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Estrogenic Regulation of the SCF/c-KIT System in Prostate Cells: a Relationship with Prostate Cancer?

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Dedicatória

Dedico esta dissertação às pessoas mais importantes da minha vida, os meus pais.

"Vocês deixaram os vossos sonhos para que eu sonhasse.
Derramaram lágrimas para que eu fosse feliz.
Perderam noites de sono para que eu dormisse tranquila.
Acreditaram em mim, apesar dos meus erros.
Jamais esqueçam que eu levarei para sempre um pedaço do vosso ser dentro do meu próprio ser…"

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Obrigada a todos!

“Algo só é impossível até que alguém decida provar o contrário.”

Albert Einstein

“Nunca tenha certeza de nada, porque a sabedoria começa com a dúvida.”

Freud

Resumo

O cancro da próstata é o tipo de doença oncológica mais comum entre os homens, a qual tem apresentado uma incidência crescente ao longo dos anos. O desenvolvimento e a progressão do cancro da próstata têm vindo a ser relacionados com o ambiente hormonal intraprostático, assim como com os níveis séricos hormonais. Apesar do papel dos androgénios como agentes estimuladores do crescimento do cancro da próstata se encontrar bem descrito, também os estrogénios parecem estar envolvidos na carcinogénese da próstata. Contudo, a dualidade dos possíveis efeitos dos estrogénios na próstata é um assunto que tem vindo a ganhar consistência nos últimos anos. Se alguns estudos defendem que os estrogénios são agentes causadores do cancro da próstata, outras evidências, igualmente fortes, indicam que estas hormonas podem ser protectoras contra o cancro da próstata. O receptor tirosina cinase c-KIT e o seu ligando, o *Stem Cell Factor* (SCF), são fortes estimuladores da proliferação celular num vasto conjunto de tecidos, e a interacção ligando/receptor parece ter um papel fundamental na carcinogénese. Para além disso, tem sido demonstrado que os estrogénios regulam a expressão do sistema SCF/c-KIT em vários tecidos, desconhecendo-se o que acontece ao nível da próstata. O presente trabalho tem como objectivo avaliar o papel dos estrogénios na regulação do sistema SCF/c-KIT em linhas celulares de próstata humana e em próstata de rato *in vivo*. Pretende-se igualmente averiguar o consequente efeito dos estrogénios na proliferação e apoptose de células da próstata.

Para isso, linhas celulares de próstata humana neoplásicas (LNCaP, DU145 e PC3) e não-neoplásicas (PNT1A) foram mantidas em cultura na presença ou ausência de 100 nM de 17β-estradiol (E_2) durante diferentes períodos de tempo. Por outro lado, ratos machos adultos da estirpe Wistar foram injectados diariamente com veículo (controlo) ou com E_2 (250 mg/dia/kg), durante 5 dias. Após o tratamento, os animais foram eutanasiados sob anestesia e removeram-se as próstatas, as quais foram pesadas e fixadas em paraformaldeído 4 % ou, alternativamente, congeladas em azoto líquido. A análise da expressão do SCF e do c-KIT em resposta ao tratamento com E_2 foi efectuada através de PCR em tempo real, Western Blot e imunocito/histoquímica. O índice de proliferação celular na próstata de rato foi estimado com base na marcação do Ki67 usando imunohistoquímica de fluorescência. O rácio proteico Bax (pro-apoptótica)/Bcl-2 (anti-apoptótica), a expressão da caspase-9, do Fas e do Fas-L, o ensaio enzimático da actividade da caspase-3 e o ensaio do TUNEL foram as metodologias usadas para avaliar a apoptose.

Os resultados obtidos revelaram uma diminuição da expressão do SCF e do c-KIT em resposta ao E_2, quer em linhas celulares de próstata humana, quer na próstata de rato *in vivo*, o que sugere uma restrição da proliferação das células prostáticas em resposta aos

estrogénios. Este facto foi confirmado *in vivo* pela diminuição do peso da próstata e pela redução do índice de proliferação Ki67 observadas no grupo tratado. Para além disso, a actividade enzimática da capase-3 encontrou-se aumentada em resposta ao E_2, o que indica que os estrogénios induziram a apoptose das células da próstata de rato. O aumento da expressão do sistema Fas nos animais tratados com E_2 sugere o envolvimento da via extrínseca na apoptose induzida pelos estrogénios.

Em conclusão, o presente trabalho demonstrou que os estrogénios diminuem a expressão do sistema SCF/c-KIT em células neoplásicas e não neoplásicas de próstata humana, assim como, na próstata de rato. Ficou ainda estabelecido que os estrogénios têm efeitos anti-proliferativos e pro-apoptóticos na próstata, os quais provavelmente dependem da diminuição da expressão do sistema SCF/c-KIT. Por fim, estes resultados constituem uma base molecular fundamental de suporte ao papel protector dos estrogénios no desenvolvimento do cancro da próstata.

Palavras-chave

Apoptose, cancro da próstata, DU145, estrogénios, c-KIT, LNCaP, PC3, PNT1A, proliferação, ratos, “Stem Cell Factor”.

Resumo Alargado

O cancro da próstata é o tipo de doença oncológica mais comum entre os homens, a qual afecta principalmente os homens com idades acima dos 50 anos. Com o envelhecimento da população e o aumento da esperança média de vida, a incidência desta doença tem vindo a aumentar, pelo que se torna necessário continuar a melhorar as suas formas de diagnóstico e tratamento.

