Multicolor qPCR Detection System (Bio-Rad, Hercules, USA) using Maxima™ SYBR Green/Fluorescein qPCR Master Mix (Thermo Scientific, Vilnius, Lithuania). The efficiency of qPCR was determined for all designated primers (Table 1) with serial dilutions (1:1; 1:10; 1:100; 1:1,000) of the cDNA. qPCR reactions were performed using 1μl of cDNA in a 20μl reaction containing 10μl SYBR Green and 300nM of specific primers. After an initial denaturation at 95 ºC for 5min, 35 cycles were carried out as follows: denaturation at 95 ºC for 30s, annealing temperature for 30s and polymerization at 72 ºC for 20s. The amplified PCR

fragments were analyzed by melting curves: reactions were heated from 55 to 95ºC with 10s holds at each temperature (0.05ºC/s). Fold differences were calculated following the mathematical model proposed by Pfaffl [26].

Western blot

Approximately 80µg of total protein from PNT1A, PNT2, PC3 and LNCaP cells were used to assess the STEAP1 protein expression on the four cell lines, and to determine protein stability. The protocol followed as previously been described by us [27]. Briefly, LNCaP and PNT1A protein extracts were resolved on 12% SDS-PAGE electrophoresis gel and then transferred into a PVDF membrane (GE Healthecare, UK). After blockage with 3% casein solution, membranes were incubated with STEAP1 rabbit polyclonal antibody against humanSTEAP1 (H105) diluted 1:300 (Santa Cruz Biotechnology, Santa Cruz). Membranes were then incubated with an alkaline phosphatase conjugated goat polyclonal antibody against rabbit IgG (GE Healthcare, UK). Finally, STEAP1 immunoreactivity was visualized using Molecular Imager FX (Biorad, Hercules) after a brief incubation with ECF substract. STEAP1 and β-actin expression levels were quantified by densitometry using Quantity One Software (Biorad). β-actin was used to normalize STEAP1 expression.

Bioinformatic analysis

mRNA and protein sequences of STEAP1, STEAP1B1 and STEAP1B2 were retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). . Alignment of amino acid (aa) sequences was performed using Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/). To determine protein homology, the online Basic Local Alignment Search Tool program (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used. Prediction of putative transmembranar domains was determined using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM). Prediction of amino acid post-translational alterations such as N-glycosylation, N-Glycation, Phosphorylation and Phosphokinase specific sites, and addition sites of O-linked β-N-acetylglucosamine (O-β-GlcNAc) were carried out using several tools available on Expasy ((http://www.expasy.org/proteomics/post-translational_modification), NetNGlyc 1.0, NetGlycate 1.0 [28], NetPhos 2.0 [29], NetPhosK 1.0 [30] and YinOYang 1.2 [31] software's, respectively.

Statistical analysis

Data from all experiences are shown as mean ± SEM of n=6. The statistical significance of STEAP1, STEAP1B1 and STEAP1B2 was assessed using One-way ANOVA followed by Bonferroni test. mRNA and protein stability experiments statistical data was obtained comparing PNT1A and LNCaP STEAP1 expression at each indicated time, using t-test.

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CHAPTER VI

Effect of STEAP1 gene knockdown in cell cycle and apoptosis of human LNCaP prostate cells

Paper in Preparation

Chapter VI

Effect of STEAP1 gene knockdown in cell cycle and apoptosis of human LNCaP prostate cells

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Abstract

Six transmembrane epithelial antigen of the prostate 1 (STEAP1) is overexpressed in numerous types of tumors, especially in prostate cancer. STEAP1 is located in the plasma membrane of epithelial cells, and several studies suggest STEAP1 as a potential biomarker and an immunotherapeutic target for prostate cancer. Recent studies have demonstrated that STEAP1 gene is down-regulated by 5α-dihydrotestosterone (DHT) in LNCaP cells and may play an important role in inter- and intracellular communication. However, the role of STEAP1 in cell proliferation and apoptosis remains unclear. Therefore, the role of STEAP1 in prostate cancer cells proliferation and apoptosis was determined by inducing STEAP1 gene knockdown in LNCaP cells. Our results demonstrate that STEAP1 gene silencing reduces LNCaP cell viability and proliferation, at the same time that induces apoptosis. In addition, the effects of STEAP1 knockdown are not reverted by DHT treatment. Overall, we showed that the effect of DHT may be dependent of STEAP1 levels in LNCaP cells, and blocking STEAP1 may reveal to be an appropriate strategy to activate apoptosis in cancer cells, as well as to prevent the proliferative effects of DHT in prostate cancer.

Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second most common cause of cancer related death in men in the Western world [1]. The relationship between hormones and the pathogenesis of PCa has been extensively studied. Androgens are known as essential hormones for the normal prostate development and growth, as well as playing an important role in prostate cancer development [2, 3].

The six transmembrane epithelial antigen of the prostate 1 (STEAP1) is up-regulated in prostate cancer and in other types of cancer, but with less magnitude [4-8]. On the other hand, STEAP1 expression in normal tissues is almost exclusively restricted to the prostate, and it appears to be a very promising biomarker and immunotherapeutic target [5, 9-13]. STEAP1 is mainly located in the plasma membrane of epithelial cells, but it can also be found dispersed in the cytoplasm [5, 6, 8]. STEAP1 role in prostate carcinogenesis and its regulation in prostate cells remains elusive. So far, it has been demonstrated that zoledronic acid inhibits STEAP1 mRNA expression in PCa cells, and that 17β-estradiol (E_2) produces the same inhibitory effect in normal breast and cancer cells [6, 14]. Recently, our research group has also identified 5α-dihydrotestosterone (DHT) and E_2 as down-regulators of STEAP1 expression in LNCaP cells, suggesting that STEAP1 may influence prostate cancer progression into an

androgen-dependent manner [8]. The main cellular functions attributed to STEAP1 are that it may act as transporter or ion channel, participating in both inter- and intracellular communication [4-6, 15].

However, a few functional studies have been performed so far, and considering that STEAP1 is over-expressed in prostate cancer and down-regulated by DHT in LNCaP cells, we aimed to investigate the oncogenic potential of STEAP1. By inducing STEAP1 gene knockdown, we analyzed the effects of STEAP1 in cell proliferation and apoptosis, as well as the effect of DHT in STEAP1-knockdown LNCaP cells. Herein, we provided evidence that STEAP1 is involved in prostate cancer cell survival, and its expression may influence the role of DHT in cell proliferation.

Material and methods

Cell culture and transfection

LNCaP prostate cancer cell line was purchased from the European Collection of Cell Cultures (ECACC, UK) and maintained in RPMI 1640 medium (Gibco, Scotland) supplemented with 10% fetal bovine serum (FBS) (Biochrom AG, Germany) and 1% penicillin/streptomycin (Invitrogen, USA), in a humidified chamber at 37ºC and a 5% CO_2 atmosphere. STEAP1 gene knockdown was performed by seeding LNCaP cells at 40% confluence in six plate multiwells. Firstly, cells were transfected using 20nM, 50nM and 100nM of a small interfering RNA (siRNA) targeting the STEAP1 (s25634) (Ambion, USA) and 5 μL of lipofectamine 2000 (Invitrogen, USA) for 24h in Opti-MEM medium (Invitrogen, USA), as recommended by the manufacturer. As control for STEAP1 specific targeting, a Scramble siRNA sequence (AM4635) (Ambion, USA) was used. STEAP1 knockdown expression was analyzed by quantitative Real Time PCR (qPCR). In order to evaluate the STEAP1 knockdown at protein level, LNCaP cells were transfected with 50nM of siRNA for 24h, after which the medium was replaced to complete medium. Cells were harvested at 0, 12, 24 and 48h. STEAP1 protein expression was analyzed by western blot.

Experimental design

After cell transfection with siRNA, the medium was changed by phenol red free RPMI 1640 medium supplemented with 5% Charcoal-stripped FBS (CS-FBS) (Gibco, USA), which was changed after 24h by the same medium containing 0nM (control) or 10nM DHT. Cells were harvested after 48h for posterior cell cycle analysis by flow cytometry, or seeded in coverslips

for TUNEL assay. For MTS assay, prior to hormonal privation with CS-FBS, $1x10^3$ LNCaP cells per well were seeded in 96 multiwell plates.

Total RNA extraction and cDNA synthesis

Total RNA from LNCaP cells was obtained using TRI reagent (Ambion, UK) according to the manufacturer's instructions. Total RNA integrity and quantification were assessed by agarose electrophoresis and measuring 260 and 280 ηm absorvances on a nanospectrometer (Pharmacia Biotech, Ultraspec 3000). cDNA synthesis was carried out using NZY First-Strand cDNA Synthesis Kit (Nzytech, Portugal) according to protocol.

Total Protein Extraction

LNCaP cells were lysed on an appropriated volume of RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris, 1 mM EDTA) supplemented with 1% protease cocktail and 10% PMSF. The total protein extract was obtained after centrifugation of the cell lysate for 20min at 12000 rpm and 4ºC. Quantification of the total protein bulk was measured using the Bradford method (Biorad, USA).

Quantitative Real-time PCR analysis (qPCR)

qPCR was used to determine the expression of STEAP1 and percentage of STEAP1 gene knockdown. qPCR reactions were performed on IQ5 Multicolor qPCR Detection System (Bio-Rad, Hercules, USA) using Maxima™ SYBR Green/Fluorescein qPCR Master Mix (Thermo Scientific, Vilnius, Lithuania). The efficiency of qPCR was determined for all designated primers with serial dilutions (1:1; 1:10; 1:100; 1:1,000) of the cDNA. qPCR reactions were performed using 1µl of cDNA in a 20µl reaction containing 10µl SYBR Green and 300nM of specific primers (Table 1). After an initial denaturation at 95ºC for 5min, 35 cycles were carried out as follows: denaturation at 95ºC for 30s, annealing temperature for 30s and polymerization at 72ºC for 20s. The amplified PCR fragments were analyzed by melting curves: reactions were heated from 55 to 95ºC with 10s holds at each temperature (0.05ºC/s). Human glyceraldehyde 3-phosphate dehydrogenase (hGAPDH) and beta-2-microglobulin (hβ2M) were used to normalize STEAP1 gene expression. Fold differences were calculated following the mathematical model proposed by Pfaffl [16].

Table 1. Sequences and Resulting Amplicon Sizes of the Specific Primers and Cycling Conditions Used in qPCR for Amplification of Human STEAP1,GAPDH and β2M.

Oligo name	Sequence (5´-3´)	Amplicon size (bp)	Anneling Temperature (ºC)
hSTEAP 619	GGC GAT CCT ACA GAT ACA AGT TGC	128	60ºC
hSTEAP 747	CCA ATC CCA CAA TTC CCA GAG AC		
hGAPDH 74	CGC CCG CAG CCG ACA CAT C	75	
hGAPDH 149	CGC CCA ATA CGA CCA AAT CCG		
hβ2M 347	ATG AGT ATG CCT GCC GTG TG	92	
hβ2M439	CAA ACC TCC ATG ATG CTG CTT AC		

Western blot

Approximately 50μg of total protein from LNCaP cells was used to determine STEAP1 protein expression. Protocol was followed as previously described by us [8]. Briefly, proteins were resolved on 12% SDS-PAGE electrophoresis gel and then transferred into a PVDF membrane (GE Healthecare, UK). After blockage with 3% casein solution, membranes were incubated with STEAP1 rabbit polyclonal antibody against human STEAP1 (H105) diluted 1:300 (Santa Cruz Biotechnology, Santa Cruz). Membranes were incubated with an alkaline phosphatase conjugated goat polyclonal antibody against rabbit IgG (GE Healthcare, UK). Finally, STEAP1 immunoreactivity was visualized using Molecular Imager FX (Biorad, Hercules) after a brief incubation with ECF substrate. STEAP1 and β-actin expression levels were quantified by densitometry using Quantity One Software (Biorad). β-actin was used to normalize STEAP1 expression.

MTS assay

MTS assay (Promega, USA) was used, according to manufacturer's instructions, to evaluate the cell viability. Briefly, 20μL of MTS solution were added at the 100μL of culture media at the same time as medium containing 0nM or 10nM DHT. Incubation at 37ºC followed, and optical density was measured at 490ηm during the following 96h. Results are depicted as absorbance at 490ηm as a function of time.

Flow cytometry

Cell cycle analysis was performed using propidium iodide (PI) staining. After being harvested, cells were washed in PBS and fixed for 72h at 4ºC in isopropanol with 10% PBS. Cells were then centrifuged and incubated for 30 minutes at room temperature in a PI staining solution constituted by PBS with 1% RNAse A 10 mg/mL, 0.1% Triton X-100 and PI (Invitrogen, UK)

1mg/mL. Stained cells were analysed by flow-cytometry on a BD Accuri C6 Flow Cytometer. Samples were prepared in triplicated and a total of 10000 events were measured per sample. The percentage of cell population in various phases of cell cycle was calculated using FlowJo software.

Tunel assay

Detection and quantification of apoptosis was determined using the *In situ* cell death detection kit, TMR red (Roche, Germany), in according to manufacturer's instructions. Briefly, cells were washed in PBS, fixed with 4% PFA for 10 minutes, and then, permeabilized in 1% Triton X-100 for 5 minutes. 50μL of TUNEL reaction mixture were added to each sample for 1h at 37°C in the dark. Cells were washed in PBS and incubated for 5 minutes in Hoechst-33342 (Invitrogen, UK) 5μg/mL. Coverslips were then mounted in Dako (Invitrogen, UK) and analysed by fluorescence microscopy. Negative controls were performed by omission of the primary antibody (Data not shown). The percentage of apoptotic cells was estimated by counting the number of TUNEL positive cells and Hoechst stained nuclei in twenty randomly selected 40x magnification fields in each coverslip. The ratio between the number of TUNEL positive cells and total number was calculated.

Statistical analysis

Data from all experiences are shown as mean ± SEM of n=3. The statistical significance of all experiments was accessed using One-way ANOVA followed by Bonferroni test.

Results

STEAP1gene knockdown using a specific siRNA

In order to optimize STEAP1 knockdown, different doses of siRNA were used to transfect LNCaP cells for 24h, and the levels of STEAP1 mRNA was determined by qPCR. As seen in Figure 1A, STEAP1 gene expression was effectively down-regulated (approximately 90%, $p<0.05$) using 50nM or 100nM of STEAP1 siRNA, when compared to control. On the other hand, 20nM of siRNA had little or no effect on STEAP1 mRNA expression. To determine the time required to observe the STEAP1 down-regulation at protein level, cells were transfected with

50nM siRNA. Western blot analysis demonstrated that STEAP1 knockdown is visible after 24h (approximately 50%, $p<0.05$) of transfection when compared to control (Fig. 1B). Hence, the selected transfection conditions for experiments, (50nM STEAP1 siRNA during 24h), allowed the STEAP1 knockdown at least until 48h after transfection.

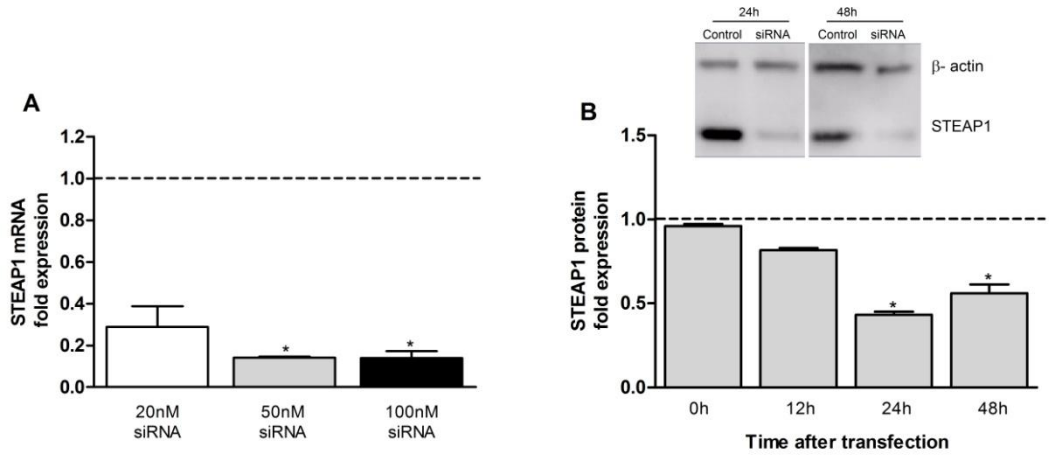

Figure 1- Analysis of STEAP1 gene knockdown by qPCR (A) and Western blot (B). mRNA expression was normalized with hGAPDH and hβ2M, and protein expression was normalized with β-actin. Error bars indicate mean ± SEM of n=3. *$p<0.05$ (One-way ANOVA followed by Bonferroni test) compared with scramble RNA treated cells (control; dashed line).

LNCaP cells viability decreases upon STEAP1 gene knockdown

Taking into account that STEAP1 is over-expressed in LNCaP cells, and DHT is a well known enhancer of LNCaP cells viability and regulates STEAP1 gene expression, it is of major importance to evaluate the role of STEAP1 in cell viability and proliferation [17, 18]. Therefore, the role of STEAP1 and DHT in cell viability was determined using a specific siRNA targeting STEAP1. As seen in Figure 2, LNCaP cells, which overexpress STEAP1, treated with 10nM DHT have an increased cell viability after 48h of treatment, reaching approximately more 50% at 72 and 96h than control (scramble siRNA 0nM DHT, $p<0.05$). When evaluating the effect of STEAP1 gene knockdown alone (0nM DHT) or in the presence of DHT (10nM DHT), a reduced cell viability is observed in both groups at all experimental periods of time, reaching approximately less 50% in comparison with control ($p<0.001$). When it is evaluated the effect of DHT in LNCaP cells with reduced levels of STEAP1, the results shows a slight more cell viability in cells treated with 10nM DHT than cells without treatment (0nM DHT). However, only at 96h is observed differences with statistical significance ($p<0.05$).

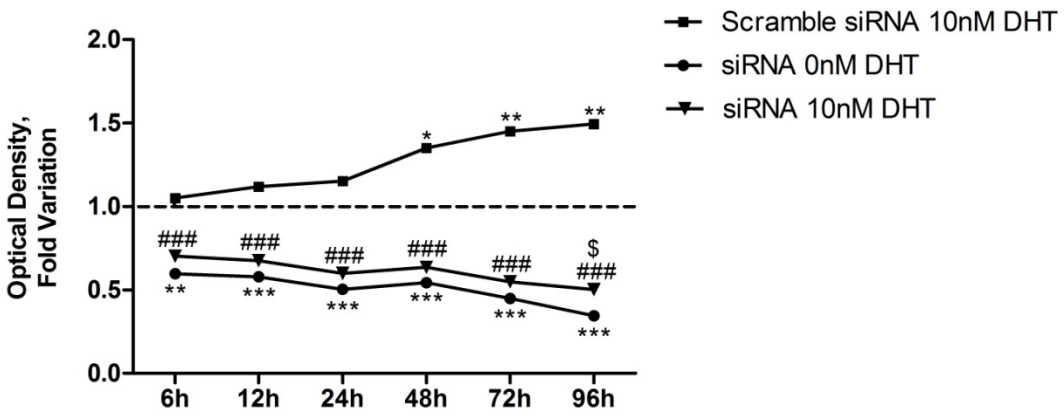

Figure 2- Analysis of cell viability by means of MTS assay. Error bars indicate mean ± SEM of n=12. *p<0.05. **p<0.01, ***p<0.001 when compared to scramble siRNA 0nM DHT treated cells (control; dashed line); ###p<0.001 when compared to scramble siRNA 10nM DHT treated cells; $p<0.05 when compared to STEAP1 siRNA 0nM DHT treated cells (One-way ANOVA followed by Bonferroni test).

STEAP1 gene knockdown induces cell cycle arrest and apoptosis in LNCaP cells, and may influence the action of DHT

Considering the results with MTS assay, it was intended to clarify the role of STEAP1 in cell cycle. Thus, PI staining was used to determine the percentage of cells in each phase of the cell cycle. As shown in Figure 3A and B, the results show a slight stimulatory effect of DHT, with a significant reduction in the % of cells in G1 and a tendency to increase in G2/M phase. Regarding the % of cells in sub-G1 and S phase, no significant differences were observed in response to DHT. The Figure 3C shows the results regarding the effect of STEAP1 gene knockdown in cell cycle. Here, it is visible that STEAP1 gene knockdown decrease the number of cells in G1 (30%) and S phase (10%) when compared to control (Fig. 3A). However, this effect is reverted by treatment with 10nM DHT in S phase, but not in G1 phase (Fig. 3D). The results also show that STEAP1 gene knockdown cells, either untreated (Fig. 3C) or treated with DHT (Fig. 3D), increase the % of cells in sub-G1 phase when compared to respective control. However, the effect of STEAP1 gene knockdown (Fig. 3C) is slightly attenuated when cells are treated with 10nM DHT (Fig. 3D).