O desenvolvimento e a progressão do cancro da próstata têm vindo a ser relacionados com o efeito das hormonas presentes na circulação, assim como, com o ambiente hormonal no interior da próstata. Os androgénios desempenham um papel crucial no desenvolvimento da próstata com acções importantes desde o período embrionário. O papel dos androgénios como agentes estimuladores do aparecimento e desenvolvimento do cancro da próstata encontra-se bem descrito, e há várias décadas que as terapias de privação androgénica são a base dos tratamentos de neoplasias prostáticas. Embora, os estrogénios sejam essencialmente conhecidos como hormonas femininas, estes estão presentes no organismo masculino, ainda que em quantidades muito pequenas. Na verdade, também os estrogénios parecem estar envolvidos na carcinogénese prostática. Contudo, a dualidade dos possíveis efeitos dos estrogénios na próstata é um assunto que tem vindo a ganhar consistência nos últimos anos. Se por um lado alguns estudos defendem que os estrogénios são agentes causadores de cancro da próstata, por outro lado, há também fortes evidências experimentais que apontam estes esteróides como protectores contra o cancro da próstata.

O c-KIT é um receptor tirosina cinase cujo ligando específico é uma citocina, o "Stem Cell Factor" (SCF). O c-KIT apresenta diferentes isoformas, as quais diferem, entre outros aspectos, pela sua localização subcelular, na membrana ou no citoplasma da célula. A forma completa do c-KIT, e com efeitos melhor documentados, corresponde à forma membranar. No entanto, o c-KIT apresenta também uma forma truncada (tr-KIT), originada por mecanismos de uso alternativo do promotor, a qual se localiza no citoplasma e contém apenas com a parte do domínio cinase e o terminal-C, e uma forma solúvel, originada por clivagem proteolítica, contendo apenas o domínio extracelular. Tal como o c-KIT, também o SCF existe na sua forma membranar (mSCF) e na forma solúvel (sSCF) libertado para o meio extracelular.

A interacção do SCF com o c-KIT provoca a dimerização do receptor, activando a sua actividade de tirosina cinase e iniciando um conjunto de vias de transdução de sinal. O sistema SCF/c-KIT está envolvido na estimulação da proliferação num vasto conjunto de tecidos, e a sua interacção parece ter um papel fundamental na carcinogénese. Para além

disso, tem sido demonstrado que os estrogénios regulam a expressão do sistema SCF/c-KIT em vários tecidos, desconhecendo-se o que acontece ao nível da próstata.

O presente trabalho tem como objectivo avaliar o papel dos estrogénios na regulação do sistema SCF/c-KIT em linhas celulares de próstata humana e em próstata de rato *in vivo*. Pretende-se igualmente averiguar o consequente efeito dos estrogénios na proliferação e apoptose de células da próstata.

Para isso, foram efectuados estudos experimentais *in vitro* e *in vivo*. Na abordagem *in vitro*, linhas celulares de próstata humana neoplásicas (LNCaP, DU145 e PC3) e não neoplásica (PNT1A) foram mantidas em cultura na presença ou ausência de 100 nM de 17β-estradiol (E_2) durante 0, 12, 24 e 48 horas. No segundo caso, ratos machos adultos da estirpe Wistar foram injectados diariamente com veículo (controlo) ou com E2 (250 mg/dia/kg), durante 5 dias. Após o tratamento, os animais foram eutanasiados sob anestesia e removeram-se as próstatas, as quais foram pesadas e fixadas em paraformaldeído 4 % ou, alternativamente, congeladas em azoto líquido. Seguidamente, procedeu-se à extracção de RNA e proteína total das linhas celulares e dos tecidos prostáticos. Sintetizou-se DNA complementar (cDNA) a partir do RNA extraído. A análise da expressão do SCF e do c-KIT em resposta ao E_2 foi efectuada através de PCR em tempo real, Western Blot e imunocitoquímica (linhas celulares) ou imunohistoquímica (ratos). Relativamente à estimulação *in vivo*, procedeu-se ainda à avaliação do efeito dos estrogénios na proliferação e apoptose celular na próstata de rato. O índice de proliferação celular na próstata de rato foi estimado com base na marcação do Ki67 usando imunohistoquímica de fluorescência. O rácio proteico Bax (pro-apoptótica)/Bcl-2 (anti-apoptótica), a expressão da caspase-9, do Fas e do Fas-L, o ensaio enzimático da actividade da caspase-3 e o ensaio do TUNEL foram as metodologias usadas para avaliar a apoptose.

Como estratégia inicial confirmou-se expressão do c-KIT e do SCF em todas as linhas celulares de próstata humana e na próstata de rato, quer ao nível do mRNA quer da proteína. Os resultados obtidos confirmaram a expressão do sistema SCF/c-KIT, e das suas várias isoformas, em todas as linhas celulares estudadas e igualmente na próstata de rato, ainda que apresentando um expressão diferencial. Relativamente às linhas celulares, verificou-se uma diminuição da expressão proteica do SCF e c-KIT (incluindo as formas membranares e truncadas) em resposta ao E_2 nas células PNT1A, LNCaP e DU145, ao contrário do que aconteceu nas células PC3, nas quais o tratamento aumentou a expressão do sistema SCF/c-KIT. Estes resultados foram obtidos por Western Blot e confirmados por imunicitoquímica. A estimulação com E_2 também diminuiu a expressão do SCF e do c-KIT na próstata de rato, contudo, este efeito só foi observado para a forma membranar; as formas truncadas do c-KIT (30 and 50 kDa) apresentaram expressão aumentada em resposta ao E_2. Ainda assim, a função destas isoformas permanece desconhecida, sendo a forma membranar a que tem sido relacionada com a estimulação da proliferação celular.

A diminuição da expressão do c-KIT e do seu ligando após estimulação com o E_2 sugere uma acção dos estrogénios na restrição da proliferação das células prostáticas. Este facto foi confirmado *in vivo*, pela diminuição do peso da próstata e pela redução do índice de proliferação Ki67 observadas no grupo tratado. Para além disso, a actividade enzimática da capase-3 também se encontrou aumentada em resposta ao E_2 o que indica que os estrogénios induziram a apoptose das células da próstata de rato. Numa tentativa de clarificar quais os mecanismos moleculares envolvidos na apoptose induzida pelo E_2 estudou-se a expressão de elementos chave nas diferentes vias apoptóticas. O aumento da expressão do sistema Fas nos animais tratados com E_2 e a ausência de alterações na expressão da caspase-9, sugerem o envolvimento da via extrínseca da apoptose.

Em conclusão, o presente trabalho demonstrou que os estrogénios diminuem a expressão do sistema SCF/c-KIT em células neoplásicas e não neoplásicas de próstata humana, assim como, na próstata de rato. Ficou ainda estabelecido que os estrogénios têm efeitos anti-proliferativos e pro-apoptóticos na próstata de rato, os quais, provavelmente dependem da diminuição da expressão do sistema SCF/c-KIT. Por fim, estes resultados constituem uma base molecular fundamental de suporte ao seu papel protector no desenvolvimento do cancro da próstata.

Abstract

Prostate cancer (PCa) is the most common type of oncological disorder in men, for which an increasing incidence has been reported. Development and progression of PCa have been highly related with the circulating and intraprostatic hormonal milieu. Despite the classical role of androgens as stimulating agents in PCa growth, currently, estrogens also have been implicated in the prostate carcinogenesis. However, a duality for the possible role of estrogens in prostate cells has been gaining consistency over the last years. If some studies defend that estrogens are potential causative agents of PCa, other strong evidences indicate that these steroids may be protective against PCa. The tyrosine kinase receptor c-KIT and its ligand, the Stem Cell Factor (SCF) are powerful agents stimulating cell proliferation in a broad range of tissues, and the SCF/c-KIT interaction seems to play a crucial role in carcinogenesis. Moreover, it has been shown that estrogens modulate the expression of SCF/c-KIT system in several tissues, except the prostate. The present work aims to evaluate the role of estrogens regulating the SCF/c-KIT expression in human prostate cell lines and in rat prostate *in vivo*. The consequent effects on proliferation and apoptosis of prostatic cells will also be determined.

Neoplastic (LNCaP, DU145 and PC3) and non-neoplastic (PNT1A) human prostate cell lines were cultured in presence or absence of 100 nM 17β-estradiol (E_2) for different time periods. Adult male Wistar rats were daily injected with vehicle (control) or E_2 (250 mg/day/kg) for 5 days. After treatment, animals were euthanized under anesthesia and prostates were collected, weighted and either fixed in 4 % paraformaldehyde or snap frozen in liquid nitrogen. The expression analysis of SCF and c-KIT in response to E_2 was performed by means of real-time PCR, Western Blot and immunocito/histochemistry. The proliferation in rat prostate cells was estimated via fluorescent immunohistochemistry of Ki67. The protein ratio of Bax (pro-apoptotic)/Bcl-2 (anti-apoptotic), the expression of caspase-9, Fas and Fas-L, the enzymatic activity of caspase-3 and a TUNEL assay were used to evaluate apoptosis.

The results obtained showed a decreased expression of both SCF and c-KIT in response to E_2-treatment either in human prostate cells or rat prostate *in vivo*, which suggested a restricted proliferation of prostate cells in response to estrogens. This fact was confirmed *in vivo* by the diminished prostate weight and reduced Ki67 proliferation index observed in the E_2-treated group. In addition, the enzymatic activity of caspase-3 was increased in response to E_2, which indicates that estrogens induced apoptosis in rat prostate. The enhanced expression of the Fas system in the prostate of E_2-treated animals suggests the involvement of the extrinsic pathway in the estrogen-induced apoptosis.

In conclusion, the present work demonstrated that estrogens down-regulate the SCF/c-KIT system in neoplastic and non-neoplastic human prostate cells and in rat prostate in vivo. Moreover, estrogens have anti-proliferative and apoptosis-inducer effects in prostate exerted likely through the down-regulation of the SCF/c-KIT system. These findings also provided a body of evidence supporting the protective role of estrogens against development of PCa.

Keywords

Apoptosis, DU145, estrogens, c-KIT, LNCaP, PC3, PNT1A, PCa, proliferation, rat, Stem Cell Factor.

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ADT	Androgen Deprivation Therapies
AR	Androgen Receptor
ATP	Adenosine TriPhosphate
BPH	Benign Prostatic Hyperplasia
CDK	Cyclin-dependent kinase
CK18	Cytokeratin-18
CS-FBS	Charcoal-Stripped Fetal Bovine Serum
DEPC	Diethylpyrocarbonate
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone Sulfate
DHT	Dihydrotestosterone
DRE	Digital Rectal Examination
DTT	Dithiothreitol
E_1	Estrone
E_2	17β-estradiol
E_3	Estriol
ED	Endocrine Disruptor
EDTA	Ethylenediamine Tetraacetic Acid
EGF	Endothelial Growth Factor
ER	Estrogen Receptor
ER-α	Estrogen Receptor Alpha
ER-β	Estrogen Receptor Beta
FBS	Fetal Bovine Serum
FGF8	Fibroblast Growth Factor 8
GPR30/GPER	G-Protein-Coupled Receptor-30
IGFs	Insulin-like Growth Factors
IGFBP	Insulin-like Growth Factor Binding Protein
JAK/STAT	Janus Kinase/Signal Transducers and Activators of Transcription
MAPK	Mitogen-Activated Protein Kinase
mSCF	Membrane Stem Cell Factor
PAP	Prostatic Acid Phosphatase
PBS	Phosphate Buffer Saline
PCa	PCa
PCR	Polymerase Chain Reaction