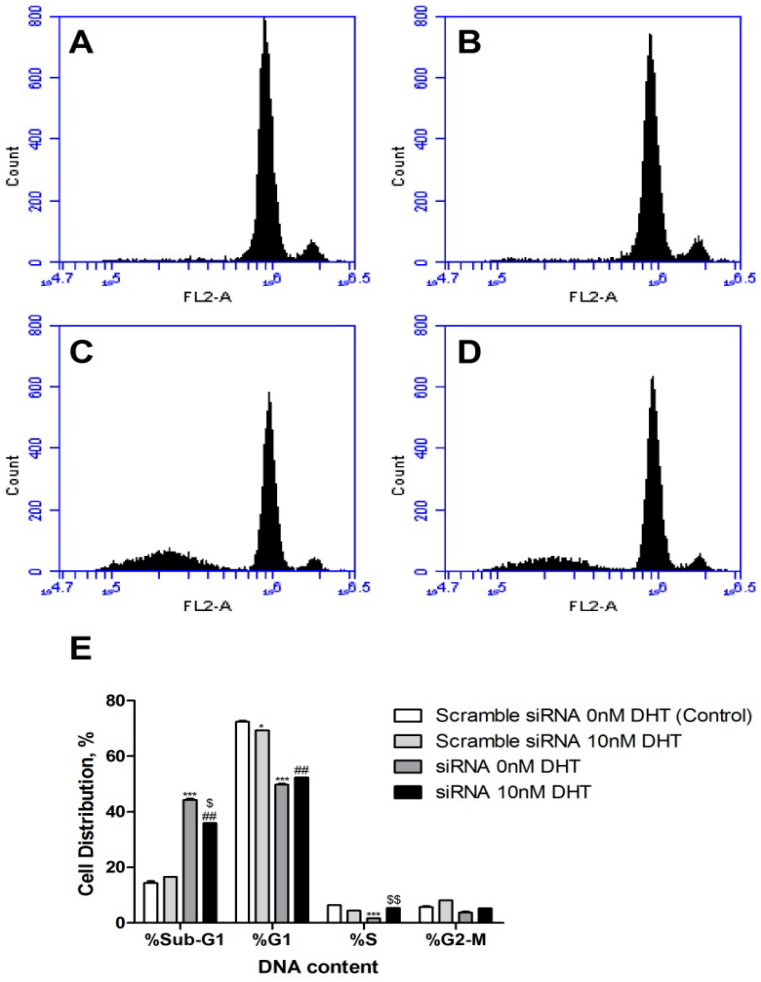

Figure 3- Analysis of cell cycle by means of flow cytometry assay, using propidium iodide. Error bars indicate mean ± SEM of n=3. Representative images of scramble siRNA 0nM DHT (A), scramble siRNA 10nM DHT treated cells (B), STEAP1 siRNA 0nM DHT treated cells (C), STEAP1 siRNA 10nM DHT treated cells (D). *p<0.05, ***p<0.001 compared with scramble siRNA 0nM DHT treated cells (control); $^{\#\#}$p<0.01 compared with scramble RNA 10nM DHT treated cells; $^{\$}$p<0.05, $^{\$\$}$p<0.01 compared with STEAP1 siRNA 0nM DHT treated cells (One-way ANOVA followed by Bonferroni test).

It is well known that cells in sub-G1 phase may indicate cells in apoptosis [19-21]. Therefore, these results suggest that STEAP1 gene knockdown may induces cell cycle arrest due to an increase in the number of cells in apoptosis. In addition, STEAP1 gene knockdown may have a suppressive effect in the stimulatory effect of DHT. In order to confirm that STEAP1 play an important role in apoptosis, TUNEL assay was performed. The number of TUNEL stained cells in relation to control is 2.2 fold increased (p<0.05) in STEAP1 knockdown LNCaP cells (Fig. 4C and E). DHT significantly reverted this effect, reducing the number of TUNEL positive cells close to 19% (Fig. 4D and E), but its stimulatory actions are still dependent on STEAP1 levels.

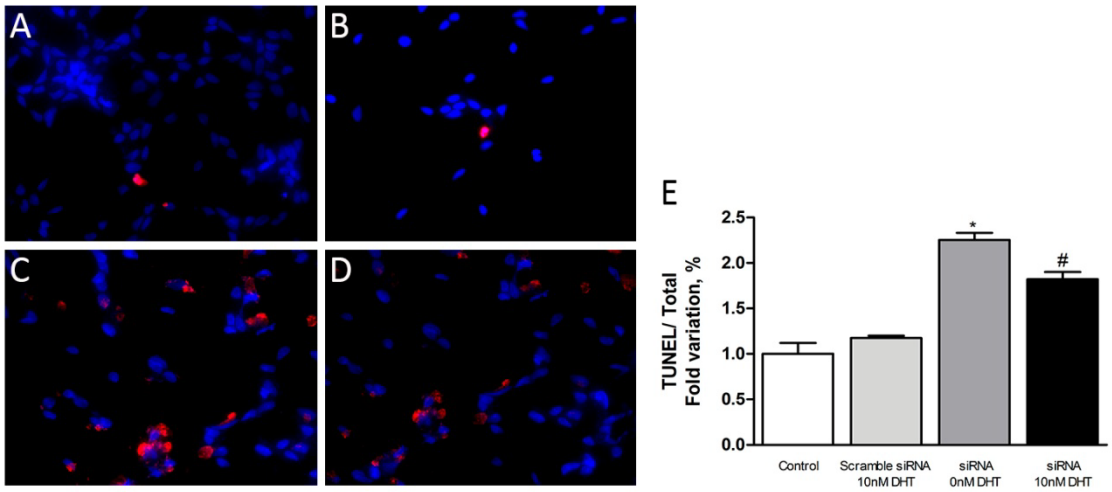

Figure 4- Detection of apoptosis based on labeling of DNA strand breaks using TUNEL assay. Representative images of merged Hoechst stained nuclei and TUNEL immunofluorescence in scramble RNA 0nM DHT treated cells (control,A), scramble RNA 10nM DHT treated cells (B), STEAP1 siRNA 0nM DHT treated cells (C), and STEAP1 siRNA 10nM DHT treated cells (D). The bar graph indicates the percentage of TUNEL positive cells relatively to total cells (E). Results are expressed as fold-variation relatively to the control group. Error bars indicate mean ± SEM (n=3). *$p<0.05$ when compared to scramble siRNA 0nM DHT treated cells (control); $^{\#}p<0.05$ when compared to scramble siRNA 10nM DHT treated cells.

Discussion

Apart from their complexity and idiopathy, most cancers share a set of capabilities that propel their development. These include self-sufficient growth, apoptosis evasion, sustained angiogenesis, unlimeted replicative potential, insensitivity to anti-growth signals and metastatic and invasion potential [22]. Understanding the mechanisms regulating PCa cellular proliferation and apoptosis would provide a basis for better managing the disease. STEAP1 is overexpressed in several types of cancer, including PCa, where we found it to be down-regulated by DHT in LNCaP cells and in rat prostate [6, 8]. The use of siRNA is now a powerful research tool for achieving gene silencing and performing functional studies [23]. To determine the role of STEAP1 in LNCaP cells viability, proliferation and apoptosis, we first induced STEAP1 gene knockdown by transfecting the cells with a specific siRNA. We confirmed that STEAP1 mRNA expression was reduced after 24h of transfection, while at protein level the reduction in STEAP1 levels is detected after 48h. The probable cause for the time-lapse needed to repress protein expression may be related to the transition period between transcription and translation, and to the high stability of STEAP1 protein in LNCaP cells [24]. Here, we showed that inducing STEAP1 knockdown expression significantly reduces LNCaP cells viability, suggesting that the communication between LNCaP cells with STEAP1 knockdown might be compromised. This is supported by a previous report which demonstrated that STEAP1 facilitates PCa cells intracellular communication and intercellular

transport *in vivo*. Furthermore, the blockage of STEAP1 using specific antibodies inhibits prostate tumor growth *in vivo* [15].

It is well characterized that DHT is involved in tumor cell proliferation, either directly acting through its cognate receptor or by activating other signaling pathways [3, 25]. Considering that DHT regulates STEAP1 expression, we aimed to evaluate the effect of DHT in LNCaP cells with STEAP1 gene knockdown. Our results demonstrated that the positive effect of DHT in cell viability is clearly blocked by STEAP1 gene silencing. On the other hand, DHT is able to increase the cell viability in STEAP1-silenced cells, but this increase is extremely low when compared to control. Taken together, our results suggest that DHT cannot reverse the effects of STEAP1 knockdown, and the DHT effect seems to be dependent of STEAP1 levels in LNCaP cells.

In an attempt to clarify the role of STEAP1 in cell cycle and apoptosis, flow cytometry was used to determine the cellular DNA content, as described by others [19]. Using this approach, we analyzed the % of LNCaP cells in each phase of the cell cycle. The results obtained are in accordance with MTS assay, where STEAP1 gene silencing also impairs LNCaP cells growth. However, the DHT-induced growth is not as visible as it is observed with MTS. This could be explained by the time point used to analyze the cell cycle phases by flow cytometry, where instead of 48h of stimuli with DHT a longer period would show an effect more pronounced. In addition, considering that the principle of MTS assay is based in a metabolic reaction it is expected that this reaction is much faster than the time required for alterations at DNA level. Nevertheless, it is clear that STEAP1 knockdown decrease the % of cells on G1 and S phases, although the effect on S phase cells could be reverted by DHT treatment. In addition, the effect of DHT on G1 phase is abolished by STEAP1 down-regulation, emphasizing that DHT-induced growth may be dependent of STEAP1 expression levels, which is in accordance to the results of MTS assays. Interestingly, it was the remarkable increase in the % of cells on sub-G1 phase, triggered by STEAP1 knockdown expression. Although the effect of STEAP1 knockdown is slightly reverted by DHT treatment, our results suggest once more that the effect of DHT may be dependent of STEAP1 levels in LNCaP cells. The population of sub-G1 cells is believed to correspond to apoptotic cells [19-21]. In order to confirm this increase in apoptotic cells, TUNEL assay was carried out as described by others [26]. Our results demonstrated that the cells on sub-G1 phase indeed correspond to apoptotic cells. Overall, we clearly evidenced that STEAP1 gene silencing induces apoptosis and decrease the growth rate in LNCaP cells. Although STEAP1 knockdown diminishes the number of proliferative cells, further functional studies are required to clarify the role of STEAP1 in cell cycle regulation. Apoptosis is a standard process intrinsic to all cells, and for decades, chemotherapeutic agents have been used to urge cell damage triggering apoptosis in cancer cells [27, 28]. Considering our results, it is possible that targeting STEAP1 may become the cancer cells more susceptible to apoptosis. In addition, it is liable to speculate that blocking STEAP1 gene may also reveal a good strategy to prevent the proliferative effects of DHT in cancer cells.

In summary, STEAP1 gene knockdown promotes apoptosis, decrease cell growth, and prevent the effects of DHT in LNCaP cells. Taken together, these results strengthen the idea that STEAP1 as a therapeutic target is a very promising strategy against hormone-dependent PCa.

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CHAPTER VII

STEAP1 is over-expressed in prostate cancer and prostatic intraepithelial neoplasia lesions, and it is positively associated with Gleason score

Original paper 4

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Original article

STEAP1 is overexpressed in prostate cancer and prostatic intraepithelial neoplasia lesions, and it is positively associated with Gleason score

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Abstract

Background: Six transmembrane epithelial antigen of the prostate 1 (STEAP1) is a transmembrane protein of epithelial cells, mostly located at cell-cell junctions, and is overexpressed in several types of tumors, particularly prostate cancer. Several studies have pointed STEAP1 as a biomarker, but the clinical significance of its overexpression is not fully understood. Therefore, we aimed to establish the association of STEAP1 immunoreactivity with histologic diagnosis and clinical data of patients.

Materials and methods: Human tissue microarrays were constructed from tissue biopsies of prostate adenocarcinoma ($n = 63$), including nonneoplastic adjacent tissue ($n = 41$), prostatic intraepithelial neoplasia (PIN) lesions ($n = 7$), and 41 prostate samples from patients diagnosed with benign prostatic hyperplasia (BPH). The histologic features of tumor specimens were classified and clinical and pathologic data were retrieved. STEAP1 expression was evaluated by immunohistochemical analysis, and a semiquantitative quantification was performed using a grade score system based on the intensity and percentage of stained cells.

Results: Overexpression of STEAP1 protein was found in both plasma membrane and cytoplasm of prostate cancer and PIN lesions when compared with nonneoplastic adjacent tissue and BPH samples. Furthermore, its expression associates positively with higher Gleason scores, but not with other clinical data, such as age, prostate-specific antigen levels, pathologic stage, and metastasis. Regarding its role as a biomarker, STEAP1 is highly liable for distinguishing malignant prostate stages from BPH. On the contrary, it lacks specificity in distinguishing PIN lesions from prostate cancer.

Conclusions: STEAP1 is consistently overexpressed in malignant prostate tissue, namely adenocarcinoma and PIN lesions. Its overexpression in PIN lesions and positive association with higher Gleason scores suggest that STEAP1 could be involved in tumor initiation and progression. The high sensitivity and specificity for detection of malignant lesions suggests that STEAP1 may be of clinical usefulness in early disease diagnosis.

Keywords: STEAP1; Prostate cancer; PIN; BPH; Biomarker; Gleason score

1. Introduction

Prostate cancer is one of the most lethal diseases among men worldwide [1]. The etiology of this disease is still far from being known [2]. Prostate pathophysiology comprises at least 3 distinct stages, starting with the appearance of benign prostatic hyperplasia (BPH), evolving to prostatic intraepithelial neoplasia (PIN), and finally to the carcinoma phase that eventually gives rise to metastasis [3,4]. The definition of an accurate distinction of prostate cancer staging is absolutely critical for an accurate diagnosis and establishment of treatment strategies. Therefore, identification and validation of novel biomarkers should be seen as a priority. The relevance of discovering molecules whose expression patterns alter owing to cancer development and progression is extremely important as it allows establishing the most effective treatment and predicting patient's outcome [5]. Six transmembrane epithelial antigen of the prostate 1 (STEAP1) is overexpressed in several cancer tissues, particularly in prostate, where its expression is higher, but with restricted expression in normal tissue

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samples [6–9]. STEAP1 is primarily localized at the plasma membrane, mostly at cell-cell junctions, but it may also be found dispersed in the cytoplasm [7,8]. Predictably acting as an ion channel or transporter protein, STEAP1 is thought to take part in intracellular and intercellular communication, modulating cancer cell proliferation, invasion, and increasing the reactive oxygen species levels [7,10–14]. Lately, several studies indicate STEAP1 as a potential biomarker and immunotherapeutic target in human tumors [15]. STEAP1 expression pattern differs significantly from prostate cancer cell lines in accordance with the cancer stage that they mimic, i.e., STEAP1 can be abundantly found on cells that mimic earlier stages of the disease rather than in those that mimic later stages [7]. Furthermore, STEAP1 messenger RNA has been detected in the serum of patients with cancer, allowing the distinction between healthy individuals and cancer-bearing patients, and its high expression in the membrane was associated with a better treatment outcome in patients with Ewing sarcoma [14,16]. However, little is known about the potential of STEAP1 as a biomarker for prostate cancer, and more studies are required to better understand the clinical significance of STEAP1 in prostate cancer. Therefore, the main goal of this work was to evaluate by immunohistochemistry the association of STEAP1 expression with the histologic diagnosis and clinical data of patients with prostate cancer.

2. Material and methods

2.1. Human samples and tissue microarray construction

The human tissue samples ($n = 104$) were provided by the pathology service of "Hospital Geral de Santo António —Porto." The patients were subjected to radical prostatectomy between 2010 and 2011 and did not receive preoperative chemotherapy or any other type of treatments. All tissue samples were obtained after informed consent from the patients and according to the local ethical committee guidelines. Among the 104 patients, 63 prostate samples were obtained from patients with prostate adenocarcinoma, including nonneoplastic adjacent tissue ($n = 41$) and PIN lesions ($n = 7$), and 41 prostate samples from patients diagnosed with BPH. The histologic features of tumor specimens were classified according to the Gleason score and using the 2010 tumor, node, and metastasis American Joint Committee on Cancer classification system [17]. The clinical and pathologic data were retrieved from the files of the pathology service of Hospital Santo António and are presented in Table 1. Some of the clinical-pathologic data were missing from patient's clinical reports, reason why they were not included.

Tissue microarrays were constructed as previously described [18]. Briefly, the predefined areas in the whole-mount prostate tissue of each patient were identified. Tissue cylinders with a core size of 2 mm and a maximum of 12 cores per case were punched from each donor tissue block and placed in a virgin paraffin block. Subsequently, sections of 4 μm in thickness were cut from the tissue microarray block and placed on positively charged slides for subsequent immunohistochemistry.

Table 1
Clinical-pathologic data of patients from whom tissue samples were obtained

Clinical-pathologic features	Prostate cancer	BPH
Patients (*n*)	63	39
Mean age, y	64 (41–83)	70 (41–88)
Total PSA, ng/ml, (mean)	8.93 (3.24–77.91)	3.73 (0.17–15.4)
Gleason (*n*), %		
<7	(16/63) 25.4	n.a.
≥ 7	(47/63) 74.6	n.a.
TNM (*n*), %		
T2a	(3/32) 9.4	n.a.
T2b and T2c	(20/32) 62.5	n.a.
T3	(9/32) 28.1	n.a.
Bone metastasis (*n*), %		
Absence	(23/25) 92	n.a.
Present	(2/25) 8	n.a.

The lack of some values is owing to the absence of some data in the clinical records of patients. n.a. = not applicable; TNM = tumor, node, and metastasis.

2.2. Immunohistochemistry

The immunohistochemical analysis was carried out on tissue microarrays of human prostate tissue samples. First, the slides were deparaffinized in xylol and rehydrated in decreasing solutions of ethanol. To block the activity of endogenous peroxidases, 200 μl of H_2O_2 (3%) was added to each slide. After 5 minutes, these were washed in phosphate buffer saline-Tween (PBS-T) for 2 minutes, and goat serum 5% (Santa Cruz Biotechnology, Santa Cruz, USA) was added to prevent any nonspecific binding and left for 30 minutes. A new wash in PBS-T was done for 5 minutes with agitation, followed by incubation with primary antibody α-STEAP1 (H-105, Santa Cruz Biotechnology, Santa Cruz, USA) diluted 1:50 in PBS with 1% bovine serum albumin for 1 hour at room temperature. The slides were washed again for 10 minutes with agitation and incubated with secondary antibody α-rabbit (diluted 1:20 in PBS with 1% bovine serum albumin) for 1 hour at room temperature. After new wash for 10 minutes in PBS-T with agitation, the slides were incubated with "ExtrAvidin-Peroxidase" for 30 minutes. Then, after washing the slides with PBS-T for 10 minutes, 100 μl of 3,3-diaminobenzidine (Sigma) was added in each slide, until the development of appropriate coloration. The slides were passed through water and immersed in Mayer hematoxylin (Richard-Allan Scientific, MI) for 1 to 2 minutes. Finally, the slides were washed with water for 10 seconds, subjected to a process of gradual dehydration, and to it 1 to 2 drops of the permanent Entellan new mounting medium were added (Merck, Darmstad,

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Germany) and covered with a cover slip. Negative controls were performed by omission of the primary antibody.

2.3. Immunohistochemical evaluation

STEAP1 immunoreactivity was assessed semiquantitatively using a grade score system, based on the intensity of staining and percentage of stained cells, as described by our research group and other authors [8,19]. Briefly, the staining intensity was classified as "0" (no staining), "1" (low staining), and "2" (high staining). The percentage of stained cells were classified as "0" (no stained cells), "1" (up to 25% of stained cells), "2" (between 25% and 50% of stained cells), and "3" (more than 50% of stained cells). Subsequently, a final score was obtained by multiplying the percentage of stained cells and intensity, originating the following scores: 0, 1, 2, 3, 4, and 6. Then, these score values were grouped into "low immunoreactivity" (scores 0 and 1), "moderate immunoreactivity" (scores 2 and 3), and "high immunoreactivity" (scores 4 and 6). When different scores were obtained in different cores of the same patient, the final score was calculated as follows: average of extension × average of intensity.

2.4. Statistical analysis

Statistical analysis was performed using SPSS16.0 software. Pearson chi-square and Fisher exact tests were used to investigate the significance of the relationship between STEAP1 immunoreactivity and individual variables of clinical-pathological data. Statistical significance was considered at $P < 0.05$.

Receiver operating characteristic curves were generated to evaluate the sensitivity and specificity of STEAP1 immunoreactivity for the diagnosis of malignant lesions and prostate cancer.