PFA	Paraformaldehyde
PIA	Proliferative Inflammatory Atrophy
PI3K/AKT	Phosphatidylinositol 3-Kinase AKT
PIN	Prostatic Intraepithelial Neoplasia
PMSF	Phenylmethylsulfonyl Fluoride
pNA	p-Nitro-Aniline
PLC-γ	Phospholipase-Cγ
PSA	Prostatic Specific Antigen
PVDF	Polyvinylidene Difluoride
qPCR	Real-time Quantitative Polymerase Chain Reaction
RIPA	Radioimmunoprecipitation
ROS	Reactive Oxygen Species
SCF	Stem Cell Factor
SDS-PAGE	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
sSCF	Soluble Stem Cell Factor
s-KIT	Soluble c-KIT
TAP	Transit Amplifying Population
TGF	Transforming Growth Factor
Tr-KIT	Truncated c-KIT
TRUS	Transrectal Ultrasound
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling
WB	Western Blot

I. Introduction

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Both authors

1. Anatomy and Physiology of Prostate Gland: Brief Overview

The prostate is an accessory gland of the male reproductive tract, with a walnut shape and size (Fig. I-1). This gland is located dorsally to the symphysis pubis, anterior to the rectum, and at the base of the urinary bladder. It surrounds the first part of urethra, known as prostatic urethra, and the two ejaculatory ducts (Lee et al., 2011;VanPutte et al., 2014).

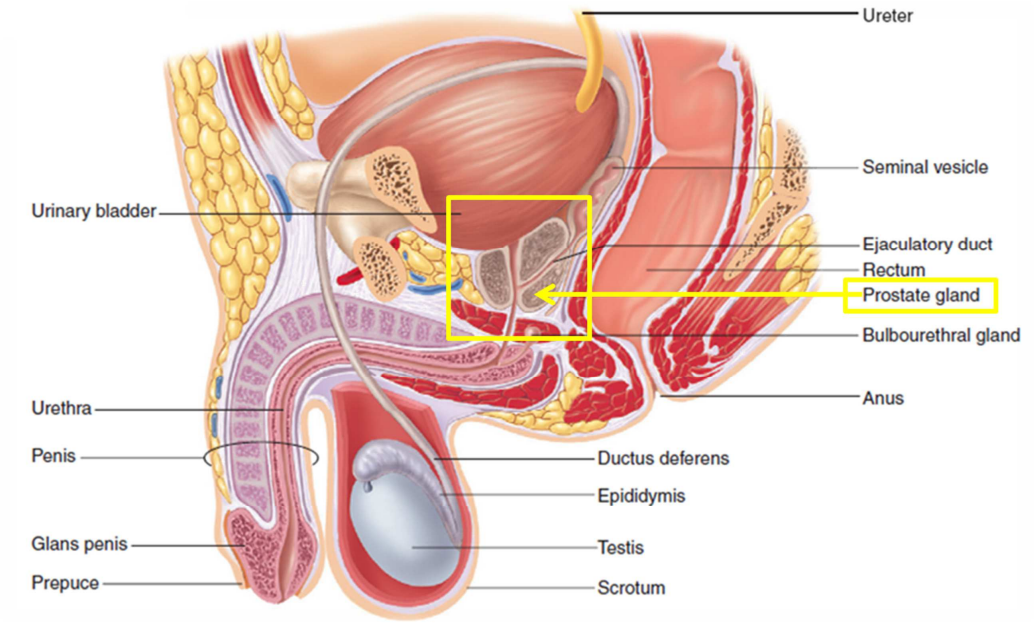


Figure I-1. Localization of the prostate gland in the male reproductive system (adapted from (VanPutte et al., 2014)).

The prostate gland consists of divided tubulo-acinar glands, surrounded by a fibromuscular stroma. It is composed by the posterior and lateral regions, enclosed by a partial capsule, and by the anterior and apical regions, enclosed by the anterior fibromuscular stroma (Sooriakumaran et al., 2012;Young et al., 2014).

Several models were proposed to characterize the prostate anatomy. The currently most accepted divides the prostate in four distinct zones (Fig. I-2)(McNeal, 1981):

- A central zone, which surrounds the ejaculatory ducts, consisting of 20% of the prostate.
- A peripheral zone, consisting of, approximately, 70% of the gland. This zone makes up the bulk of the prostate.
- An anterior fibromuscular stroma, composed by fibromuscular tissue contiguous with the bladder, without glandular tissue.
- A transition zone, surrounding the proximal prostatic urethra, and consisting of about 5% of the glandular tissue.