3. Results

3.1. STEAP1 protein is overexpressed in human prostate cancer and PIN lesions

To evaluate whether STEAP1 immunoreactivity differs between histologic diagnoses, several cases were analyzed using the immunochemistry method. STEAP1 immunoreactivity was classified according to a score scale established for this parameter, which takes into account the intensity of the signal and percentage of stained cells. Representative images of prostate cancer cases with positive results showing low, moderate, and high STEAP1 immunoreactivity are shown in Fig. 1. In the 104 cases investigated in this study, distinct STEAP1 immunoreactivity was identified. Representative pictures of STEAP1 staining in prostate cancer, nonneoplastic adjacent tissue, PIN lesions, and BPH are shown in Fig. 2. Strong staining was observed in the cytoplasm (Fig. 2A) and membrane (Fig. 2B and C) of prostate cancer epithelial cells. However, the final score for STEAP1 immunoreactivity in cell

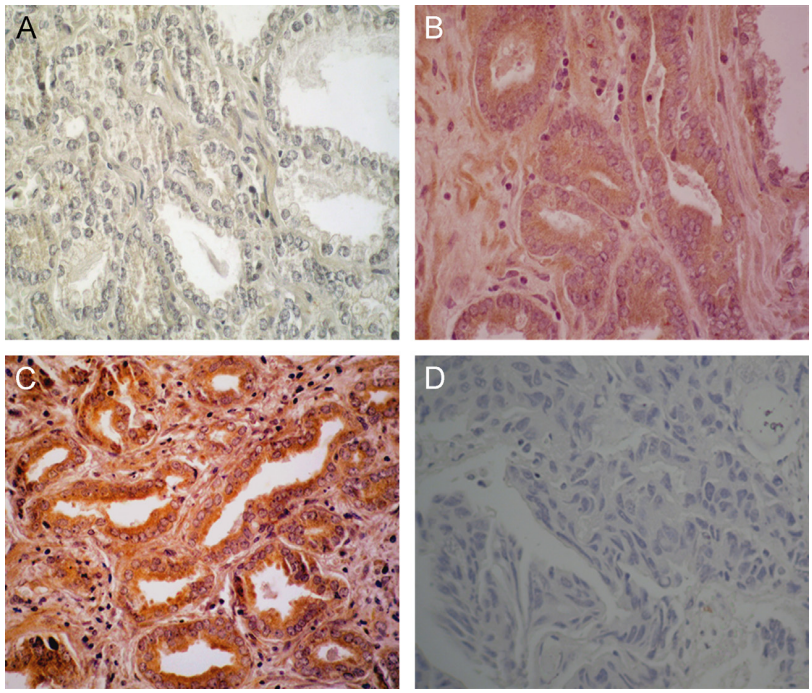

Fig. 1. STEAP1 immunoreactivity in TMAs of prostate cancer. Representative images of low (A), moderate (B), and high (C) STEAP1 immunoreactivity in prostate specimens. Negative control (without primary antibody) was done in prostate cancer (D). These images were observed in an optic microscope, at a magnification of 40×. (Color version of figure is available online.)

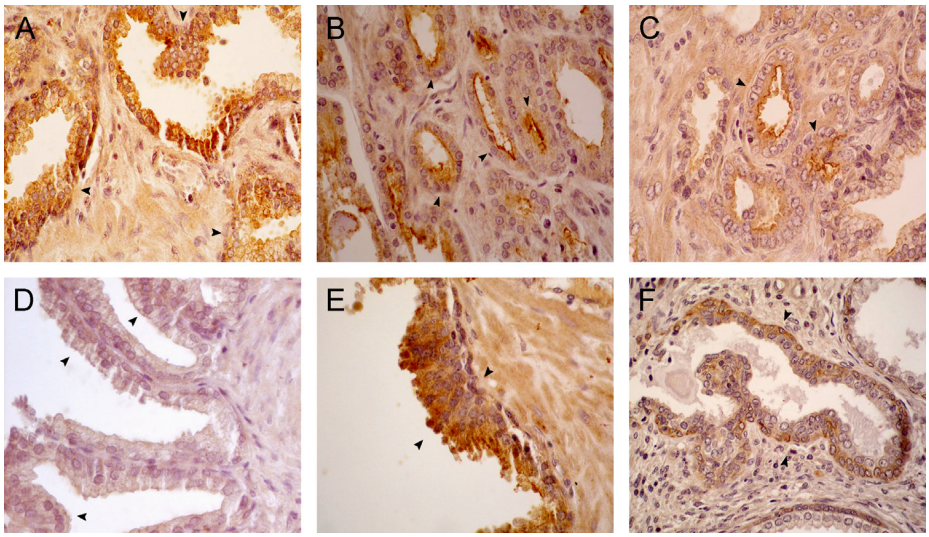

Fig. 2. Representative pictures of the immunohistochemical expression of STEAP1 in prostate cancer (A–C), nonneoplastic adjacent tissue (D), PIN lesions (E), and BPH (F). These images were observed in an optic microscope, at a magnification of 40×. (Color version of figure is available online.)

membrane is similar to that found in cytoplasm. Therefore, no statistical difference between cell membrane and cytoplasm was obtained (data not shown). Taking into account that STEAP1 is a membrane protein, we assessed STEAP1 immunoreactivity in cell membrane. In several cases, a great heterogeneity of STEAP1 staining in the same specimen of cancer was noticed, as shown in Fig. 2C. The pattern of cellular localization was similar between tumors and PIN lesions, preferentially at the apical membrane (Figs. 1C, 2A and B, 2C and E). Although only 2 cases of BPH showed positive results for STEAP1, both cases show that STEAP1 is also expressed in basal cells (Fig. 2F). In some prostate tissue samples, STEAP1 expression was also detected on stromal cells (Fig. 2A and E). The grading of 104 specimens showed that STEAP1 levels were significantly increased in malignant tissues when compared with nonmalignant tissues ($P < 0.0001$). The differences in STEAP1 immunoreactivity between prostate cancer and BPH ($P < 0.0001$), prostate cancer and nonneoplastic adjacent tissues ($P < 0.0001$), PIN and BPH ($P < 0.0001$), PIN lesions and nonneoplastic adjacent tissues ($P < 0.0001$), and BPH and nonneoplastic adjacent tissues ($P < 0.021$) were significant. On the contrary, no differences between prostate cancer and PIN lesions ($P = 0.230$) were found. As shown in Table 2, from 63 cases of prostate cancer, 56 showed moderate (35.51%) or high (52.38%) STEAP1 immunoreactivity. Besides prostate cancer, the results of STEAP1 staining in PIN lesions indicate that all cases present moderate (14.29%) or high (85.71%) immunoreactivity. In contrast, 73.17% of nonneoplastic adjacent tissue shows low STEAP1 immunoreactivity and 19.51% and 7.32% show moderate or high immunoreactivity, respectively. Regarding BPH, our results demonstrate that 39 (95.12%) of 41 present low STEAP1 immunoreactivity.

3.2. Association of STEAP1 immunoreactivity with clinical-pathological data, and its putative role as a biomarker

We further assessed the association between STEAP1 expression and clinical-pathologic data of the patients, such as age, total prostate-specific antigen (PSA), percentage of free PSA levels, Gleason score, pathologic stage, and presence of bone metastases. As shown in Table 3, there was no significant relationship between STEAP1 immunoreactivity and age, total PSA levels, percentage of free PSA, pathologic stage, or bone metastasis. The percentage of cases with high STEAP1 immunoreactivity was significantly different ($P = 0.004$) between patients with Gleason scores 5 to 6 and 7 to 9. Most of the patients with higher Gleason score (7–9) displayed high STEAP1 immunoreactivity, whereas patients with lower Gleason score (5–6) presented moderate STEAP1 immunoreactivity.

As a first approach to evaluate the potential role of STEAP1 as a biomarker for malignant lesions of prostate, sensitivity and specificity were determined (Table 4). The

Table 2
Distribution of STEAP1 immunoreactivity in prostate cancer, nonneoplastic adjacent tissue, PIN lesions, and BPH

Histologic diagnosis	STEAP1 immunoreactivity		
	Negative or low	Moderate	High
Prostate cancer	11.11% (7/63)	36.51% (23/63)	52.38% (33/63)
Nonneoplastic adjacent tissue	73.17% (30/41)	19.51% (8/41)	7.32% (3/41)
PIN	0% (0/7)	14.29% (1/7)	85.71% (6/7)
BPH	95.12% (39/41)	4.88% (2/41)	0% (0/41)

Parentheses indicate the number of cases over total number of specimens in each group.

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Table 3
Associations between STEAP1 expression in prostate cancer samples and clinicopathologic data

Variable	STEAP1 immunoreactivity			*P* value
	Low	Moderate	High	
Age, y				
<55	0% (0/9)	11.11% (1/9)	88.89% (8/9)	
55–65	9.09% (3/33)	48.48% (16/33)	42.42% (14/33)	0.076
>65	19.05% (4/21)	28.57% (6/21)	52.38% (11/21)	
Total PSA, ng/ml				
<4	16.67% (1/6)	16.67% (1/6)	66.67% (4/6)	
4–10	16% (4/25)	36% (9/25)	48% (12/25)	0.892
>10	20% (1/5)	40% (2/5)	40% (2/5)	
Percentage of free PSA, %				
<10	10% (1/10)	30% (3/10)	60% (6/10)	
10–15	0% (0/8)	37.5% (3/8)	62.5% (5/8)	0.490
15–20	25% (1/4)	50% (2/4)	25% (1/4)	
20–25	40% (2/5)	20% (1/5)	40% (2/5)	
Gleason				
<7	31.25% (5/16)	43.75% (7/16)	25% (4/16)	0.004
≥7	4.26% (2/47)	34.04% (16/47)	61.7% (29/47)	
pTNM				
T2a	33.33% (1/3)	33.33% (1/3)	33.33% (1/3)	
T2b and T2c	5% (1/20)	55% (10/20)	45% (9/20)	0.372
T3	11.11% (1/9)	22.22% (2/9)	66.67% (6/9)	
Bone metastasis				
Absence	37.21% (2/23)	32.56% (10/23)	30.23% (11/23)	0.908
Present	0% (0/2)	50% (1/2)	50% (1/2)	

TNM = tumor, node, and metastasis.

cutoff value was determined for STEAP1 immunoreactivity based on the receiver operating characteristic curve (Fig. 3). The STEAP1 immunoreactivity showed a great discriminatory power to distinguish between malignant lesions and BPH, with sensitivity and specificity of 100% and 95.1%, respectively. Regarding the STEAP1 immunoreactivity to detect prostate cancer but not PIN lesions, sensitivity and specificity of 47.6% and 85.7%, respectively, were obtained.

4. Discussion

The introduction of biomarkers as tools in oncology revolutionized this field of expertise, as robust biomarkers can indicate a patient's potential for developing the disease; can allow a better screening, diagnosis, outcome prediction, and monitoring; and may indicate the most efficient treatment strategy [20]. Owing to the high incidence of prostate cancer worldwide, there is a demand for identification of robust biomarkers. The most clinically used biomarker for prostate cancer screening has been the PSA. Its usefulness derived from the fact that elevated serum levels of PSA were a sign of the development of prostate abnormalities [21]. However, the PSA levels in serum present several limitations, such as poor sensitivity and specificity, leading to excessive false-positive diagnosis [21–25]. Regarding the usefulness of STEAP1 as a biomarker, several studies have been performed to assess its clinical significance as well as its role in prostate pathophysiology [6,14,16,26]. Taking into account that STEAP1 is mainly localized in the cell membrane, a differential expression between cell membrane and cytoplasm could be expected. However, no differences were found between STEAP1 immunoreactivity in the cytoplasm and the cell membrane of samples that showed positive results for STEAP1. Moreover, in bladder and colon cancer tissues, a light immunoreactivity of STEAP1 is found in the cytoplasm of bladder and colon cancer tissues, suggesting that STEAP1 may be associated with some intracellular organelles [7]. As previously reported, STEAP1 expression appears to be dependent on the tumor development, i.e., it is more intense in malignant tissues than in nonmalignant ones [7,16]. Accordingly, we showed that STEAP1 expression is significantly higher in PIN lesions and prostate cancer tissues than in BPH and normal adjacent tissue. This differential expression of STEAP1 may point to its putative roles in prostate cancer, enabling cellular communication and facilitating prostate cancer initiation and progression [7,9].

Although STEAP1 is overexpressed in malignant tissues, we found 2 cases of BPH with moderate STEAP1 immunoreactivity. Interestingly, although STEAP1 is mainly localized in luminal cells, in BPH, it is found in basal cells. Regarding the PIN lesions, STEAP1 is found in both luminal and basal cells. Besides the localization of STEAP1 in epithelium of prostate, some staining was also observed in stroma cells in some tissues with cancer and PIN lesions. These results may suggest that STEAP1 localization may be dependent of histologic diagnosis and could be involved in cell communication between different types of cells. In fact, several studies show that basal cells have a great potential for tumor development, and a reservoir of prostate stem cells can be found on the basal layer of prostate gland [27,28]. Taking into account that STEAP1 was previously

Table 4
Determination of sensitivity and specificity of STEAP1 immunoreactivity as a biomarker in malignant lesions of prostate

Diagnosis	AUC (95% CI)	Cutoffs	Sensitivity (95% CI)	Specificity (95% CI)
Malignant cases (n = 70) vs. BPH (n = 41)	0.989 (0.94–1.00)	Low[a]	100 (94.9–100.0)	95.1 (83.5–99.4)
Cancer (n = 63) vs. PIN lesions (n = 7)	0.675 (0.55–0.78)	Moderate[b]	47.6 (34.9-60.6)	85.7 (42.1–99.6)

AUC = area under curve; CI = confidence interval.
[a]The cutoff means that individuals with moderate or high STEAP1 immunoreactivity would be considered as having a malignant lesion.
[b]The cutoff means that individuals with low or moderate STEAP1 immunoreactivity would be considered as having a PIN lesion.

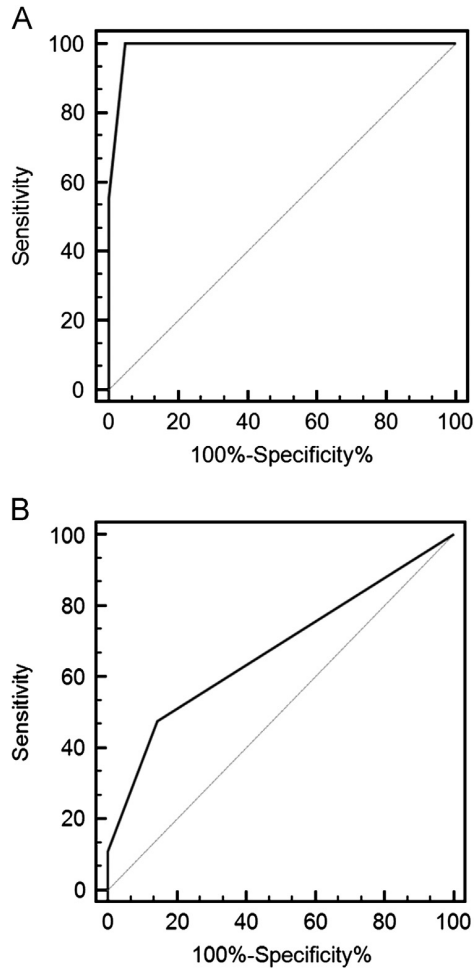

Fig. 3. Receiver operating characteristic (ROC) curves analyses for evaluating diagnostic properties of STEAP1 immunoreactivity to distinguish malignant lesions from BPH (A) and between prostate cancer and PIN lesions (B).

identified as expressed on mesenchymal stem cells from the bone marrow [29], it is liable to speculate that STEAP1 may also be expressed in prostate stem cells and could be involved in cancer development and progression.

So far, the clinical significance of STEAP1 overexpression in prostate cancer remains unclear. Although a previous report suggest that there is no difference between low-grade, high-grade, and metastatic tumors [7], the results are limited by the reduced number of samples. Nonetheless, the same authors noticed that in prostate cell lines, STEAP1 expression is increased in early prostate cancer stages (LNCaP) in opposition to advanced stages (PC3). According to the data reported here, STEAP1 immunoreactivity is positively associated with higher Gleason, providing evidence that advanced stages could be distinguished from earlier ones. These results are in accordance with those previously described in Ewing Sarcoma, where STEAP1 immunoreactivity is associated with invasive phenotype [14,26]. Interestingly, it was found that Ewing Sarcoma with high expression of STEAP1 is associated with better outcome of patients [14,26]. On the contrary, in patients with colorectal cancer, diffuse large B cell lymphoma, and acute myeloid leukemia, high STEAP1 expression is associated with lower survival rates [30]. As far as the clinical outcome is concerned, the results published in human tumors suggest a high variability between tumors. Therefore, further studies are required in patients with prostate cancer to clarify the role of STEAP1 in clinical outcome.

As a first approach to using STEAP1 as a biomarker, we determined the sensitivity and specificity of antibody anti-STEAP1 to distinguish malignant prostate tissues from BPH, which is the main nonmalignant alteration that occurs in prostate. The results of immunohistochemical staining shows that STEAP1 presents high sensitivity (100%) and specificity (95.1%) to distinguish malignant tissues (prostate cancer and PIN lesions) from BPH, suggesting that STEAP1 could be pointed as a biomarker for early detection of malignant prostate tissue. In addition, the possibility to misdiagnose BPH as prostate cancer is reduced. Regarding prostate cancer and PIN lesions, it is not possible to distinguish between them because a strong staining of STEAP1 is also observed in PIN lesions, which drastically decrease the sensitivity (47.6%) and specificity (85.7%) of this protein to detect only prostate cancer.

If on one hand the specificity of STEAP1 to detect prostate cancer is low, on the other hand, its overexpression in PIN lesions suggests that the increased levels of STEAP1 may occur before tumor initiation. Taking into account that PIN lesions are precancerous lesions, which will develop to prostate cancer, these results suggest that STEAP1 may be involved in tumor initiation and its usage in clinical practices may become useful. In conclusion, our study points to the consistent overexpression of STEAP1 in prostate cancer, and for the first time also shown in PIN lesions, suggesting that STEAP1 overexpression may occur even before cell transformation and could be involved in tumor initiation. Despite the limitations of some clinical data, it was possible to obtain a positive association between STEAP1 immunoreactivity and Gleason score, but not with other clinical variables. The assessment of STEAP1 as a biomarker, our results shows high sensitivity and specificity to distinguish malignant prostate tissues from BPH, which may help to prevent the diagnosing of BPH as prostate cancer. Although further studies are needed, STEAP1 is an interesting protein that could be used as biomarker for prostate cancer early diagnosis, monitoring, screening, and treatment outcome prediction.

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CHAPTER VIII

Conclusions and Future Perspectives

Chapter VIII

Conclusions and Future Perspectives

The prostate gland is a key structure for the male reproductive system. Its morphogenesis, development and malignant transformation are dependent of androgens and estrogens actions [1-6]. The molecular pathways involved in prostate carcinogenesis are extremely complex, and the quest for new biomarkers and immunotherapeutic targets is demanding.

STEAP1 is overexpressed in PCa and in several other types of cancer, but in normal tissues, its expression is almost restricted to the prostate [7, 8]. The regulation of STEAP1 expression is an intricate process, which may involve several molecular mechanisms yet unidentified. In this thesis, it was demonstrated for the first time that both DHT and E_2 down-regulate STEAP1 expression at mRNA and protein level. The effects were time- and dose-dependent, and it seems to require the activation of nuclear AR and a membrane ER. However, our results showed that the activation of STEAP1 gene transcription should not depend directly from an interaction between AR and promoter region of STEAP1, which is supported by *in silico* analysis. The activation of ER at the membrane level may be a result of translocation of ERs to the membrane or possibly due to activation of GPR30, a membrane receptor with estrogen affinity found in prostate epithelium [9]. Thus, studies in order to determine what kind of receptor is involved in the regulation of STEAP1 should be addressed in the future. Considering the role of STEAP1 in cell growth, it is liable to speculate that STEAP1 down-regulation may be a mechanism to protect cells from stimulatory effects of sex steroid hormones [7, 10]. Additionally, it was verified that STEAP1 overexpression in malignant cells may be tightly related to the high stability of STEAP1 mRNA and protein and the harboring of potential post-translational modification sites, which may be responsible for modulating protein activity. Although an homolog of STEAP1 gene, STEAP1B, has been previously identified, no studies have been conducted regarding the expression of STEAP1B in human prostate cells [11]. Resorting to *in silico* analysis, we demonstrated that STEAP1B isoforms, STEAP1B1 and STEAP1B2, share high structural homology with STEAP1, and that they are also differentially expressed in prostate cell lines. Although we have clarified some mechanisms underlying the regulation of STEAP1 gene expression, the main mechanism responsible for STEAP1 overexpression in PCa remains unexplained. Therefore, studies in order to identify and characterize the transcription factors involved in STEAP1 transcription as well as putative mutations should be addressed. In addition, several studies have pointed out that epigenetic deregulation can affect gene expression and play an important role in PCa [12, 13]. Recently, it was demonstrated that STEAP4, which is located on the same chromosome arm as STEAP1, is also differentially expressed in PCa and its expression is dependent of the methylation pattern of CpG islands present in the promoter region of the gene [14, 15]. Therefore, epigenetic analysis of the promoter region of STEAP1 would also be of great value to better understand the regulation of STEAP1 expression in PCa. Regarding the

putative post-translational modifications identified in this study, several experiments are required to demonstrate their existence, as well as their role in carcinogenesis. Due to its high homology to STEAP1, STEAP1B expression patterns should also be investigated *in vivo*, as well as its function in prostate carcinogenesis.