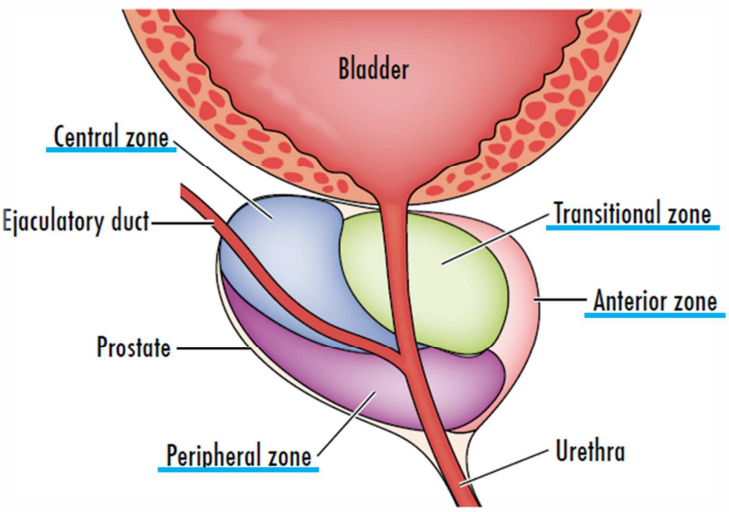


Figure I-2. The zonal anatomy of the prostate gland (Eylert and Persad, 2012).

The epithelium of the prostate consists of three main types of cells: the secretory epithelial cells, the basal cells and the stem cells (Frick and Aulitzky, 1991). The presence of cells morphologically different from the smooth muscle was detected, more recently, and these cells were called interstitial cells of Cajal (Van der Aa et al., 2003;Shafik et al., 2005). The non-cellular stroma and connective tissue compose the extra-cellular matrix presenting elements that control prostatic development and function (Frick and Aulitzky, 1991).

The prostate is an exocrine gland, because it has the function of secrete components of seminal fluid, making this a nutritive and lubricating fluid medium in which spermatozoa are transported (Young et al., 2014). The prostatic fluid has alkaline properties that help to protect the spermatozoa from the acidic vas deferens fluid and the acidic environment of the female reproductive system (Guyton and Hall, 2006).

The prostatic secretions constitute, approximately, 30% of the semen (Guyton and Hall, 2006) and are related with its gelation, coagulation, and liquefaction, and some of the secreted proteins are involved in the coating and uncoating of spermatozoa and in interactions with cervical mucus (Aumuller and Seitz, 1990). Secreted products include ions like zinc and calcium, citrate, phosphate, citric acid, spermine, prostaglandins, cholesterol, seminin, clotting enzyme, profibrinolysin, the acid phosphatase, the prostatic acid phosphatase enzyme (PAP), used to assess prostate function, and the well-known prostatic specific antigen (PSA), the mainly indicator of prostatic disease (Frick and Aulitzky, 1991;Hayward and Cunha, 2000;Guyton and Hall, 2006). PSA is detected in bloodstream in case of pathology of the prostate, like chronic inflammation or cancer (Hayward and Cunha, 2000).

2. Prostate Cancer

Prostate cancer (PCa) is the third most frequent type of cancer after the cancers of the female breast and colorectal. Furthermore, PCa is the most common type of oncological disorder among men, in Europe, with an incidence of 214:1000 which has been increasing in last years. Moreover, 416.700 new cases has emerged and 92.200 men died, in Europe, in 2012, with PCa (Heidenreich et al., 2011;Ferlay et al., 2013). In the same year, only in Portugal, 6620 new cases of PCa emerged and it were estimated 1580 deaths (Ferlay et al., 2013;Miranda et al., 2013). The incidence and mortality of PCa differ with the geography, with many factors contributing to these variances (Ferlay et al., 2013;Tewari et al., 2014), but in all over the world, men that suffer from this terrible disease are losing its life quality.

PCa is a highly heterogeneous disease, both in terms of its pathology and clinical presentation, and presents several developmental stages. It starts to be localized in the prostate, with genetic aberrations, local invasion of extracellular matrix, but subsequently can progress to invasion of secondary organs. At the initial stages PCa is modulated by hormonal milieu and in later stages becomes resistant to hormonal modulation.

When cancer develops, the prostate structures become progressively less organized with smaller ducts and ultimately the gland may lose these structures entirely. With further growth, the tumors invade surrounding tissues and metastasize to the lymph nodes, bladder, and bone (Miller et al., 2003). In addition to structural changes, many molecular changes occur in PCa initiation and progression. The expression profile of many genes, namely, genes related with proliferation, apoptosis, and response to stress, is changed, due to genetic events that include epigenetic changes, chromosomal alterations, somatic mutations and alternative splicing (DeMarzo et al., 2003;Reynolds, 2008;Cheng et al., 2012). The cellular metabolism is also changed in PCa (Vaz et al., 2012;Zadra et al., 2013). Nevertheless, the metastatic potential of PCa is the most alertness problem of this type of cancer. Proliferation, neovascularization and extravasation at the primary site are events involved in metastasis (Clarke et al., 2009).

The majority of PCa cases are adenocarcinomas, which arise from the peripheral zone (approximately 70 %). On the other hand, the anterior fibromuscular stroma and the central zone are less related with cancer development, with, respectively, 25 % and 1-2 % of the cases (Hammerich et al., 2009;Lee et al., 2011;Sooriakumaran et al., 2012).

There are evidences that PCa arises slowly from proliferating stem cells or the transit amplifying population (TAP) from the basal compartment (Isaacs and Coffey, 1989). These stem cells differentiate into luminal cells in response to androgens. The TAP seems to expresses androgen receptor (AR). Moreover, *in vitro* stimulation of TAP with androgens promotes cells differentiation by inducing the expression of markers of luminal cells, such as

cytokeratin-18 (CK18), AR, PAP and PSA. Additionally, androgens down-regulate the $\alpha_2\beta_1$ integrin expression, a molecule implicated in the maintenance of the immature basal cell phenotype (Heer et al., 2007). However, there is conflicting evidence regarding whether the tumors arise from basal cells or from the luminal epithelial cells (Wang et al., 2009;Lawson et al., 2010;Wang et al., 2013).

Indeed, PCa etiology remains largely unknown. Many factors can contribute to the development of this disease, but the best characterized are aging, ethnicity and familiar factors (Heidenreich et al., 2011;Tewari et al., 2014). The principal risk factor for PCa development is the aging (Tewari et al., 2014). Moreover, PCa is more frequently diagnosed in older men and they are more likely to die from the disease than younger men (Hoffman, 2012). In childhood, the human prostate is small, weighing around 2 g. Then, it undertakes exponential growth, at puberty, increasing to about 20 g of weight, due to the rise in serum testosterone levels, characteristic of this phase of life. After that, the weight of the prostate tends to become stable and remains constant until the 40-50 years old, age in which prostatic weight may begin to rise slowly (Berry et al., 1984;Guyton and Hall, 2006). This slowly increase in prostate weight frequently leads to the onset of Benign Prostatic Hyperplasia (BPH) (Berry et al., 1984), a condition characterized by the benign growth of the prostate gland from its normal size to a larger size that can be larger than 150 g (Powers and Marker, 2013). Although there are differences between BPH and PCa, these diseases have some commonalities, and there are evidences that men with BPH have an increased risk of PCa and PCa-related mortality (Orsted and Bojesen, 2013). The race/ethnicity also seems to be a PCa high risk factor, since African men present higher PCa incidence than other groups (Burks and Littleton, 1992;Mordukhovich et al., 2011). The heredity has an important role in PCa development, because the presence of affected first-degree relatives increases the PCa risk in men (Hemminki and Czene, 2002;Zeegers et al., 2003). Although these three factors are the main risk factors related with PCa, other may contribute to the disease only or in combination. Some of them include environmental factors, as the use of pesticides, dietary habits, lifestyle, inflammation and hormones (Giovannucci et al., 1997;Karan et al., 2008). In fact, exposure to pesticides showed to be related with PCa development (Lemarchand et al., 2014). Although some reports did not found correlation between obesity and PCa (Habel et al., 2000), others showed the opposite (Calle et al., 2003). Accordingly, it has been reported that a high-fat rich diet may contribute to PCa (Brasky et al., 2013). Some of the previous numbered factors like environmental, dietary and hormonal factors may contribute to PCa through inflammation (De Marzo et al., 2007;Karan et al., 2008). Some studies reported inflammation as a high risk factor for PCa (Roberts et al., 2004;Atala, 2014). Inflammation may contribute to the pathogenesis and development of cancers, since it can cause infiltrating cell, like leucocytes to release reactive oxygen species (ROS) that may destroy cellular structures and damage genome, which leads to activation of cell proliferation in order to supplant damaged cells, and to release of many cytokines that promote cell division,

angiogenesis and tissue repair. It can also contribute to destroy cell-cell and cell-matrix adhesion, important factors in cancer progression (Coussens and Werb, 2002;Alberti, 2008;Acharya et al., 2010). Moreover, inflammation damages the prostatic epithelium, cause lesions with atrophic morphology, but the cells remain able to proliferate. This condition is named proliferative inflammatory atrophy (PIA) (De Marzo et al., 1999). Morphological transitions can lead to histological lesions causing prostatic intraepithelial neoplasia (PIN), and it was shown that 40% of high-grade PIN lesions arise directly from PIA (Putzi and De Marzo, 2000). Thus, it is clear that PIA may be the linkage between inflammation and PCa.

PCa remains asymptomatic for a long time, which hinders the early screening of the disease. When symptoms become evident, it include urinary symptoms including hematospermia and hematuria, in localized PCA, and rectal obstruction, bone pain, and more systemic features of malignancy, in advanced disease (Tewari et al., 2014).

The PCa screening tests include, at first line, the physical examination with a digital rectal examination (DRE) and measurement of serum PSA. Based on these results, if there are suspicious findings, more sophisticated diagnostic techniques as transrectal ultrasound (TRUS) and guided systematic biopsy are used (Heidenreich et al., 2011;Tewari et al., 2014). The biopsy allows to determine the Gleason score, that measures the aggressiveness of the cancer, predicting prognosis and guiding treatment (Gleason and Mellinger, 1974). In fact, PSA is the mostly used biomarker to screen PCa (Ercole et al., 1987;Phillips, 2014). However, this test lacks some important features needed to a correct diagnosis, namely, sensitivity and specificity (Thompson et al., 2004;Eylert and Persad, 2012). This is a problem that may affect the successfully diagnosis and treatment with serious consequences. In last years, many studies aimed to found new biomarkers for PCa (Crawford et al., 2014).

Some therapeutic options are available to treat PCa. These include radical prostatectomy, brachytherapy, focal therapies (e.g. high intensity focused ultrasound, cryotherapy) and androgen deprivation treatment for local PCa (Eylert and Persad, 2012). In some cases of men with small tumors that have not spread beyond the gland it is recommend close monitoring but without applicate aggressive treatment, a strategy named “watchful waiting” (Eylert and Persad, 2012). In the case of metastasis, it may be used hormone therapy, chemotherapy or radiotherapy (Drudge-Coates and Turner, 2012). Nevertheless, these options are not always effective and investigation to better understand the molecular and cellular mechanisms involved in PCa, is still needed.

3. Hormonal Actions and Prostate Carcinogenesis

The hormonal factors, namely, steroid hormones have important roles on prostate development and physiology, but are also a predisposition factor to PCa development. According to this, several studies reported that administration of male sex hormones can induce PCa in animal models (Noble, 1977).