As previously mentioned, STEAP1 biological functions are still ambiguous [10, 16, 17]. Hence, by investigating the potential oncogenic effects of STEAP1 we uncovered that STEAP1 promotes an increase in cell proliferation and inhibits apoptosis in LNCaP cells. In addition, the proliferative effect of DHT in LNCaP cells seems to be dependent of STEAP1 levels. Further analysis should include the evaluation of the molecular mechanisms involved in regulation of cell cycle and apoptosis, such as the expression of cyclins, p53 and caspases. In addition, identification of proteins interacting with STEAP1 gene and protein would help to understand its role in PCa and to unveil downstream signaling pathways activated by STEAP1.

STEAP1 has been identified as a potential PCa biomarker and a strong target for cancer immunotherapy [7, 18-22]. By performing an extensive immunohistochemistry analysis of STEAP1 immunoreactivity in several human prostate tissue sections, we demonstrated that STEAP1 is persistently overexpressed in PIN lesions and adenocarcinomas, and its immunoreactivity is positively associated with higher Gleason score. Taken together, these results suggest that STEAP1 may be involved in PCa initiation and progression, as well as its potential use as a biomarker of neoplastic lesions. Nevertheless, future studies are needed to determine with more accuracy the role of STEAP1 in the clinical outcome of PCa patients. Considering that STEAP1 mRNA was previously detected in serum from patients with PCa [22], it also becomes mandatory to evaluate if STEAP1 mRNA levels in serum could be used for diagnosis and/or prognosis of PCa.

As schematized on figure 1, we propose that in androgen-dependent tumors STEAP1 expression is negatively regulated by both DHT and E_2, through activation of nuclear AR and membrane ER, respectively, possibly by decreasing STEAP1 mRNA and protein stability. Even so, the cells continue to have high levels of STEAP1, meaning that steroid hormones are not able to reduce it to the levels seen in normal prostatic tissue. This slight down-regulation may result from a negative feedback in response to DHT stimulatory effects. Although more studies are required, the high levels of STEAP1 may regulate the activity of AR. The high STEAP1 levels in hormone-dependent PCa appear to inhibit apoptosis and induce cell proliferation, which promotes tumor progression. The positive association between STEAP1 immunoreactivity and Gleason score, and its differential expression between malignant and non-malignant lesions, emphasize its potential use as a biomarker.

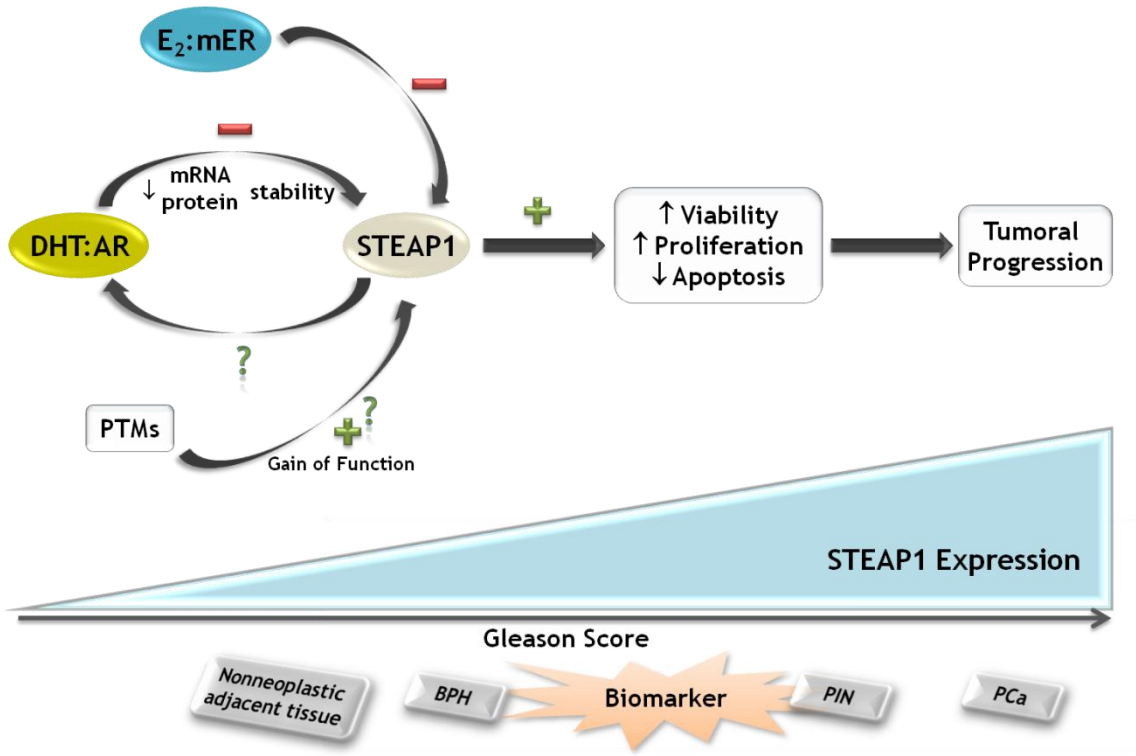

Figure 1- Schematic representation highlighting the proposed mechanisms of regulation, function and clinical significance of STEAP1 in human prostate cancer.

In summary, this thesis highlights some mechanisms underlying the regulation of STEAP1 expression in PCa, as well as its pivotal role in the initiation and/or progression of PCa. In addition, the expression pattern in PCa strengthens the potential use of STEAP1 as a biomarker and immunotherapeutic target.

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Attachments

Attachment I

Estrogens and prostate cancer: from biosynthesis to physiological effects

Original Book Chapter

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Chapter 4

Estrogens and Prostate Cancer: From Biosynthesis to Physiological Effects

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Abstract

Although the most impressive characteristic of prostate cancer is its androgen dependence, several studies support that 17β-estradiol (E_2) also play an important role in onset and progression of prostate cancer. Regarding the effects of E_2 in prostate carcinogenesis, we can highlight the carcinogenic properties of several products derived from E_2 metabolism, which play an important role on cell malignant transformation. Several polymorphisms in estrogen-related genes such as estrogen receptors (ERs) and enzymes involved in E_2 biosynthesis and metabolism have been described to favour carcinogenesis. Also, both isoforms of ERs (ERα and ERβ), which act on cells in order to maintain the normal physiology of prostate gland, are differentially expressed between neoplastic and non-neoplastic prostate cells. Therefore, this deregulation may conduct to alterations on normal gene expression in prostate cells, which may favour the progression and migration of cancer cells. On the other hand, other studies have been pointing the anti-carcinogenic activity of E_2 in prostate. These contradictory effects are based on the role of ERβ, which has been shown to exhibit anti-proliferative and anti-oxidant functions. Taking into account the scientific evidences that relate E_2 with prostate cancer, several therapeutic approaches based on ERs are being explored. This chapter summarizes the main knowledge on how E_2 may contribute to the pathophysiology of prostate gland.

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In developed countries, prostate cancer (PCa) is the most frequently diagnosed malignancy in men [1]. The aetiology of this disease is complex and remains unclear. Risk factors for a high morbidity of PCa can be classified as endogenous (family history, hormones, race, aging and oxidative stress) or exogenous (dietary factors, physical inactivity, obesity, environmental factors, occupation, smoking, sexual activity, vasectomy). However, a positive family history is the strongest epidemiological risk factor for PCa [1-3].

The relationship between hormones and the pathogenesis of PCa has been extensively studied. PCa is generally considered a paradigm of androgen-dependent tumour. However, estrogens role appears to be equally important in both normal and malignant prostate. Recent epidemiologic and experimental data have clearly pinpointed the key roles of estrogens in PCa development and progression [4, 5]. PCa risk in adulthood could be determined by exposure to estrogens during embryonic, perinatal/neonatal, or peripubertal development, a phenomenon referred to as ‘‘estrogen imprinting’’ [6]. Estrogens action should be considered both at a systemic endocrine level, because these steroid hormones are able to act through the pituitary gland to indirectly lower androgens, and locally within the prostate tissue [4, 7] . Salonia and collaborators demonstrated for the first time that the circulating 17β-estradiol (E_2, the most potent subtype of estrogens) levels are associated with a greater incidence of high grade PCa. Patients with high E_2 levels have an increased risk to develop PCa [8]. Another study also demonstrated that E_2 exposure could neoplastically transform the rat prostatic epithelial cells *in vitro* [9]. These results support the idea that estrogens have long been implicated in the prostate carcinogenesis. In addition, high estrogens levels and low testosterone levels induce the development of inflammation upon aging and the onset of premalignant lesions [10].

Understanding how estrogens are synthesized and act on prostate cells, will indubitably contribute to better understand the molecular mechanisms underlying PCa onset and progression, as well as to delineate novel strategies in diagnosis and treatment of PCa.

Biosynthesis and Metabolism of 17β-Estradiol in Prostate Cells

Estrogens are stereogenic-derived hormones which encompass natural and synthetic compounds. Natural estrogens are composed of 18-carbons steroids with an aromatic ring containing the CH_3 group in the 13^{th} carbon. These natural estrogens can be divided into three basic and biologically active subtypes: estrone (E_1), E_2 and estriol (E_3) [11].

Using cholesterol as precursor, steroidogenic enzymes are responsible for the biosynthesis of various steroid hormones (Figure 1). Some of these steroids include glucocorticoids, mineralocorticoids, progestins and androgens which will lead to the biosynthesis of estrogens. These enzymes belong to the family of cytochrome P450 (CYPs), hydroxysteroid dehydrogenases (HSDs), and steroid reductases [12].

Androgen precursors for the biosynthesis of estrogens can be derived from various pathways. 17α-hydroxylase/17,20-lyase (P450c17, CYP17) is considered a crucial enzyme in the steroidogenic pathway, possessing dual enzymatic activity, namely 17α-hydroxylase and 17,20-lyase, that converts C21-steroids (pregnenolone and progesterone) into 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, which are the precursors of cortisol

biosynthesis in the human, as well as into C19-steroids (dehydroepiandrosterone (DHEA) and 4-androstenedione) that are precursors of potent sex steroid hormones [13, 14]. Biosynthesis of estrogens precursors is achieved through the high level of circulating adrenal pro-hormones DHEA and DHEA sulphate (DHEAS) in humans. This pathway is well recognized as an additional source of active sex steroids in peripheral tissues [13-16]. Many of these steroidogenic enzymes, involved in estrogen biosynthesis and metabolism, are expressed in the human prostate [17], suggesting that E_1 and E_2 may be produced locally within the prostate via aromatization of androstenedione and testosterone, respectively, catalyzed within the prostate stroma by aromatase [18-20]. This conversion occurs due to the chemical structure of testosterone and androstenedione which allows these two precursors to act as substrates for aromatization to estrogens [20], and this process is irreversible under biological conditions [21].

Aromatase is a CYP-dependent enzyme which requires NADPH as a coenzyme. Aromatase action involves oxidation, removal of the methyl group at the carbon 19 position of testosterone or androstenedione, and removal of the hydrogen at the carbon 1β-position. For aromatase activity, three molecules of oxygen are required per molecule of steroid. Aromatase is encoded by the CYP19 gene, which encodes a protein that in many species consists of approximately 500 amino acids. The coding region of the gene spans nine exons beginning with exon II, and expression of the gene is determined by tissue-specific promoters, which lead to transcripts that are always the same regardless of the site of expression [21].

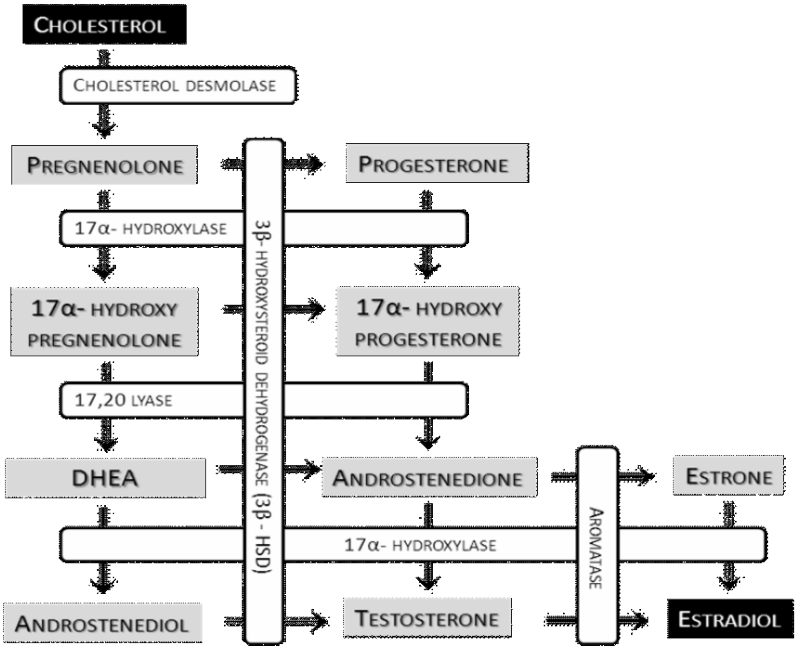

Figure 1. Biosynthesis of steroids hormones, starting with the oxidation of the side chain of cholesterol, which is catalyzed by the enzyme cholesterol desmolase. The next steps in the 17β-estradiol synthesis can proceed via different routes that are outlined in the figure. Abbreviation: DHEA – dehydroepiandrosterone.

The testis are the main source of testosterone that is delivered into all tissues of the body through the blood circulation, and it is believed that circulating testosterone is the main precursor for the biosynthesis of estrogens, namely E_2, via aromatase [22]. However, alternative pathways for the biosynthesis of E_2 have been suggested. Although aromatase uses testosterone as a substrate, it has been shown that 4-dione acts as a better substrate for this enzyme [23, 24] leading to the conversion of androstenedione to E_1, whereas reductive 17β-HSDs, namely the types 1, 7, and 12, convert E_1 to E_2 [25-27]. On the other hand, the oxidative 17β-HSDs types 2 and 4 convert E_2 into a weak estrogen, E_1. These two estrogens are biochemically interconvertable by the enzyme E_2 dehydrogenase. Although controversial, several reports [17, 28, 29] show that enzymes involved in the metabolism of estrogens are expressed in both epithelial (including basal cells) and stromal cells.

After the biosynthesis of estrogens, these can be eliminated from the body by metabolic conversion to hormonally inactive (or less active) water soluble metabolites. This metabolic deposition of estrogens includes oxidative metabolism and conjugative metabolism by glucuronidation, sulfonation and/or *O*-methylation [30]. The phase I enzymes, CYP1A1 and CYP1B1, catalyze the conversion of E_2 to 2-hydroxyestradiol and 4-hydroyestradiol, respectively. These two metabolites are also called catechol estrogens. An evidence has arisen that local E_2 metabolism may contribute to PCa and play an important role in tumour progression because some metabolites have been linked to free radical generation and direct genomic damage [31]. Studies have shown that the 2- and 4-hydroxyestradiol can be rapidly oxidized to 2,3- and 3,4-semiquinones and quinines [20, 32] , respectively, which are capable of forming DNA adducts, leading to DNA damage including single- and double stranded DNA breaks. In general, 2-hydroxyestradiol is considered less damaging, while 4-hydroxyestradiol and its respective quinine have greater toxicity. The catechol estrogens resultant from the CYP1A1 and CYP1B1 catalytic conversion are activated by conjugating reactions, such as glucuronidation and sulfonation [20]. To maintain intracellular homeostasis, the phase II enzyme, catechol-O-methyl transferase (COMT) converts these metabolites to 2- and 4-methoxyestrone, respectively, which are rapidly excreted. Importantly, while the human prostate expresses these enzymes [32], estrogens are capable of markedly increasing CYP1B1 expression in a variety of tissues, including prostate [33].

It is well characterized the association between androgens and the development of PCa, but recently, several studies have shown that aromatase expression is altered in PCa tissues [34], giving further support to a role of estrogens in this disease. Abnormal levels of E_2 and/or estrone and, especially, some of their hydroxylated derivates have also been implicated in tumour onset and progression [35]. In non-malignant prostate tissues, aromatase expression is confined to the stroma cells, while it is expressed throughout microdissected epithelial tumour cells and PCa cell lines [18].

In addition, it is important to have in consideration that polymorphisms of genes encoding the enzymes involved in estrogens biosynthesis, along with their epigenetic silencing or structural alterations may result in a profound disturbance of enzyme expression and activity. To date, at least three different polymorphisms in the CYP1A1 gene, encoding the 2-hydroxylase enzyme, have been associated with an increased risk of PCa [36]. Comparable findings were obtained for the CYP1B1 gene that encodes the 4-hydroxylase enzyme [37]. These polymorphisms usually induce a prolonged half-life and activity of both enzymes, and, hence, a sustained carcinogenic potential of their products, respectively 2- and 4-hydroxyestradiol. Moreover, a single nucleotide polymorphism (SNP) linked to the

CYP19A1 aromatase gene, is mildly through significantly associated with PCa risk, suggesting that this SNP may encode for a variant enzyme having higher activity and, hence, resulting in lower androgen levels [38].

Estrogens Receptors and Estrogens-Regulated Genes in Prostate Cells

Expression of ERs and Their Regulation in Prostate Cancer

The miscellaneous effects of estrogens in prostate may be explained by different expression patterns of estrogens receptors (ERs) in different cellular compartments of the prostate tissue. The classical ER, ERα, has been detected almost exclusively on the stroma and in subsets of basal cells [39-44]. Both in normal, high-grade prostatic intraepithelial neoplasia (HGPIN) and prostatic adenocarcinoma tissues, ERα maintains this restricted cellular expression. Interestingly, some studies show a positive correlation between ERα expression and high grade tumours (Gleason 4 and 5) as well as recurrent and metastatic tumours, leading to the assumption that the increase of receptor expression accompanies the development of PCa [45]. However, this is slightly controversial because although ERα is expressed in benign prostatic hyperplasia (BPH) and PCa cells, no clear or significant differences were found between the two pathologies, or even between hormone-responsive and refractory prostate carcinomas [46]. Epigenetic modifications have been pointed as one of the mechanisms involved in the regulation of ERα expression. It has been showed that the transcriptional activity of ERα gene is under control of methylation patterns, which is responsible to decrease ERα expression in higher grades of PCa [47].

ERβ expression, on the other hand, seems to be restricted to the luminal and basal epithelial cells, with lower or none expression in stromal cells [44, 48-51]. However, *in vitro* studies using normal and malignant prostate epithelial cell lines, show that this receptor is highly expressed [52]. In human prostate tissue specimens, ERβ expression pattern alters significantly across PCa progression, suggesting that it may have an important role on prostate carcinogenesis and metastasis. Diminished levels of ERβ have been associated with the appearance of HGPIN, and this receptor expression is progressively lost on primary PCa stages. Nonetheless, ERβ expression appears to be retained on tumours with higher rates of recurrence and regained in metastasis [44, 50, 53, 54]. The regulation of ERβ expression appears to be through epigenetic mechanisms, specifically by hypermethylation CpG islands in the ERβ promoter leading to gene silencing [52, 55]. This event seems to be in accordance with the proposed role of ERβ as a putative tumour suppressor gene [56].

Characterization and Expression of ER Splice Variants, and Their Association with Prostate Cancer Risk

There are at least five distinct mRNA isoforms of ERα, ERα-A-E, indicating that more than one promoter may control the ER transcriptional activity [57-59]. All these variants show deletions at the C-terminal ligand-binding domain (LBD), a domain essential for

receptor dimerization, and partial or complete deletion leads to absence of transcriptional activity [59]. ERα-A and ERα-B mRNA transcripts originating from the proximal promoter A and distal promoter B, respectively, have distinct 5' untranslated regions and their transcriptional activity is tissue-specific [57, 60-63]. Common to these two promoters is the absence of TATA- and CAAT-boxes consensus sequences. The ERα-C isoform, has an even different 5' untranslated exon, with specific localization to the liver [58]. All these isoforms were found to have their expression controlled by epigenetic mechanisms in the prostate, namely through methylation of the gene promoter region. Of the three isoforms, ERα-A and ERα-B expression could only be noticed upon treatment with a demethylation agent. This methylation pattern associated to each isoform suggests that each one plays a specific role in prostate carcinogenesis [64]. Likewise, all other isoforms present low expression levels in PCa cell lines. Nonetheless, this fact does not exclude the possibility that these isoforms could be key regulatory elements or interact with other protein factors, regulating gene expression patterns and hormone sensitivity in normal and malignant prostate tissues [59].

In what concerns ERβ, five different human mRNA isoforms have been identified (ERβ1-5), which either have truncation or insertion in the LBD [44, 65]. Some studies have been performed to determine the expression and role of these splice variants in prostate tissue. Initially, it was thought that all isoforms acted individually, but recent data show that they depend of each other to enhance their transactivation activities and exert their physiologic functions, although only ERβ1 displays a fully functional activity as an ER [66]. The ERβ2 isoform (ERβcx), a C-terminal truncated splice variant, has been evaluated. This isoform is inversely associated with the expression pattern of the wild type ERβ, i.e., ERβ2 has no significant immunoreactivity in benign prostate epithelial cells, but it is greatly expressed in malignant lesions. Furthermore, its expression correlates with increasing Gleason scores and poor survival, indicating that ERβ2 may potentiate PCa progression [44].

Genetic Polymorphisms of ER and Prostate Cancer Risk

The presence of polymorphisms in a gene sequence may reflect the risk of disease development. Several polymorphisms in ERα and ERβ have been identified, but not all have implicated in PCa development. One of these polymorphisms in the ERα gene, which differs significantly from healthy and tumour bearing individuals, is located in intron 1 (GGGA sequence) and it is apparently associated with increased PCa risk. The length of this repeated sequence in the ERα gene could possibly alter the ability to bind transcription factors, and therefore the receptor transcriptional activity [67]. The genotypes of ERα *PvuII* (C/C, C/T or T/T) and *XbaI* (A/A, A/G or G/G) polymorphisms in intron 2 were analyzed on a Japanese population. Of these, the T/T genotype of ERα *PvuII* increased the risk of PCa development [68]. Previously, the G/G genotype of *XbaI* polymorphism and C/C genotype of *PvuII* polymorphism were also associated with increased risk of PCa [69]. Finally, the detected genotypic alterations (C/C, T/T or T/C) in codon 10 of the first exon of ERα gene were also associated with higher susceptibility to develop PCa [70]. Regarding ERβ, several SNPs were evaluated using the population-based case-control study CAPS (CAncer Prostate in Sweden). The selected SNPs were genotyped and one SNP located in the promoter region (-13950 C/T) was found to be associated with increased PCa risk, suggesting a role of ERβ promoter in PCa aetiology [71].

Animal Models to Study Gene Expression

Several animal models have been used to conduct studies in order to understand the role of estrogens and ERs in PCa. Present data lead us to believe that estrogens have a major role in prostate development and pathophysiology. Nowadays, there are distinct animal models with altered estrogenic response, ERα knockout (αERKO), ERβ knockout (βERKO), and the double ERα and ERβ knockout (DERKO), and estrogens-deficiency, the aromatase knockout (ArKO). Each animal model displays a unique phenotype and its usage to understand the role of estrogens in prostate is of great value [72]. To what it is known, only the βERKO mice display an abnormal prostate phenotype. These mice tend to develop severe epithelial hyperplasia with increasing age and display higher expression of androgen receptor [73-75]. The characteristics of the ArKO model include augmented androgen serum levels and total absence of E_2, and have also the particularity of being enabled to develop PCa [76]. Using this model, researchers are able to determine the rate of *de novo* E_2 synthesis from testosterone, through aromatase pathway [77].

Effects of Estrogens in Cell Cycle and Invasion of Prostate Cancer

It has been established the importance of estrogens in the normal growth, differentiation and development of prostate as well as in the uncontrolled growth and transformation observed in PCa [78, 79]. Experimental animal models support the view that estrogens, alone or in combination with androgens are potent inducers of cell growth and differentiation in PCa [80, 81]. The administration of estrogens at pharmacological doses, alone or with androgens, results in the development of benign lesions, known as squamous metaplasia in the prostates of various mammalian species, including humans [82, 83]. The treatment of Noble rats with estrogens plus androgens promoted the appearance of a pre-cancerous lesion similar to PIN and a high incidence of prostatic adenocarcinomas [84, 85]. In addition, estrogens lead to progression of human PCa and metastasis at distant organs in nude mice treated with E_2 and testosterone and transplanted with a recombinant tissue composed of mouse mesenchyme and human prostatic epithelial cell line [86]. *In vitro* studies also referred that both androgen and estrogens affect proliferation of cultured PCa cells. More specifically, physiological concentrations of estrogens stimulate LNCaP cell proliferation, and may decrease in a dose-dependent manner the growth of PC3 cells [87, 88]. The mechanisms underlying the action of estrogens in PCa development involve several growth factors-dependent signalling pathways that promote aberrant cell growth, e.g., TGFα/EGF receptor [89] ; TGFβ [90]; IGF-1 and VEGF [91], ER [92], prolactin-receptor [93] and mitogen-activated protein kinase (MAPK) activation [94]. Furthermore, estrogens appear to have a stimulatory effect in the Src/Raf-1/Erks signal transducing pathway in LNCaP cells, allowing PCa cell survival [95].

Evasion of apoptosis is another mechanism of estrogens action, in which estrogens promote the overexpression of anti-apoptotic mediators, such as metallothionein and TRPM-2/clusterin [96, 97]. In addition, changes in gene-expression profiles related to cell

proliferation, DNA damage, activation of proto-oncogenes and transforming factors are well established in *in vivo* studies [98].

The effect of estrogens in controlling cell growth appears to be mainly receptor-mediated [88]. ERα and ERβ seem to exert opposite effects in prostate cell proliferation (Figure 2). ERα stimulation has been described to result in hyperplasia, inflammation and dysplasia of prostate, and ERα inhibition prevents PCa progression [99-101]. On the other hand, ERβ exerts an anti-proliferative effect in the prostate, directly or through ERα inhibition, and a loss of ERβ expression has been related with PCa progression [102]. *In vivo* studies using the βERKO mice showed that these animals develop prostatic hyperplasia with aging, which is not observed in wild-type or αERKO mice [103]. In addition, treatment of neonatal rats with diethylstilbesterol (DES) leads to prostatic hyperplasia and dysplasia, which probably results from ERα upregulation and ERβ downregulation [104]. *In vitro* studies are well correlated with these data, as in prostate cells, ERβ caused a marked reduction of proliferation [56]. This mechanism involves, besides the ER activation, the recruitment of specific co-regulators, the interaction of the complex with estrogen-responsive elements (EREs) on the DNA and subsequently the activation of target gene transcription [105]. Furthermore, ERα and ERβ can interact with other DNA binding sites, such as AP-1 and Sp-1, which may regulate gene transcription in opposite directions [105].

The estrogen-ER complex regulates a set of genes involved in cell proliferation and cell cycle progression. It has been described that estrogens may exert their mitogenic effects regulating cyclin D1 expression, which increased expression causes aberrant G1 to S transition and lead to cell cycle progression [106]. In LNCaP cells, which are androgen-dependent, it was observed that ERβ expression increases from the G1 to the S phase and decreases previously to entry in the G2/M phase, and ERβ can activate transcription via ERE during the S phase in a ligand-dependent manner. In addition, ERβ overexpression caused an arrest in early G1 with a decrease in c-Jun phosphorylation and cyclin D1 expression [107]. Others refer that estrogens induce the expression of Cyclin D1 via ERβ by increasing c-fos and c-jun expression in PC3 cells. In PCa tissues, cyclin D1 is overexpressed and it is positively correlated with ERβ expression, while ERα was almost undetectable [108]. Pravettoni et al. showed that E_2 or a ERβ selective agonist, diarylpropionitrile, decrease DU145 cell proliferation, which is accompanied by an increase of p21, a nuclear protein and a key point in control of cell cycle progression due to its effect inhibiting cyclin-dependent kinases [109]. Also, another study using a flavonoid, shows that genistein arrests the cell cycle at the G2/M phases and also suppresses cyclin B and p21, and p21 is induced via both p53-dependent and p53-independent pathways [110].

ERα and ERβ can also regulate cell proliferation through the activation of specific kinase activity, regulating EGFR and the activation of PI3K and MAPK signalling pathway, or induction of calcium influx [111-113]. Previously, we identified regucalcin (RGN), a calcium-binding protein that maintains intracellular calcium levels, as an ER regulated gene in human prostate cell line [114]. Moreover, it was observed that RGN levels are diminished in human cases of PCa [115]. These findings suggest that ER may have a role controlling PCa progression by regulating proteins, such as RGN, which are involved in maintenance of calcium homeostasis.

ERβ inhibits cell growth and invasiveness of PCa cells, and strongly stimulates apoptosis regulating the expression of Bax, Poly(ADP-ribose) polymerase and caspase-3 [56]. In addition, ERβ induces apoptosis via caspase-8 in prostatic stromal, luminal and castrate-

resistant basal epithelial cells of ArKO mice, in Gleason score 7 xenografted tissues and in PC3 and DU145 PCa cell lines [116].

The upregulation of AIB1 expression, an ER co-regulator associated with cell proliferation stimuli and cell motility and invasion, was significantly correlated with lymph node metastasis of PCa [117]. AIB1 directly regulates the transcription of matrix metalloproteinase (MMP)-2 and MMP-13 [117]. Interestingly, E_2 treatment leads to MMP2 production through stimulation of TGFβ1 expression in prostatic stromal cells which in turn promotes PCa cell invasion [118].

Although the effects of estrogens are known to be mainly mediated by ERα and ERβ, other alternative mediators may play important actions in PCa progression. ERβ2 and ERβ5 isoforms are reported to promote metastasis and their expression was associated with shorter metastasis in patients with PCa [119]. Recently, G-protein-coupled receptor-30 (GPR30) was referred to have estrogens-binding affinity, regulating cell growth [120]. Activation of GPR30 by its specific ligand G-1, inhibits PCa cell growth by activating Erk1/2 and also c-jun and c-fos, which in turn upregulates p21 that mediates G2-M arrest, suggesting GPR30 as a novel mediator of estrogenic actions in the prostate [120].

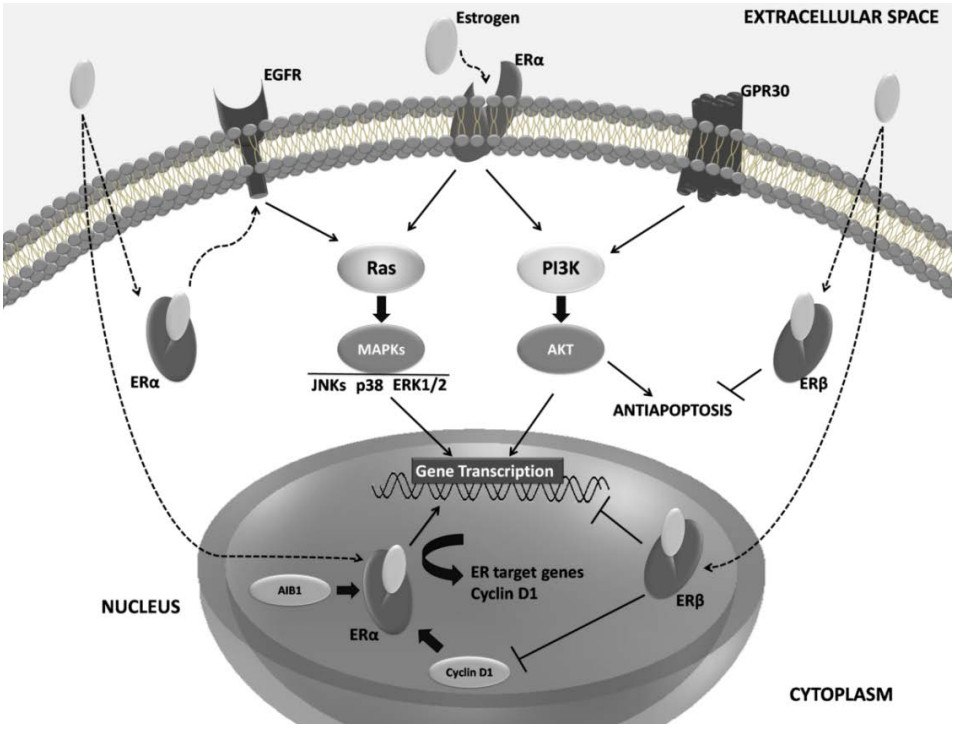

Figure 2. Schematic diagram of estrogen signalling in prostate cancer. Binding of Estrogen to the Estrogen Receptor α (ERα) promotes the translocation of this complex to the nucleus and the transcription of ER target genes along with a set of independent genes, including Cyclin D1. Estrogen-ERα complex also activates Ras-MAPK and PI3K signalling pathways, which in turn triggers cell proliferation and anti-apoptotic routes. Estrogen may stimulate the transcription of anti-apoptotic genes, via PI3K-AKT pathway, upon activation of G protein-coupled receptor 30 (GPR30). In addition, Cyclin D1 and AIB1 potentiate the transcription of ER-target genes. Contrarily, the binding of estrogen to ERβ seems to inhibit cell growth and anti-apoptotic events.

Estrogen Signalling as a Hallmark of Hormonal Therapy

The action of estrogenic therapeutic compounds has long been observed on several PCa cell lines, *in vivo* models and in clinical trials. From the two most expressed ER subtypes in prostate tissue, it is believed that only ERβ could mediate the anti-tumoural action in response to these compounds [52, 121]. Nonetheless, their action is not confined to restrain PCa development, when the tumour is only confined to the prostatic tissue, but also have anti-metastatic properties. The ultimate aim would be to prevent PCa by selectively induce apoptosis or restrain malignant cell proliferation, preferably by activation of ERβ signalling pathways avoiding undesirable side-effects. Nowadays, several new estrogenic/anti-estrogenic compounds are being studied.

Estrogens and Derivates

Estrogens-based hormonal therapy for PCa have been effectively used for over seventy years, since in 1941 Huggins et al launched the first clinical trial to ascertain the effects of endocrine manipulation with the xenoestrogen, DES, in patients with advanced and metastatic prostate adenocarcinoma [122-124]. Since then, estrogens therapy became the standard treatment for PCa, minimally evolving for half a decade. The most commonly used was in fact 1-3mg of DES, but other compounds such conjugated estrogens, poly-E_2 phosphate, ethinyl E_2, the synthetic estrogen chlorotrianisene or estramustine, a nor-nitrogen mustard carbamate derivate of E_2 phosphate were also considered. Although the majority of them presented mild to high toxicity, and the development of serious cardiovascular and thromboembolic complications, they showed relatively high efficacy on the treatment outcome, rivalling with those from surgical interventions [125]. Nonetheless, upon testing DES on a randomized controlled trial setting, its usage as a first-line medical treatment was discontinued on behalf of less toxic and equally effective agents [126]. Regardless, the European Organization for the Research and Treatment of Cancer (EORTC) established that 1mg of DES was effectively and safe enough for clinical practices [127]. Several studies using cohorts of patients with advanced PCa recognized DES as an active drug and could still be seen as a useful treatment option, particularly when palliative chemotherapy is inappropriate or the life expectancy is poor [128-130]. The first proposed mechanism of action was based on a direct action of estrogens on the prostatic epithelium through the hypothalamic-pituitary axis by a negative feedback exerted on the pituitary, diminishing the secretion of gonadotropins leading to limited testicular testosterone production [123, 131]. Briefly, the hypothalamus production of the luteinizing hormone-releasing hormone (LHRH) induces the release of luteinizing hormone (LH) from the pituitary and consequently, the production of testosterone by the Leydig cells in testis [132]. Increasing estrogens levels exert a negative feedback on this pathway, impairing the release of LH and thus decreasing serum testosterone [123]. In the case of DES, a prolonged therapeutic approach will turn into a decline of serum LH levels and serum testosterone to a castrate level, due to irreversible damage of Leydig cells [133-135]. In fact, DES anti-tumoural effects do not involve just the direct regulation of androgen levels through negative feedback on the hypothalamic-pituitary

axis, but also by induction of cytotoxicity [136]. More recently, DES was found to inhibit the activity of telomerase, synergistically with androgens in the case of the androgen-responsive LNCaP cells, disclosing a new possible therapeutic approach and showing that abolition of androgens is not always clinically beneficial [137]. Other estrogenic compounds are found to induce apoptosis through c-jun recruitment, increase the number of cells on the sub-G1 phase or by induction of death receptors, bind to tubulin causing microtubules depolymerization and impair cell cycle progression at G2/M phase [138-142].

Selective Estrogen Receptor Modulators (SERMs)

Selective ER modulators (SERMs) are synthetic compounds with weak estrogenic activity. According to the target tissue or cell, SERMs act either as agonists or antagonists, depending on the expression pattern of ERs and interaction with co-regulators that ultimately alter gene transcription [143, 144]. Different generations of SERMs have been used to suppress PCa progression, both on androgen- dependent and castration resistance stage. Pre-clinical studies using animal models revealed that SERMs such toremifene and raloxifene tend to repress the androgen-dependent development of ventral prostate and seminal vesicles, reduce the appearance of HGPIN lesions and PCa incidence, as well as improve survival rates [99, 145]. Apparently, these effects are a reflection of ERα signalling activation and do not dependent on androgen action [99]. Usually, estrogens-based therapies are thought to be mediated by direct action on the hypothalamic-pituitary axis, inhibiting proliferation due to testosterone decline and preventing epigenetic silencing of anti-tumour genes. Nonetheless, several SERMs compounds act through pathways independent of this axis [5]. Their ability to suppress PCa development engages several events that ultimately culminate on anti-proliferative and pro-apoptotic activity [5, 146]. Accordingly, some of the described mechanisms of action include induction of apoptosis through increased nuclear fragmentation levels and induction of death receptors, proliferation arrest by inhibiting protein kinase C and induction of p21 (waf1/cip1) [121, 147, 148]. The differential expression of ERs is also central for SERMs action, as multiple signalling pathways leading to cell death and proliferation arrest are activated according to the expression levels of ERα and ERβ [149]. Recently, two novel SERMs were found to selectively activate ERβ, recruitment of TGFβ1 and members of the TNF family (Fas/Fas-L), increase of bax/bcl-2 ratio, decrease of ERα, AR and EGF receptor and impairment of IGF-I signalling and PARP cleaving [150]. The Food and Drug Administration (FDA) has approved the use of several SERMs in clinics. Unfortunately, these present some limitations and further studies are needed to undoubtedly prove that SERMs are the future for PCa prevention and as substitutes to DES in terms of efficiency [77, 151-156].

Phytoestrogens

Complementary and alternative medicine (CAM) strategies have been used for quite a while as potential adjuvant or alternative therapeutics on hormonal-dependent cancers, in order to improve the tolerance and efficacy of more conventional treatments [156]. Phytoestrogens are weak natural estrogens that display a similar structure to mammalian

estrogens and can be found in a broad range of plants. These compounds are divided into three classes: isoflavones, flavones and lignans; being the isoflavones the most described and which major source is soybean, mainly in the form of genistein [155, 157]. Human diet has been pointed as an aetiological factor for PCa and the increased and widespread consumption of phytoestrogens provenient from soy appears to reduce the PCa risk [155, 158-160]. Studies suggest that genistein can activate ERβ, eliciting apoptosis or inhibiting cell proliferation and modulating genetic and epigenetic mechanisms [161-164]. Its ability to prevent PCa is believed to be achieved through downregulation of 5α-reductase, tyrosine-specific protein kinase, aromatase, and topoisomerase II activity and NF-kB activation, but also by increasing the levels of sex hormone-binding globulin, which decrease the free testosterone levels and induce G1 cell cycle arrest [155, 165-168]. Genistein induces the transcription of anti-angiogenic and anti-metastatic genes, as well as increases cell adhesion by stimulating the focal adhesion kinase pathway in PCa cells [169, 170]. Genistein is available in the market and it has been tested in clinical trials, although the results were not the most favourable [171]. Other phytoestrogens have been listed as PCa protectors, mostly by eliciting apoptosis and inhibiting proliferation [77, 172-174]. However, recent *in vivo* data produced contradictory effects of genistein compared to *in vitro*, requiring therefore a careful use [175-178]. Regardless of what is known, the use of CAMs for PCa prevention will require a more thorough control as they are usually unregulated commercially available formulations, with uncertain mechanisms of action, unconvincing safety, efficacy and effectiveness.