3.1. Androgens

Androgens are the most abundant sex steroids in men. They are responsible for the development of male organs and secondary sexual characteristics. The most well-known androgens are testosterone and its 5α-reduced metabolite, the potent androgen 5α-dihydrotestosterone (DHT). Testosterone and DHT are considered potent androgens, since even in low dosages they stimulate androgen-dependent structures, like prostate. Testosterone is the most important androgen in men, being mainly produced by Leydig cells on the testis, but also adrenal glands secrete low quantities of this steroid. Other common androgens include the dehydroepiandrosterone (DHEA), its metabolite the dehydroepiandrosterone sulfate (DHEAS) and androstenedione. These are weak androgens produced by the adrenal glands (Jones and Lopez, 2006).

Androgens exert their actions through binding to the intracellular AR, a member of the nuclear receptor superfamily that acts as a ligand dependent transcription factor (Mangelsdorf et al., 1995). This binding activates AR, allowing it to bind DNA and to recruit co-regulators for induction or repression of downstream gene transcription (Heinlein and Chang, 2002).

In the prostate, the biologically active androgen is the DHT that is produced by the local reduction of testosterone produced from the testis by the 5α-reductase enzyme. As other male organs, the prostate development and growth is regulated by androgens since the embryonic development to adulthood (Sensibar, 1995). Testosterone regulates the glandular morphogenesis in the developing prostate and the prostatic function and glandular maintenance in the adult prostate (Sensibar, 1995). Androgens are required for normal prostate development, acting directly on prostate epithelial cells to induce terminal differentiation, or acting at indirect way on prostate epithelium proliferation, through induction of secretory growth factors by the adjacent stroma (Isaacs and Coffey, 1989). In normal prostate, epithelial AR acts predominantly to produce the proteins secreted by the prostate and the stromal AR promotes growth (Lai et al., 2012).

Since androgens are so important in prostate development, hormonal changes may be related with prostate diseases, namely with PCa. Huggins and Hodges were the first to postulate that androgens promote prostate carcinogenesis (Huggins, 1967;Huggins and

Hodges, 1972). In fact, strong evidences reported a correlation between androgens and PCa, for example, African men are thought to have higher androgenic levels, leading to the higher incidence of PCa (Ross et al., 1998). Other evidences showed that estosterone and androstanediol glucuronide were statistically significant positively associated with PCa (Gann et al., 1996).

A comparative study of the effects of DHEA, testosterone and DHT in LNCaP cells revealed that all the androgens increased cell proliferation and PCa biomarkers, however, the DHEA showed delayed effects compared to the other two hormones (Arnold et al., 2005). Furthermore, animal studies also support this role: testosterone caused adenocarcinomas in the dorsolateral prostate of rats and a PCa incidence increase in different rat species (Bosland and Mahmoud, 2011).

The balance between proliferation and apoptosis is crucial to maintain the normal function of prostate. Androgens seem to act as regulators of the both processes through several mechanisms. One of the most common genetic alterations in PCa is the fusion between two genes, namely, the TMPRSS2 gene and the ETS transcription factor genes, ERG or ETV1. TMPRSS2 gene codifies a membrane-bound serine protease, which is regulated by androgens and overexpressed in PCa, and ETS transcription factor genes are involved in multiple processes, including cell proliferation and cancer cell invasion (Lin et al., 1999;Hsu et al., 2004;Tomlins et al., 2005). There are evidences that androgens may induce the translocation of TMPRSS2-ERG and TMPRSS2-ETV1 (Lin et al., 2009;Cai et al., 2010), suggesting a potential mechanism by which androgens promote prostate carcinogenesis through inducing gene translocation. Moreover, androgens regulate the expression of growth factors and their receptors, namely, insulin-like growth factors (IGFs), fibroblast growth factor (FGF8) and endothelial growth factor (EGF), that are involved in prostate cell proliferation, migration, and tumor angiogenesis, thereby facilitating prostate carcinogenesis and cancer progression (Rudra-Ganguly et al., 1998;Torring et al., 2003;Wu et al., 2007).

Androgens also seem to crosstalk with the downstream effectors of growth factor signaling, such as phosphatidylinositol 3-Kinase/AKT (PI3K/AKT), that plays a critical role in prostate carcinogenesis and its progression (Vivanco and Sawyers, 2002). At cell cycle, androgens may increase cyclin expression and promote the assembly of active cyclin/cyclin-dependent kinase (CDK) complexes (Xu et al., 2006;Balk and Knudsen, 2008). Androgens also may increase the cell proliferation through induction of autophagy and intracellular lipid accumulation (Shi et al., 2013) and also through increase of ROS in PCa cells to a level that potentiates cell growth (Lin et al., 2010;Lu et al., 2010).

Relatively to apoptosis, an important role of the androgens in its prevention has been also reported (Raclaw et al., 2008). Androgen treatment of the LNCaP hormone-dependent human PCa cell line induces increased expression of the anti-apoptotic Bcl-2 protein

(Berchem et al., 1995). The expression of the cyclin-dependent kinase inhibitor p21 gene was shown to be up-regulated by androgens and functioned as an apoptosis inhibitor to promote LNCaP cell growth (Lu et al., 1999). Moreover, androgens may suppress the TNFR family (TNF-α/Fas)-induced apoptosis through inhibition of p53 expression and caspase-2 activation (Rokhlin et al., 2005). Other studies also showed that androgen could block apoptosis induced by Fas activation and TNF-α and reduce the Bax expression (Kimura et al., 2001).