Conclusion

Estrogens are well known mitogenic agents whereas cancer is characterized by an uncontrolled cell proliferation and increased resistance to apoptosis. Thus, it is not surprising that several studies have pointed estrogens as players in prostate carcinogenesis. Several experimental studies showed that early exposure to estrogens predisposes individuals to later development of PCa and demonstrated the extremely genotoxic activity of several estrogens-derived metabolites. Moreover, the proliferative and anti-apoptotic role of ERα in prostate cells was defined. On the other hand, it was noticed that ERβ may act on prostate cells as an anti-proliferative agent and/or to favouring the apoptosis of PCa cells. Presently, experimentally and clinical evidences support the potential therapeutic application of estrogens-related targets which have a relevant role in prostate pathophysiology. Targeting the ERα and ERβ selectively, or in combination, with other anti-cancer molecules may represent a successful strategy as chemoprevention or treatment of PCa.

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Attachment II

STEAP proteins: From structure to applications in cancer therapy

Original Paper 1

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Molecular Cancer Research

STEAP Proteins: From Structure to Applications in Cancer Therapy

Inês M. Gomes, Cláudio J. Maia and Cecília R. Santos

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Review

STEAP Proteins: From Structure to Applications in Cancer Therapy

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Abstract

The human 6-transmembrane epithelial antigen of prostate (STEAP) family comprises STEAP1, STEAP2, STEAP3, and STEAP4. All of these proteins are unique to mammals and share an innate activity as metalloreductases, indicating their importance in metal metabolism. Overall, they participate in a wide range of biologic processes, such as molecular trafficking in the endocytic and exocytic pathways and control of cell proliferation and apoptosis. STEAP1 and STEAP2 are overexpressed in several types of human cancers, namely prostate, bladder, colon, pancreas, ovary, testis, breast, cervix, and Ewing sarcoma, but their clinical significance and role in cancer cells are not clear. Still, their localization in the cell membrane and differential expression in normal and cancer tissues make STEAP proteins potential candidates as biomarkers of several cancers, as well as potential targets for new immunotherapeutic strategies for disease attenuation or treatment. This review brings together the current knowledge about each STEAP protein, giving an overview of the roles of this family of proteins in human physiology and disease, and analyzes their potential as immunotherapeutic agents in cancer research. *Mol Cancer Res; 10(5); 573–87. ©2012 AACR.*

Introduction

The 6-transmembrane epithelial antigen of prostate (STEAP) family of proteins includes 4 members, named 6-transmembrane epithelial antigen of prostate 1 to 4 (STEAP1–STEAP4). They all have in common a 6-transmembrane domain, a COOH-terminal domain with significant homology to the yeast FRE family of b-type cytochrome metalloreductases, and an *N*-terminal with homology to the archaeal and bacterial $F_{420}H_2$:$NADP^+$ oxidoreductase (FNO)–binding proteins (Figs. 1–4; ref. 1). STEAP proteins uptake iron and copper because of 2 conserved histidine residues predicted to bind at least an intramembranar heme group (2, 3). The heme-binding 6-transmembrane domain is also present in the Nox and YedZ family, in which only 2 histidine residues are present and received the designation of apoptosis, cancer, and redox-associated transmembrane (ACRATA; ref. 4). In mammals, STEAP proteins contain an exclusive FNO-like domain, enabling them to use intracellular flavin adenine dinucleotide– or flavin mononucleotide–derivate flavins as electron donors for iron and copper reduction (1, 3). Other features common to most STEAP proteins are the presence of the YXXØ consensus sequence (in which Ø is a large hydrophobic amino acid) that is responsible for targeting transmembrane proteins to lysosomes and endosomes, and the Rossman fold (GXGXXG/A motif), a feature of proteins with oxidoreductase and dehydrogenase functions (3, 5). The first role attributed to this family of proteins was their contribution to metal homeostasis by reducing iron and copper, thereby allowing their uptake. The only exception is STEAP1, which does not reduce metals, possibly owing to the absence of the FNO-like domain and the Rossman fold. Nevertheless, the partial colocalization of STEAP1 with transferrin (Tf), transferrin receptor 1 (TfR1), and endosomes specialized in iron uptake suggest that STEAP1 may also have a role in iron metabolism (1). This review updates the available data for the STEAP proteins and discusses their role in pathophysiology, with a particular focus on carcinogenesis.

Structural Features of STEAP Genes and Proteins

STEAP1 was the first member of the STEAP family to be identified. The STEAP1 gene (Table 1) is located on chromosome 7q21.13 and comprises 10.4 kb, encompassing 5 exons and 4 introns. Transcription of the STEAP1 gene gives rise to 2 different mRNA transcripts of 1.4 kb and 4.0 kb. However, only the 1.4-kb transcript is processed into the mature protein, which contains 339 amino acids with a predicted molecular weight of 36 kilodaltons (6, 7). The 4.0-kb transcript contains a large intron of 2,399 bp, and it is not translated into a mature protein. The protein contains 6-transmembrane domains with the COOH- and *N*-terminals located in the cytosol, and 3 extracellular and 2 intracellular loops (6).

The STEAP2 gene (Table 1), also known as 6-transmembrane protein of the prostate 1 (STAMP1), is located on

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Table 1. Characterization of STEAP genes, mRNA transcripts, and proteins

STEAP gene	Chromosome location	Gene size (kb)	Exon/intron	mRNA transcripts (kb)	Amino acids	MW (kilodaltons)	Reference
STEAP 1	7q21.13	10.4	5/4	1.4 4.0	339	36	(6, 9)
STEAP 2	7q21.13	26	6/5	2.2 4 4.5 6.5 (7.5?)	490	56	(7, 8)
STEAP 3	2q14.2	42	6/5	4.3	488	50–55	(3, 9, 10)
STEAP 4	7q21.12	26	5/4	4	459	52	(9, 11)

Abbreviations: MW, molecular weight.

chromosome 7q21.13 and contains 6 exons and 5 introns, corresponding to a full-length cDNA that spans around 26 kb, owing to the large size of intron 2 (12,713 bp; refs. 7, 8). The transcription of STEAP2 seems to be TATA- or CAAT-box independent. Human tissues express 4 different mRNA transcripts, 3 of them resulting from alternative splicing of the last exon, with estimated sizes of 2.2, 4.0, 4.5, and 6.5 kb (7). However, there is some controversy about the size of the larger transcript, which has also been identified as having 7.5 kb (8). No significant rearrangements of the gene have been detected in LNCaP, PC3, and DU145 prostate cancer cell lines, but 4 sites of base variations were identified in prostate cancer cell lines and in normal prostate tissue. These cDNA variations originate from a neutral substitution from CTC to CTT with no amino acid change at codon 272 and 3 missense substitutions: TTT to TGT, leading to Phe17Cys; CGA to CAA, leading to Arg456Gln; and ATG to ATT, resulting in Met475Ile. The open reading frame of the STEAP2 gene is located within the third exon and gives rise to a 490–amino acid protein with a predicted molecular weight of 56 kilodaltons (7, 8).

STEAP3 (Table 1), also known as tumor-suppressor activated pathway-6 (TSAP6) or dudulin-2, is located on chromosome 2q14.2. Human multitissue analysis detected a single STEAP3 transcript of 4.3 kb, which originated from a 42-kb STEAP3 gene with 6 exons and 5 introns (3, 9). The promoter region of the STEAP3 gene contains functional p53-binding sites and interacts with Nix and myt1 proteins, an interaction that increases apoptosis and delays cell-cycle progression (10). STEAP3 is composed of 488 amino acids and 6-transmembrane domains at the COOH-terminal region and a cytoplasmic *N*-terminal oxireductase domain with free access to electrons transported by NAD(P)H, essential for iron and copper uptake (3, 10). The expected molecular weight of this protein is around 50 to 55 kilodaltons, with the detection of 2 isoforms in human epithelial cells from cervical carcinoma, HeLa-39, and HeLa-Tet cell lines, which overexpress STEAP3 (10).

STEAP4 (Table 1), also known as 6-transmembrane protein of prostate 2 (STAMP2), is located on chromosome 7q21, and contains 5 exons and 4 introns. Partly because of the large size of intron 1 (22,516 bp), the genomic sequence comprises around 26 kb. The gene is translated into a single mRNA transcript of 4.0 kb, with a 5′–untranslated region (UTR) of about 1.7 kb. The STEAP4 protein has 495 amino acids and 6-transmembrane regions near the COOH-terminal domain. In the *N*-terminal domain, 3 conserved motifs have been identified, corresponding to a dinucleotide-binding domain, a NADP oxireductase motif, and a motif similar to pyrroline 5-carboxylate reductase (11).

Tissue Expression and Cellular Localization

STEAP1 is overexpressed in several types of human cancer tissues and cell lines, namely prostate, bladder, colon, pancreas, ovary, testis, breast, cervix, and Ewing sarcoma (Tables 2–4; refs. 6, 12). Among normal tissues, the prostate gland is where STEAP1 expression is more abundant. Other nontumoral human tissues, such as ureter, fallopian tubes, uterus, pituitary, pancreas, stomach, colon, and breast show diffuse and low-intensity staining (6, 12). In the prostate, STEAP1 is primarily expressed in the plasma membrane of the epithelial cells, particularly at cell–cell junctions (6).

STEAP2 was identified as an upregulated gene in normal and malignant prostate cells using suppression subtraction hybridization and cDNA array hybridization (Table 3; ref. 8). Analysis of its expression in several human tissues showed that STEAP2 mRNA expression is more abundant in the prostate (Table 2; ref. 7). STEAP2 is mainly located in epithelial cells of the prostate, particularly in the plasma membrane and Golgi complex, in association with the *trans*-Golgi network (TGN) and early endosomes. Besides the prostate, its expression is also detectable in other normal human tissues, for example, heart, brain, pancreas, ovary, skeletal muscle, mammary gland, testis, uterus, kidney, lung, trachea, and liver (7, 8). In addition, in mouse embryos, STEAP2 has strong expression in the epithelium of the gastroduodenal junction, fetal liver, and in the choroid plexus (1).

STEAP3 is expressed in hematopoietic tissues (Tables 2 and 3), supporting important physiologic functions related to iron metabolism, especially in erythroid precursors. In addition, STEAP3 mRNA has been detected in the fetal liver of mouse embryos and in the bone marrow, placenta, liver,

Table 2. Expression of STEAP mRNA and proteins in normal and cancer tissues

	STEAP 1		STEAP 2		STEAP 3		STEAP 4		Reference
	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein	
Normal tissue									
Brain	+	−	++		+		−		(1, 3, 6–8, 11, 14)
Pituitary gland		+							(6)
Fetal liver	++		+		++		+		(1, 3)
Liver	++	−	+		+++		+		(1, 3, 6, 8, 10, 11, 14)
Heart	+		+		+		++		(1, 3, 7, 8, 10, 11, 14)
Trachea			+						(8)
Lungs	+	−	+		+		++		(1, 3, 6, 8, 11, 14, 15)
Thymus	+	−	+				+		(1, 6)
Lymph node		−							(6)
Bone marrow	+	−	+		++		+++		(1, 3, 6, 64)
Thyroid gland		−							(6)
Adipose tissue							+++		(14, 41)
Pancreas	+	+	++		++		++		(1, 3, 6–8, 11, 14)
Spleen	−	−	−		−		−		(3, 6, 8, 14)
Adrenal gland		−							(6)
Stomach	+	+	+				+		(1, 6)
Duodenum	+		++		+		−		(1, 3)
Small intestine			+				+		(8, 11)
Colon	+	+	+		+		−		(1, 3, 6, 8, 11)
Kidney	++	++	+		+		+		(1, 3, 7, 8, 14, 78)
Bladder	+	++							(6, 15, 78)
Breast	++	++	+						(8, 12)
Placenta	+	−	−		++		+++		(1, 3, 6, 8, 10, 11, 14)
Ovary		−	++				−		(6–8, 11)
Fallopian tubes		+							(6)
Uterus		+	+						(6, 8)
Prostate	+++	+++	+++		+		++		(1, 3, 6–8, 11, 15)
Testis		−	+				+		(6, 8, 11, 14)
Ureter		+							(6)
Skeletal muscle	+	−	+		+		+		(1, 3, 6, 8, 10, 11, 14)
Skin		−							(6)
Cancer tissue									
Liver					++	++			(69, 70)
Lungs		++							(15)
Colon		+							(6)
Kidney		++							(78)
Bladder		++							(6)
Breast		++							(12)
Prostate		+++	++		++				(6, 27, 77)
Prostate lymph node metastasis		+++							(15)
Prostate bone metastasis		+++							(15)

NOTE: Low levels, +; medium levels, ++; high levels, +++; not detectable, −.

skeletal muscle, and pancreas of adult mice. A similar distribution of STEAP3 mRNA is seen throughout human tissues, with higher expression levels in the liver and much less expression in the skeletal muscle, fetal liver, pancreas, bone marrow, placenta, and heart (3, 10). The cellular localization of STEAP3 is the plasma membrane, near the nucleus, and in vesicular tubular structures (13).

The STEAP4 gene is mainly expressed in adipose tissue, placenta, bone marrow, lung, pancreas, and heart, followed by prostate, liver, skeletal muscle, pancreas, testis, small intestine, and thymus, with no detectable expression in brain, kidney, spleen, colon, and peripheral blood leukocytes (Tables 2 and 3; refs. 1, 11, 14). The intracellular localization of STEAP4 resembles that of STEAP2; it is found in the

Table 3. Expression of STEAP mRNA and proteins in cell lines

		STEAP 1		STEAP 2		STEAP 3		STEAP 4		
Cell line		**mRNA**	**Protein**	**mRNA**	**Protein**	**mRNA**	**Protein**	**mRNA**	**Protein**	**Reference**
Hepatocellular carcinoma	HepG2							+		(11)
	TTC 1105	−								(75)
Lungs	NCI-H661							−		(11)
Melanoma	Mel 526	+								(75)
	Mel 624	+								
	Mel 888		+							(78)
Myeloid Leukemia	KCL-22	+								(6)
	LTR 6						+			(10)
Lymphoma	Jurkat		−							(78)
	BxPC-3	+	−							(6)
Pancreas	HPAC	++	−							
	Capan-1	++	−							
	Colo 205	++	+							(6)
Colon	CaCo-2	+	+							
	LoVo	++	+							
	T84	++								
	Caki-1		+							(78)
Kidney	SW839		+							
	ACHN		+							
	SMKTP3		−							
	UM-UC-3	++	++							(6, 78)
	5637	+++	++							
	EJ-1		+							(64)
Bladder	T24		+							
	TCCSUP	+								(6)
	HT1197	−								
	SCABER	+								
	J82	−								
	MCF 7[a]	+						−		(6, 11, 81)
	MCF 7-LCC1							−		(11)
	MCF 7-LCC2							−		
Breast	MDA-MB-435[a]	+						−		(6, 11, 82)
	CAMA-1[a]	−								(6, 83)
	DU4475	−								(6)
	SKBr3[a]		−							(70, 85)
	OV-1063	++	++							(6)
Ovary	PA-1	+	+							
	SW 626	++	+							(6, 75)
	CAOV-3	+								(6)
Cervix	HeLa	+	−					−		(6, 11)
	A431	+								(6)
	LNCaP[a]	+++	+++	+++		+		+		(6–8, 11, 77, 84)
	PC3[b]	+	+	+		+		−		
	DU145[b]	+	+	+		−		−		
	CA-HPV10[b]							−		(11, 39, 85)
Prostate	PZ-HPV7[b]							−		
	YPEN-1							−		(11)
	22Rv1[a]			+						(8, 86)

(Continued on the following page)

Table 3. Expression of STEAP mRNA and proteins in cell lines (Cont'd)

		STEAP 1		STEAP 2		STEAP 3		STEAP 4		
Cell line		**mRNA**	**Protein**	**mRNA**	**Protein**	**mRNA**	**Protein**	**mRNA**	**Protein**	**Reference**
	NCI-H660[b]			+						(8, 87)
	NTERA-2	+								(6)
Testis	NCCIT	−								
	TERA-1	+								
	TERA-2	+								
Ewing sarcoma	RD-ES	++	+							(6)
	A 673	++								(75)
	TC 32	++								
Myotube	C2							−		(11)

NOTE: Low levels, +; medium levels, ++; high levels, +++; not detectable, −.
[a]Androgen receptor positive.
[b]Androgen receptor negative.

plasma membrane, near the nuclear region where it colocalizes with the Golgi complex and the TGN and is dispersed in the cytoplasm as bright spots with the appearance of vesicles or tubular-shaped structures associated with vesicular tubular structures, or with reticular shapes associated with the endoplasmic reticulum (11).

Physiologic Roles, Regulation, and Implications in Cancer

STEAP1

Because of its localization on the cell membrane and its predicted secondary structure as a 6-transmembrane protein, it is believed that STEAP1 acts as an ion channel or transporter protein in tight junctions, gap junctions, or in cell adhesion, taking part in intercellular communication. As STEAP1 is overexpressed in cancer, it has been suggested that STEAP1 may facilitate cancer cell proliferation and invasion, perhaps through modulation of concentration of ions such as Na^+, K^+, and Ca^{2+} and small molecules (15–17). Higher levels of voltage-gated Na^+ channels confer a highly invasive phenotype to prostate cancer cells and the presence of these channels seems to be linked with the loss of androgen receptor expression and function and the progression to androgen-independent cell stages (17–20). In addition, modulation of Ca^{2+} and K^+ levels seems to be very important for the progression of prostate tumors toward androgen-insensitive stages, by conferring an apoptotic-resistant cellular phenotype (21–24). Therefore, the relationship between STEAP1 and ion channels should be addressed in the future. STEAP1 also plays an important role in intercellular communication. Blocking STEAP1 with specific monoclonal antibodies in LNCaP cells increases cell death, suggesting that STEAP1 may promote proliferation of cancer cells or prevent apoptosis (15). The pathways underlying these effects are still unknown and need to be studied. On the other hand, STEAP1 seems to facilitate cell growth by raising the intracellular level of reactive oxygen species (ROS), showing that STEAP1 acts both on inter- and intracellular pathways (Fig. 1; ref. 25). 17β-Estradiol seems to be the only known regulator of STEAP1 expression to date. This hormone downregulates STEAP1 expression both *in vivo* in rat mammary glands and *in vitro* in MCF-7 breast cancer cells (12).

STEAP2

STEAP2 works as a shuttle between the Golgi complex and the plasma membrane, moving in both directions, in the endocytic and exocytic pathways, suggesting that it may act as a receptor for endogenous and exogenous ligands, such as lipids and proteins, or as a regulator of protein delivery and sorting mechanisms (7). Taking into account its colocalization with Tf and TfR1, STEAP2 may have a role in the endosomal Tf cycle of erythroid cells, contributing to iron and copper uptake by reducing Fe^{3+} to Fe^{2+} and Cu^{2+} to Cu^+ (Fig. 2). STEAP2 may also regulate iron and copper availability in the choroid plexus, the site of cerebrospinal fluid synthesis and traffic control of molecules and ions between the blood and the cerebrospinal fluid, and in the gastrointestinal tract, through absorption of iron and copper by enterocytes of the proximal duodenum (1, 26).

As prostate cancer progression is androgen dependent and STEAP2 is overexpressed in prostate tissue, the involvement of androgens in the regulation of STEAP2 expression has been investigated. Both *in vivo* and *in vitro* studies suggest that STEAP2 is not an androgen-regulated gene. Androgen-dependent CWR22 tumors (derived from primary human prostate cancer) grown in mice significantly regressed following castration, without any alterations in STEAP2 mRNA expression (7). In addition, LNCaP cells cultured with dihydrotestosterone (DHT) or with a synthetic androgen R1881 did not show any significant differences in STEAP2 expression when compared with nontreated cells (7, 8). Although these results were in agreement with animal experiments, the fact that STEAP2 expression has only been assessed at a single time point of R1881 treatment and only

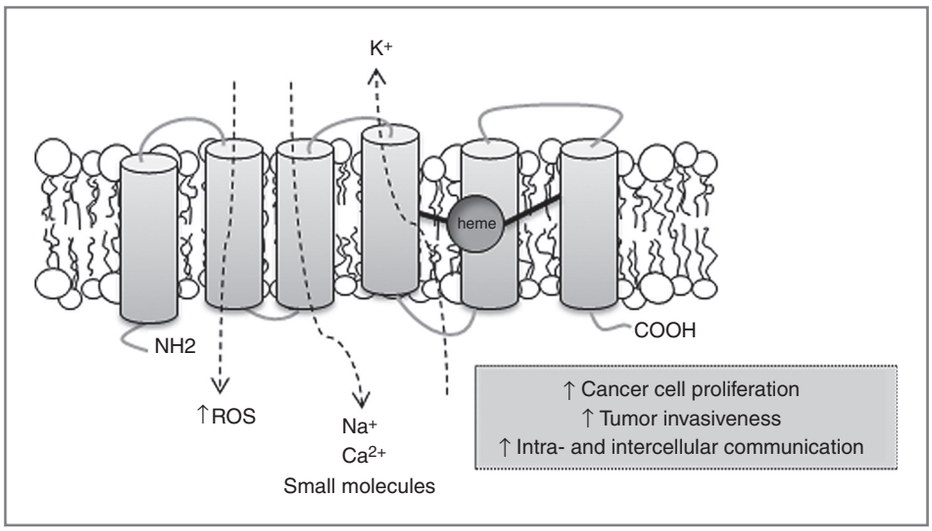

Figure 1. Schematic of STEAP1 protein structure, cellular localization, and physiologic functions. Similar in structure, presenting a 6-transmembrane structure, intracellular COOH- and *N*-terminal, and intramembrane heme group, STEAP1 lacks the innate metalloreductase activity conferred by the presence of the FNO-like domain. STEAP1 actively increases intra- and intercellular communication through the modulation of Na^+, Ca^{2+}, and K^+ concentration, as well as the concentration of small molecules. It stimulates cancer cell proliferation and tumor invasiveness.

using a single concentration of this androgen posed several limitations to the study above. Analyzing STEAP2 expression in response to several androgen concentrations at different time points would give a more comprehensive view of its putative regulation of STEAP2. Furthermore, it would also be of interest to analyze STEAP2 expression at the protein level, as androgens may regulate translation itself. Therefore, until further studies are carried out, it remains unclear if STEAP2 is indeed an androgen-independent gene, especially because STEAP2 expression seems to be responsive to the presence of an active androgen receptor, and this receptor seems to act on the regulation of STEAP2

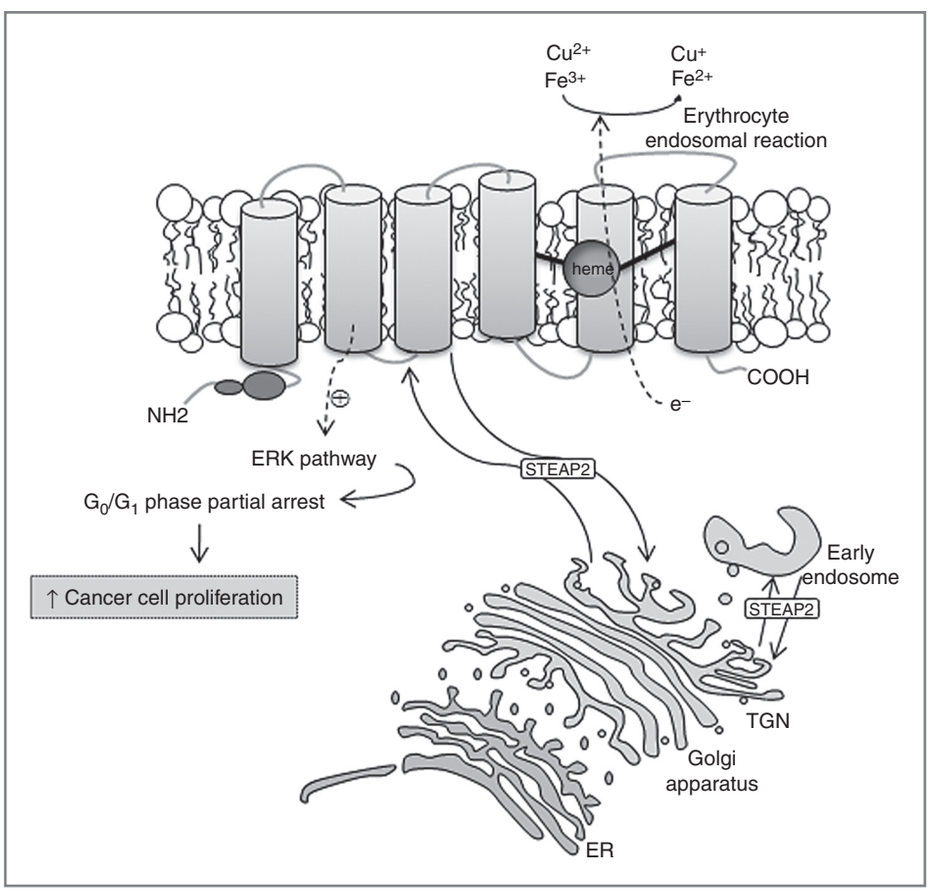

Figure 2. Schematic of STEAP2 protein structure, cellular localization, and physiologic functions. Because of its metalloreductase activity, STEAP2 contributes to proper erythrocyte Fe^{3+} and Cu^{2+} uptake and their reduction to Fe^{2+} and Cu^+, facilitating the progression of the Tf cycle. By activating the ERK pathway, STEAP2 induces partial arrest on the G_0–G_1–cell-cycle phase in cancer cells, thereby increasing cell proliferation and tumor development. STEAP2 can be found in association with early endosomes and the TGN, suggesting that it acts as a receptor for both exogenous and endogenous ligands or as a regulator of protein delivery and sorting mechanisms.

Table 4. Regulation of STEAP proteins and involvement in human diseases

STEAP protein	Regulation	Involvement in disease	Reference
STEAP 1	Androgens (?)	Progression of several cancer types	(6, 12, 15–17, 25)
	17β-E_2	Regulation of the MSC differentiation process (?)	
STEAP 2	Androgens (?)	Prostate cancer progression	(1, 7, 8, 26, 27)
STEAP 3	p53-activated protein	Hepatocellular carcinoma progression (?)	(3, 10, 13, 40)
		Involvement in several types of anemia (?)	
STEAP 4	Androgens	Prostate cancer development and progression	(11, 14, 41–43, 45)
	TNF-α	Obesity-related insulin resistance	
	IL-6	Inflammatory processes	
	Leptin		
	IL-1β		

expression along the progression of prostate cancer. These observations were made in PC3 and DU145 cells, and in 4 relapse derivatives of CWR22 tumors. In PC3 and DU145 cells, STEAP2 expression was not detected, and in CRW22R tumors, the expression of STEAP2 was significant, although lower than in LNCaP cells. Both PC3 and DU145 cell lines represent androgen-independent advanced prostate cancer and do not express androgen receptor, whereas CWR22R tumors do, possibly explaining the differences in STEAP2 expression. Furthermore, as no major genomic rearrangements, mutations, or promoter methylation have been found, loss of STEAP2 expression may be due to a deregulatory mechanism occurring during cancer progression (Table 4; refs. 7, 8).

In vitro and *in vivo* studies show that STEAP2 increases prostate cancer cell proliferation, regulating several genes involved in the cell cycle, causing a partial cell-cycle arrest at the G_0–G_1 phase.

This proliferative activity of STEAP2 seems to be coordinated through the activation of the extracellular signal-regulated kinase (ERK) pathway. Combined with its proliferative features, STEAP2 also acts as a prosurvival factor, as its knockdown increases the number of apoptotic events in prostate cancer cells (Fig. 2; ref. 27). However, the pathways by which STEAP2 inhibits apoptosis are not known and should be explored in the future (Table 4).

STEAP3

STEAP3 was first identified in studies searching for the gene responsible for the hypochromic microcytic anemia in the *nm1054* mouse mutant (Table 4; refs. 3, 5, 28). This autosomal recessive trait that results from a deletion in both STEAP3 alleles is characterized by an inefficient supply of iron to erythrocytes, leading to impairment of hemoglobin synthesis (28). In fact, the anemic phenotype is completely reversed when STEAP3 expression is restored (3).

The role of STEAP3 goes beyond iron metabolism in erythroid precursors (Table 4). It is upregulated upon p53 activation in the LTR6 myeloid leukemia cell line and in MCF7 breast cancer cells, increasing cell death. In addition, 2 other proteins involved in apoptosis interact with STEAP3 both *in vitro* and *in vivo*: the Nix protein, a mitochondrial proapoptotic protein associated with apoptotic cardiomyopathy, terminal erythroid differentiation, reticulocyte maturation, and Parkinson disease; and Myt1 kinase, a regulator of cyclin-dependent kinase activity. Therefore, STEAP3 may be involved in apoptosis and in cell-cycle progression, especially in G_2–M progression (10, 29–33). Nix seems to intensify the apoptotic effect of STEAP3 alone, whereas the interaction between STEAP3 and Myt1 implies modulation of the Myt1 phosphorylation state. When overexpressed, STEAP3 keeps Myt1 dephosphorylated and functional, possibly by recruiting specific phosphatases, by protecting the phosphorylation sites, or by maintaining $p34^{cdc2}$ phosphorylated. Therefore, STEAP3 could be viewed as a positive regulator of Myt1, and together, STEAP3 and Myt1 cause a pronounced effect on the cell cycle, delaying the G_2–M progression (Fig. 3; ref. 10).

STEAP3 also interacts with the translationally controlled tumor protein (TCTP), a Ca^{2+}- and microtubule-binding protein implicated in cell-cycle progression and malignant transformation (Fig. 3; refs. 34, 35). TCTP participates in the inflammatory response triggered by parasites, such as *Plasmodium falciparum* and *Schistosoma mansoni* and has antiapoptotic activity (36–38). TCTP is a nonclassical secreted protein, which is exported to the extracellular milieu independently of the endoplasmic reticulum–Golgi complex pathway. Like TCTP, STEAP3 is also present near the cell nucleus and the plasma membrane, suggesting that in certain circumstances they have simultaneous localization and distribution. In fact, secretion of TCTP is mediated by STEAP3, at least in epithelial and hematopoietic cell lines (293T, HepG2, and K562 cell lines). Moreover, TCTP is also present in exosome preparations derived from the 293T cell line, in which under the influence of STEAP3 overexpression, both endogenous and exogenous TCTP levels are raised (13). The expression of MHC-I was also increased by the overexpression of STEAP3 (13). Exosomes derived from dendritic cells express high levels of MHC-I, MHC-II, and cytosolic proteins likely involved in the function and biogenesis of exosomes (hsc73, annexin II, and Gi2). In addition, they express membrane proteins associated with cell targeting (MFG-E8, Mac-1, and CD9) and T-cell activation (B7.2; ref. 39). Other exosomes derived from

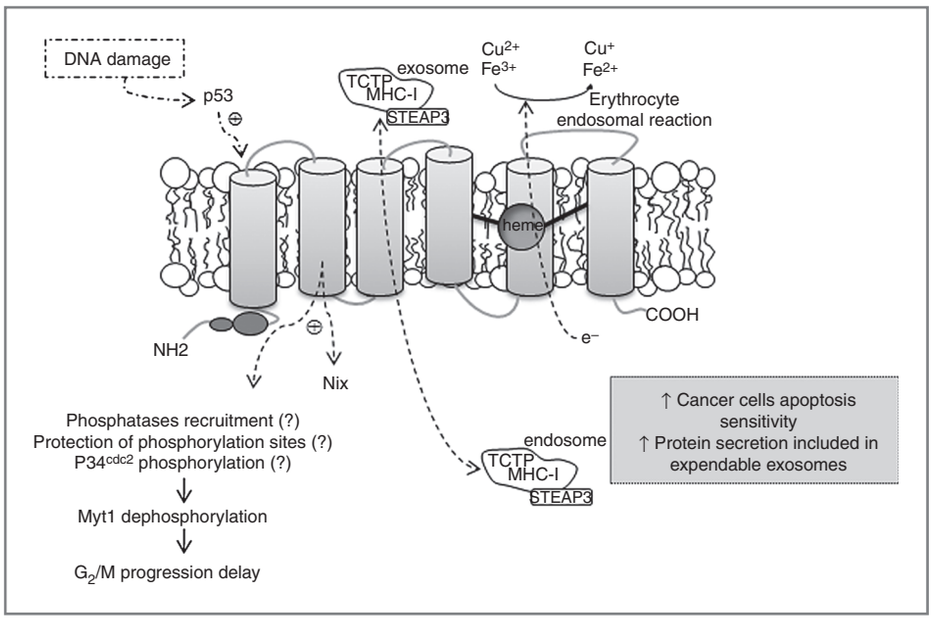

Figure 3. Schematic of STEAP3 protein structure, cellular localization, and physiologic functions. Taking part in the erythrocyte Tf cycle with the reduction and uptake of iron and copper, STEAP3 expression in the endosomal membrane grants an efficient hemoglobin synthesis. Upon p53 activation, upregulation of STEAP3 and its interaction with Nix and Myt1 intensifies cell death events. STEAP3 activates Myt1 through dephosphorylation, by recruiting phosphatases, protecting Myt1 phosphorylation sites, or inducing $p34^{cdc2}$ phosphorylation, thereby preventing cells to proceed adequately into G_2–M phase and leading to apoptosis. STEAP3 colocalizes with TCTP leading to MHC-I and its own overexpression. It takes part in vesicular trafficking and secretion in a way independent of the Golgi complex.

bone marrow erythroblasts and peripheral blood reticulocytes are important for these cells to mature, a process delayed in STEAP3 knockout mice. The induction of DNA damage and the consequent p53 activation in mouse embryo fibroblasts and in adult mice splenocytes stimulated the production of exosomes in a STEAP3-dependent manner. Accordingly, STEAP3 is thought to be the mediator of protein secretion in exosomes that are no longer needed for maturation and survival of the cells. The STEAP3 induction of TCTP secretion could also be seen as a strategy for making cancer cells more sensitive to apoptosis (40). Overall, STEAP3 could have broad functions as a regulator of vesicular trafficking and secretion and in intercellular communication (Table 4).

STEAP4

The localization of STEAP4 also suggests involvement in molecular trafficking (Table 4). STEAP4 moves packaged vesicular tubular structures from the cytoplasm to the periphery of the cell, and its colocalization with the early endosome protein EEA1, in the cell periphery and close to the nucleus, suggests that STEAP4 is either involved in the secretory pathway or in the endocytic pathway (Fig. 4). The link between STEAP4 and the endoplasmic reticulum is not well understood, but STEAP4 may reach the endoplasmic reticulum in an unfolded state and then acquire its active biologic conformation; and/or STEAP4 may actually have a functional role inside the endoplasmic reticulum (11).

STEAP4 has also been associated with obesity, insulin resistance, inflammation, and prostate cancer progression (11, 41–45). The STEAP4 mouse homolog, TNF-α–induced adipose-related protein (TIARP), was first identified as a plasma membrane protein overexpressed in both white and brown adipose tissues, with increased expression during preadipocyte differentiation and strongly induced by TNF-α, interleukin-6 (IL-6), growth hormone, and interleukin-1β (IL-1β). It is clear that TIARP is an important modulator of inflammation and nutrition, implicated in systemic metabolic homeostasis and obesity-related insulin resistance. However, the role of STEAP4 in humans is still uncertain (Fig. 4; refs. 46–51). Unlike TIARP, STEAP4 does not regulate the differentiation of preadipocytes *in vitro* (42). In adipocytes, STEAP4 is present in the plasma membrane and its expression is downregulated in patients who are obese (44). STEAP4 also has an important role in cell viability, proliferation, and apoptosis. The treatment of preadipocytes with an antibody against STEAP4 promotes the appearance of apoptotic cellular morphology, with mitochondrial damage causing swelling and pyknosis (44). STEAP4-induced apoptosis pathways seem to be mediated by caspase-3 and caspase-8. On the other hand, STEAP4 enhances cell proliferation, possibly acting as a signaling molecule. Preadipocyte treatment with anti-STEAP4 antibody decreased the proliferation rate and the number of cells that proceed to the S phase of the cell cycle (44). In primary cultures of adipose tissue, treatment with TNF-α increases its expression in a dose-dependent manner, disclosing a role for STEAP4 in inflammation processes in the adipose tissue. Nevertheless, the effects of TNF-α *in vitro* may not be quite the same as *in vivo*, because patients with higher TNF-α serum levels have lower levels of STEAP4 than controls. TNF-α, along with IL-6, is also responsible for increased STEAP4 expression during adipocyte differentiation and in mature adipocytes. Although the effects are time dependent, long-term exposure of mature adipocytes to IL-6 tends to decrease STEAP4 expression. Leptin, an adipocyte-secreted hormone linked to obesity-related insulin resistance, also regulates STEAP4 expression in mature adipocytes, decreasing both mRNA and protein levels (14, 41, 42). Furthermore, STEAP4 expression is also

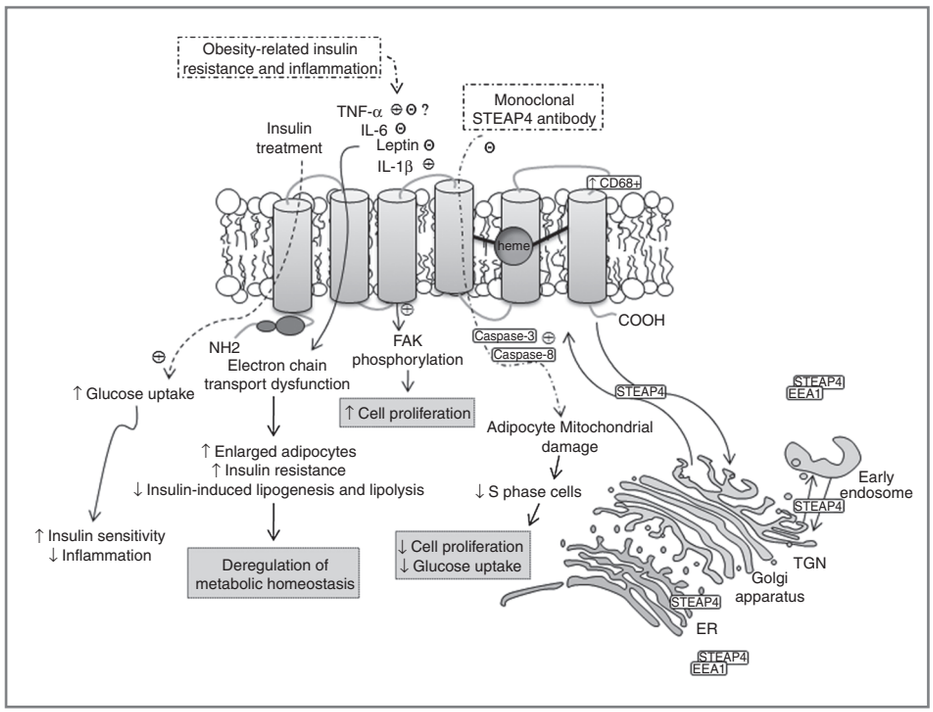

Figure 4. Schematic of STEAP4 protein structure, cellular localization, and physiologic functions. STEAP4 shuttles between the nucleus and the plasma membrane and colocalizes with EEA1, endoplasmic reticulum (ER), TGN, and early endosomes, suggesting its involvement in the endocytic and exocytic pathways. It mediates cell proliferation, apoptosis, glucose uptake, and inflammation in obesity-related insulin resistance cases. Proliferation is induced upon FAK activation. STEAP4 blockage triggers caspase-3 and caspase-8 and causes mitochondrial damage in adipocytes, decreasing cell proliferation and glucose uptake. IL-6 and leptin (repressors) and TNF-α and IL-1β (inductors) regulate STEAP4 actions. The macrophage marker $CD68^+$ colocalizes with the overexpressed STEAP4 in the plasma membrane in patients with rheumatoid arthritis.

induced by another proinflammatory cytokine, IL-1β. In human mesenchymal stem cell (MSC)–derived adipocytes, IL-1β treatment significantly increased STEAP4 mRNA and protein levels in a time-dependent manner. Overall, the effects of proinflammatory cytokines on STEAP4 could potentially indicate that STEAP4 has a protective role, restraining inflammation and insulin resistance (42). Correspondingly, STEAP4 induces glucose uptake after insulin treatment in human mature adipocytes, indicating that STEAP4 augments insulin sensitivity. The same happens in preadipocyte cultures, in which lower glucose uptake upon treatment with anti-STEAP4 antibody and insulin stimulation can be observed (42, 44). One hypothesis for the effects of STEAP4 in adipose tissue is that, similar to other members of its family, STEAP4 has an *N*-terminal domain with NADP-oxireductase activity that allows cellular uptake of iron and copper. Both are essential for glucose and lipid metabolism and could be associated with electron transport in mitochondria and regulation of ROS-related pathologic pathways, which are major players in obesity and obesity-related insulin resistance (52–54). Alteration of STEAP4 expression could eventually lead to dysfunction of these 2 processes and, consequently, disruption of metabolic homeostasis (1, 11, 14, 41, 44, 52, 55). Likewise, inflammation occurring in obesity and rheumatoid arthritis has TNF-α as a critical player (Fig. 4; refs. 56–58). The colocalization of STEAP4 with $CD68^+$ in the joints of patients with rheumatoid arthritis and its overexpression in the synovia of patients with rheumatoid arthritis and osteoarthritis indicate the role of STEAP4 in these diseases (43). Still, further studies are required to support the involvement of STEAP4 in the inflammatory process in the joints of patients with rheumatoid arthritis. In addition, STEAP4 may have a physiologic function in other organs such as placenta, lung, and heart, where its expression is more abundant (Table 4).

The mouse counterpart of STEAP4 has been associated with the appearance of a metabolic syndrome (MetS) phenotype in the TIARP knockout mouse, but the role of STEAP4 in MetS in humans is not clear (51). Two inconclusive epidemiology studies were conducted with the aim of establishing a correlation between STEAP4 gene polymorphisms and MetS associated with insulin resistance (59, 60). Of the several single-nucleotide polymorphisms (SNP) identified in regions that included introns, exons, and UTRs of the STEAP4 gene, only 3 were chosen for genotyping: 224 A/G (rs1981529, Gly75Asp) at exon 2; 364 G/A (rs34741656, Ala122Thr) at exon 2; and 7414 G/A (rs8122) at 3′-UTR. The polymorphism at exon 2 was previously identified as being a missense mutation. Although the first 2 had been initially described by Miot and colleagues (59) as having no effect on the prevalence or incidence of MetS, it was seen later that the rs8122 and rs1981529 SNPs had significant levels of association with MetS, in females but not in males (59, 60). Also, 2 common STEAP4 gene haplotypes identified as H1 (rs8122-rs1981529-rs34741656, G-A-G) and H2 (rs8122-rs1981529-rs34741656, A-G-G) were significantly associated with MetS phenotype in females. The reason why these associations were only found in the female population is still unknown (60). To continue exploring the effect of STEAP4 in MetS, $CD14^+$ monocytes were analyzed in the peripheral blood of 97 unrelated Chinese subjects (48 with MetS and 49 controls) to determine the relation between

cardiovascular alterations of patients with MetS and STEAP4 expression. The results showed that mRNA STEAP4 levels were lower in patients with MetS, and more significantly in women. Downregulation of STEAP4 was then positively correlated with high-density lipoprotein and negatively with body mass index, low-density lipoprotein, insulin, and fasting blood glucose, thereby suggesting that diminished STEAP4 levels may play a role in obesity, dyslipidemia, and hyperglycemia, all risk factors for MetS development. Furthermore, STEAP4 downregulation was also associated with 2 cardiac malfunctions that are repercussions of MetS; carotid atherosclerosis and left ventricular diastolic function (61).

As STEAP4 is expressed in the prostate, the role of androgens as possible regulators of its expression has been investigated (Table 4). Exposing LNCaP cells to DHT, testosterone, and to the synthetic androgen R1881 for different time periods increased the expression of STEAP4 mRNA in dose- and time-dependent manners, indicating that STEAP4 is an androgen-regulated gene (11). Further analysis of several other cell lines showed that STEAP4 mRNA expression is absent in prostate cell lines that do not express androgen receptor (PC3, DU145, CA-HPV10, PZ-HPV7, and YPEN-1), breast cancer cell lines (MCF7, MCF7-LCC1, MCF7-LCC2, and MB435), the hepatocellular carcinoma cell line HepG2, C2 myotube cell line, and HeLa cervical carcinoma cell line. As STEAP4 mRNA has only been found in androgen-dependent and androgen receptor–expressing LNCaP cells and not in the androgen receptor–negative prostate cancer cell lines, an active androgen receptor seems to be required. In addition, there seems to be a possible involvement of STEAP4 in prostate carcinogenesis due to the higher abundance of STEAP4 in prostate cancer samples compared with normal tissue (11). However, the pathway by which the active androgen receptor regulates STEAP4 expression needs to be elucidated.

The differential expression of STEAP4 in androgen receptor–positive and androgen receptor–negative prostate cancer cell lines, possibly associated with progression to a more aggressive phenotype, was attributed to an epigenetic mechanism (45). In LNCaP and DU145 cell lines, CpG islands were detected next to the STEAP4 gene promotor region, in the 5′-upstream sequence and part of the first exon, but were only methylated in the DU145 cell line, especially at the 5′-upstream sequence. Methylation reversal allowed an increase in both mRNA and protein expression in DU145 cells. Therefore, it seems that methylation of the STEAP4 promotor region is associated with the absence of STEAP4 expression in prostate cancer cells. Nevertheless, CpG methylations may not be the only epigenetic alterations occurring in the STEAP4 gene, and, therefore, histone modifications and interaction with transcription factors should also be evaluated, as well as its significance in prostate cancer development (45). Following the observed differential expression of STEAP4 between androgen receptor–positive and androgen receptor–negative prostate cancer cell lines, the potential role of STEAP4 in cancer progression was examined (11). In a colony formation assay using adherent cell-culture conditions, overexpression of STEAP4 in transfected PC3, DU145, and COS-1 originated larger and higher number of colonies compared with controls transfected with the empty vector (11). In contrast, in soft agar cultures, the number of colonies formed by 239T cells transfected with STEAP4 decreased in comparison to controls, and inhibition was reversed by adding a monoclonal antibody against STEAP4, indicating an inhibitory effect of STEAP4 in the proliferation rate of anchorage-independent cell cultures. The ability of STEAP4 to inhibit anchorage-independent cell proliferation was linked to the phosphorylation of focal adhesion kinase (FAK), a molecule implicated in carcinogenesis (Fig. 4; refs. 45, 62). STEAP4 interacts with FAK in immunoprecipitation assays, and 239T cells overexpressing STEAP4 show reduced FAK phosphorylation compared with mocked transfected cells, which in turn had time-dependent increased phosphorylation of FAK. Therefore, it is plausible to attribute the inhibition of cell growth in suspension to an inadequate activation of FAK by STEAP4. Concurring with the proliferative effect of STEAP4 in adherent cell cultures is the fact that these cells showed higher levels of FAK phosphorylation. However, the mechanism by which STEAP4 regulates FAK phosphorylation remains unclear. STEAP4 itself is predicted to have several putative phosphorylation sites that could be associated with its functions and signaling pathways in cancer cells (45).

STEAP Proteins as Biomarkers of Disease

Initial studies on STEAP1 expression during the development of prostate cancer did not find any significant alterations along the different cancer stages (6). However, a later analysis of prostate cancer cases, benign prostate hyperplasia, and nonprostatic malignancies disclosed a negative correlation between STEAP1 expression and histologic grading of prostate cancer cells (63). In addition, STEAP1 protein expression is higher in primary colon and bladder cancer when compared with colon and bladder cell lines derived from metastatic cancers (6). So, more studies are required to clarify the association of STEAP1 expression with histologic grading in different cancer types, to understand the clinical significance of STEAP1 and its importance in cancer progression, particularly in prostate cancer initiation, development, and metastasis. Recently, STEAP1 mRNA has been identified by real-time PCR in serum of patients with cancer (64). This highly sensitive and specific method allowed the distinction between 50 patients bearing pancreatic, bladder, breast, prostate, colon-rectal, lung, or stomach tumors and healthy subjects. External factors like age, histologic type, and clinical stage of cancer and therapy were evaluated, but none of them had a direct influence on STEAP mRNA levels. Therefore, the idea that STEAP1 may be a useful marker for several types of cancer, as well as its potential for cancer diagnosis, was reinforced (64–66).

Although STEAP1 and STEAP2 have been almost exclusively associated with prostate cancer, both are also

differentially expressed in murine and human MSCs, and in human bone marrow cells (67). As MSCs can differentiate into several types of cells that compose bone marrow and, due to the cell-surface localization of STEAP1 and STEAP2, these epithelial antigens could also be useful markers for isolation and purification of MSCs, distinguishing between normal and abnormal populations of bone marrow cells. In addition, its possible involvement in the regulation of the differentiation process of the MSCs should be further investigated (68).

Expression of STEAP2 also differs between normal and prostate cancer tissue. *In situ* hybridization analysis of 18 normal and 25 tumoral prostate sections showed that STEAP2 expression is about 2.5-fold higher in prostate cancer cells than in normal cells, suggesting that STEAP2 could be implicated in prostate cancer progression (7). Moreover, the immunohistochemical analysis of 17 benign and 67 cancer specimens of prostate tissue was in agreement with mRNA expression data (27). Despite the higher levels of STEAP2 mRNA and protein in prostate cancer, the only study that attempted to establish an association of STEAP2 expression with the Gleason score of prostate tumors was unsuccessful (27). However, this question should be further analyzed as the expression of ERK, 1 of the proteins that may mediate the proliferative and antiapoptotic functions of STEAP2, augments from normal to benign prostate hyperplasia and prostate cancer stages (27). If ERK expression is associated with advanced stages of prostate cancer and mediates STEAP2 functions, is STEAP2 expression really independent of the tumor grade? Nonetheless, STEAP2 may be a good and useful marker for the detection of prostate cancer progression.

STEAP3 could be used as a marker of the transition from cirrhosis to hepatocellular carcinoma (HCC; refs. 69, 70). Of the different causes that lead to hepatocellular carcinoma, hepatitis B and C virus, chronic alcohol abuse, and cirrhosis are the most common (71). It is known that HCC is associated with inhibition of transcription of a wide range of genes. These events in the adult liver also occur in the late fetal liver development, implying that some genetic changes are common to these conditions. Another common feature of these 2 liver stages is that active proliferation becomes more prominent than apoptosis, a consequence of the downregulation of proapoptotic genes. STEAP3 is one of these genes, and its expression is remarkably diminished in HCC nodules compared with cirrhotic peritumoral tissues. STEAP3 mRNA levels in peritumoral cirrhosis are significantly lower than in healthy liver (70). Its expression is dependent on tumor differentiation stage, with lower levels of protein associated with moderately or poorly differentiated tumors. In HCC-free cirrhosis, STEAP3 protein expression levels are notably increased when compared with healthy liver. Overall, STEAP3 expression is associated with the liver health status, that is, if the tissue is healthy or cirrhotic, if HCC is present or not. In the case of HCC-free cirrhosis, STEAP3 expression also differs according to the stage of differentiation. Therefore, analysis of STEAP3 expression levels could help identify the transition from HCC-free cirrhosis into a cancer-developing liver. Therefore, the use of STEAP3 as a new marker for hepatic carcinogenesis should be taken into account (69, 70). Furthermore, as STEAP3 is highly expressed in fetal liver and has an important role in hemoglobin production in erythroid precursors, it could be of interest to investigate whether STEAP3 could be considered as a marker of viability for fetal development and for other types of anemia besides the recessively inherited hypochromic microcytic anemia (3).

No studies have been conducted with STEAP4 to establish its usefulness as a biomarker of disease.

Its involvement in prostate cancer progression and clinical significance should be further clarified, especially in the androgen-dependent phase of tumor development. The possibility that STEAP4 could be a new prostate cancer biomarker of great importance for early detection of disease is reasonable due to its androgen-regulation dependence. The role of STEAP4 as a biomarker of inflammation in a variety of tissues, in obesity, and other metabolic and cardiac disorders should also be of interest, as well as understanding the clinical significance of STEAP4 overexpression in placenta and lung.

STEAP Proteins as Immunotherapeutic Targets

STEAP1 has been considered to be a good target for T-cell–based immunotherapy, with applications in prostate, colon, pancreas, bladder, Ewing sarcoma, breast, testicular, ovarian, and melanoma cancers, as it has the required features of tumor-associated antigens (TAA), specifically, cell-surface localization, high expression levels in several types of tumors, especially in the prostate, and absence of expression in vital organs (6, 72). Good immunotherapy techniques require the increase of expression or cross-presentation of self-peptides to naïve T cells. Therefore, the ultimate purpose of tumor immunotherapy is the production of an effective vaccine containing epitopes that elicit both $CD8^+$– and $CD4^+$–T-cell immune responses, leading to tumor regression. This vaccine should be administrated to patients with cancer without using invasive techniques (73, 74).

The identification of STEAP1 epitopes has been directed toward prostate, renal, and bladder cancers with some success. The first STEAP1 epitopes used to trigger an antitumor immune response were $STEAP_{292}$ (MIAVFLPIV), a naturally processed peptide, and its modified version, $STEAP_{292.2L}$ (MLAVFLPIV). Their selection was based on their strong binding to HLA-A*0201 molecules and ability to elicit a sustained cytotoxic T-lymphocyte (CTL) response. In fact, $STEAP_{292}$, and especially $STEAP_{292.2L}$, induced naïve $CD8^+$ T cells into CTL capable of recognizing peptide-loaded cells with high specificity. Moreover, CTL induced by $STEAP_{292.2L}$ peptide not only recognized peptide-loaded cells, but also tumor cells from prostate, colon, bladder, Ewing sarcoma, melanoma, and embryonic rhabdomyosarcoma that expressed STEAP1 (75). Two additional nonameric STEAP1 epitopes ($STEAP_{86–94}$ and

$STEAP_{262–270}$) were found to be HLA-A*0201–restricted epitopes. Both can be found in human and mouse, but with slightly different constitutions; human $STEAP_{86–94}$ differs at position 9 from the mouse peptide (FLYTLLRE**V**→-FLYTLLRE**I**) and human $STEAP_{262–270}$ differs at position 6 from the mouse peptide (LLLGT**I**HAL→LLLGT**V**HAL). The latter has been initially identified as a TAA of STEAP3 (76, 77). Despite the differences mentioned, both $STEAP_{86–94}$ and $STEAP_{262–270}$ peptides are immunogenic *in vivo*, in HLA-A*0201 transgenic mice (HHD), and *in vitro*, in peptide-specific human $CD8^+$ T cells from healthy donors. Furthermore, $STEAP_{86–94}$ and $STEAP_{262–270}$ from human and mouse $CD8^+$ T cells were able to recognize STEAP1 expressed in human tumor cells in an HLA-A*0201–restricted manner. Also, specific CTL-expressing $STEAP_{86–94}$ were amplified *ex vivo,* from the peripheral blood of 3 out of 5 patients with non–small cell lung carcinoma and 2 out of 3 patients with prostate cancer, reinforcing the protective role of STEAP1. However, the contribution of tumor cells expressing STEAP1 to the observed immunologic response generated by the naturally processed STEAP1, as well as the quantitative and qualitative discrimination of the T cells expressing $STEAP_{86–94}$ and $STEAP_{262–270}$ remain to be identified (76). As a TAA, STEAP1 was also thought to be able to trigger an immune response to eliminate a tumor by specifically eliciting $CD4^+$ helper T cells. Consequently, 2 specific synthetic STEAP1 peptides, $STEAP_{102–116}$ (HQQYFYKIPILVINK) and $STEAP_{192–206}$ (LLNWAYQQVQQNKED), which strongly bind to different classes of HLA-DR, can also be presented by those different classes to $CD4^+$ helper T cells (74, 78). These naturally processed epitopes are both presented to $CD4^+$ helper T cells by tumor cells such as PC3 prostate cells, m697 melanoma cells, and Epstein Barr virus–transformed lymphoblastoid cell lines, but only $STEAP_{102–116}$ is presented to $CD4^+$ helper T cells by antigen-presenting cells (APC; ref. 74). The reactive $CD4^+$ helper T cells present in the different types of cancer may play a crucial role in the immune response that follows their activation; these cells would initiate the immune response sending the appropriate signals to APCs and CTL, resulting in CTL expansion, maturation, costimulation, and generation of memory CTL populations (79). In fact, both of these peptides were identified as being processed endogenously, through direct presentation of STEAP1 peptides by HLA-DR molecules, and exogenously, by APCs that process STEAP1 peptides derived from cell lysates, as seen *in vitro* with renal and bladder cancer (78).

Application of therapeutic vaccination, using STEAP1 as a target, that efficiently attenuates or even stops cancer progression is still in its early steps. No commercial formulations are available, particularly in prostate cancer, as the efficacy of the developed vaccines relies on the immunosuppressive state of the patients included in clinical trials and their tumor microenvironment, which prevents triggering of an immune response. Two studies that applied a mouse STEAP1 DNA prime/Venezuelan equine encephalitis virus-like replicon particles boost vaccine showed the efficacy of this therapeutic strategy against prostate cancer (73, 80). The capacity of this vaccine to trigger an immune response could be seen by the increasing number of $CD8^+$ and $CD4^+$ T cells and by the production of cytokines such as TNF-α, IFN-α, IL-2, and IL-12 following vaccination of C57BL/6 mice injected with a cell line derived from the transgenic adenocarcinoma mouse model, TRAMPC-2 (73). Protection against prostate cancer dramatically increases when vaccination occurs in mice mimicking earlier stages of cancer. Immunosuppressive mechanisms that lead to a reduction of Th1 and Th2 function, reduction of proinflammatory cytokines, and increased expression of immunosuppressive factors activated during prostate cancer progression tend to interfere with the efficacy of the vaccine (80). Avoiding the establishment of an immunosuppressive tumor microenvironment seems, therefore, to be the key to the success of therapeutic vaccination in later cancer stages. Thus, all together, these observations offer new possibilities for novel immunotherapeutic strategies, not only directed toward prevention but also for the treatment of patients with cancer.

The ability of STEAP3 to elicit an immune response was assessed *in vitro* in resensitized splenocytes against peptide-loaded RMA-*S*-HHD-B7.1 cell line and against peptide-loaded dendritic cells. STEAP3 binds to HLA-A2.1 molecules in a restricted and stable manner, and the activated CTL cells promote a dose-dependent peptide-specific cell lysis. Using the HLA-A2.1–positive LNCaP and the HLA-A2.1–negative PC3 cell lines as targets for peptide-loaded RMA-*S*-HHD-B7.1 cells, the lysis pattern promoted by the stimulation of CTL confirmed that STEAP3 is indeed presented by MHC-I molecules expressed on the tumor cell surface and that it is processed inside the cell. As an MHC-I–presented peptide on tumor cells *in vitro*, it was assumed that *in vivo*, STEAP3 could have antitumor reactivity. In fact, subcutaneous administration of autologous CTL cells around a previously induced tumor led to an expressive tumor regression (77). The confirmation that human CTL precursors specific for STEAP3 have the ability to initiate an antitumor response in healthy and prostate cancer–bearing individuals highlights the potential of $STEAP_{262–270}$ for immunotherapeutic procedures against tumors in which STEAP3 is expressed (77). Together with the STEAP3 coexistence with MHC-I and MHC-II in exosomes derived from dendritic cells and its dependence on p53, its use for exosomal immunotherapy could be the next step.

Although no studies have been done to establish an immunotherapeutic strategy using STEAP2 or STEAP4, their contribution to molecular trafficking and involvement in prostate cancer progression make them promising targets. The unique features of STEAP4 could also be directed toward adipose tissue to prevent inflammation and reduce insulin resistance, both associated with obesity (44). In addition, the FNO-like domain, which is only absent in STEAP1, could also be seen as a potential target in STEAP2, STEAP3, and STEAP4, with the advantage of its exclusive presence in this family of proteins in mammals. Overall,

future immunotherapeutic strategies may include all STEAP family members, with promising results. Furthermore, their use as vaccine components could also lead to a major improvement in prostate cancer, anemia, metabolic disorders, and antiinflammatory therapies, in which these STEAP proteins are overexpressed.

Conclusions and Future Prospects

All members of the STEAP family share common features in their structure and act as metalloreductases in human cells, with the exception of STEAP1, because of the absence of the FNO-like domain and Rossman fold. Overall, STEAP1, STEAP2, STEAP3, and STEAP4 are overexpressed in several human cancers, and some studies suggest that these proteins, particularly STEAP1 and STEAP2, increase the proliferation of cancer cells, suggesting their potential as therapeutic targets. Still, the mechanisms by which each STEAP protein contributes to cell proliferation and cancer progression in different organs and tissues remain largely elusive. Thus, more data are required to clarify the role of STEAP proteins in the cell cycle, proliferation, and apoptosis. Furthermore, the regulation of STEAP expression should also be addressed, particularly in those tissues in which STEAP is overexpressed in malignancies. These studies should encompass detailed analysis of hormones, particularly androgens in the prostate, and cytokines, and the analysis of the epigenetic mechanisms involved in STEAP regulation, not only in animal and cellular models, but also in a clinical context in patients with cancer.

The fact that STEAP proteins, particularly STEAP1 and STEAP2, localize at the cell membrane, are overexpressed in cancer tissues, and are absent in vital organs, underlines their potential as biomarkers of disease and as potential immunotherapeutic targets against prostate, bladder, kidney, and liver cancer. More studies to establish clear associations of STEAP expression with clinical data, and the effects on cancer treatments, in animal models and in patients with cancer should be entailed to provide further support for their use as biomarkers of disease and to ascertain their immunotherapeutic potential.

A comprehensive analysis of the functions and clinical importance of STEAP in cancer is still in its early days, but the information gathered to date is promising and definitively encourages further research exploring the expression, regulation, and role of STEAP proteins in cancer pathophysiology, diagnosis, and therapeutic approaches.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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