Nevertheless, despite the pro-proliferative and anti-apoptotic effects of androgens, studies that report the opposite effect have been emerging (Wen et al., 2014).

The AR signaling pathway has been implicated in early PCa growth, metastatic disease, development of hormonal resistance and disease relapse. In the majority of PCa cases, the AR is expressed through all stages of development (Debes and Tindall, 2002).

Since AR is a critical effector of PCa development and progression, androgen deprivation therapies (ADT) by ablation of AR function through ligand depletion and/or the use of AR antagonists are the first line of therapeutic intervention. This triggers the cell death or cell cycle arrest of PCa cells (Knudsen et al., 1998;Agus et al., 1999;Koksal et al., 2010). Although these strategies are initially effective, recurrent tumors may arise as a result of inappropriately restored AR function (Feldman and Feldman, 2001).

Many AR alterations are related with PCa progression to more advanced stages, namely, the androgen-independent PCa. Some of these mechanisms involve the response of AR even at low levels of androgens, namely due to mutations, overexpression/amplification, activation by growth factors and cytokines, altered expression co-activators, calpain proteolysis or even by the increased local production of androgen by prostate cells (Linja and Visakorpi, 2004;Devlin and Mudryj, 2009;Saraon et al., 2011).

Somatic mutations of AR are mostly gain-of-function, which can lead to androgen hypersensitivity or decreased ligand specificity, contributing to PCa development (Han et al., 2005). In fact, AR gene mutations are rare in patients with primary PCa but have been reported with a higher frequency in bone marrow specimens from patients with advanced disease (Taplin and Balk, 2004). AR amplification/overexpression sensitizes cells to low concentrations of the ligand (Waltering et al., 2009), and is a process mainly present in tumors that recur after ADT (Bubendorf et al., 1999;Ford et al., 2003). Constitutively active AR splice variants, generated by gene splicing or genomic rearrangement, were detected in PCa cell lines and tumor samples (Guo et al., 2009;Hu et al., 2009), and may be responsible for recurrent PCa through alternative transcriptional output (Hu et al., 2012a). Posttranslational modifications of AR command activity, structure, and stability, including phosphorylation, ubiquitylation, acetylation and methylation. Overall, the majority of these modifications results in AR activation and is related with progression of PCa (McCall et al.,

2008). Other important question related with PCa progression is that, in some cases, the enzymes necessary for androgen synthesis are present and some of them elevated in PCa metastasis or recurrent tumors (Montgomery et al., 2008).

3.2. Estrogens

Estrogens are mainly known as "female sex hormones". However, they are also present in male serum at low levels and their physiological action has been deserved attention. There are different estrogens, including the natural and synthetic compounds. The natural estrogens include estrone (E_1), 17β-estradiol (E_2) and estriol (E_3). The most well-known and potent is the E_2 (Krolik and Milnerowicz, 2012).

In men, the estrogens production results from the testosterone conversion by aromatase. This phenomenon occurs mainly in the Sertoli cells of the testis, but also in other tissues, including the prostate, which express many of the steroidogenic enzymes, involved in estrogen biosynthesis and metabolism (Takase et al., 2006). In that way, E_1 and E_2 may be produced locally within the prostate via aromatization of androstenedione and testosterone, respectively (Ellem and Risbridger, 2006). Thus, estrogens also seem to have an important role in normal development of prostate gland.

Despite the classical role of androgens as stimulating agents in PCa growth, currently, estrogens also have been implicated in the onset and progression of PCa. However, a duality for the possible role of estrogens in prostate cells has been gaining consistency over the last years. If some studies defend that estrogens are potential causative agents of PCa other strong evidences indicate that these steroids may be protective against PCa.

Estrogens have been associated with the uncontrolled growth and transformation observed in PCa (Carruba, 2007;Ho et al., 2011). In fact, the estrogens/androgens ratio increases with age in parallel with a decrease in testosterone levels. That coincides with an increasing prevalence of PCa in older men, suggesting, in fact, a role of estrogens in prostate carcinogenesis. This increased ratio seems to induce the development of inflammation upon aging and the onset of premalignant lesions (Vermeulen et al., 2002;Ellem and Risbridger, 2010).

An increased risk to develop PCa was found in patients with high E_2 levels (Salonia et al., 2011). Moreover, E_2 exposure seems to neoplastically transform the rat prostatic epithelial cells *in vitro* (Yu et al., 2011b). Other studies confirm that estrogens, alone or in combination with androgens are potent inducers of cell growth and differentiation in PCa (Ricke et al., 2007), and may induce squamous metaplasia of prostatic epithelium (Risbridger et al., 2001). Moreover, estrogens in combination to androgens showed to increase the PCa incidence in rodents in comparison to androgens alone (Bosland et al., 1995), and this

combination was thought to be necessary to PCa development (McPherson et al., 2001;Setlur et al., 2010), but now is known that even alone, estrogens may contribute to PCa development. Furthermore, estrogens also contribute to PCa progression and metastasis in nude mice (Ricke et al., 2006).

Also *in vitro* studies support these adverse effects of estrogens. Physiological concentrations of estrogens stimulate LNCaP cell proliferation and PSA expression (Castagnetta et al., 1995;Arnold et al., 2005). Accordingly, proliferation was also increased in normal prostate stromal cells (PrSC) and LNCaP cells treated with E_2, and the ratio estrogens/andogens seems to influence this proliferation (King et al., 2006). E_2 treatment also showed effects in cell invasion by up-regulating the production of matrix metalloproteinase 2 in PrSC and WPMY-1 cells, which was mediated by the TGFß1 (Yu et al., 2011a) and induced stromal cell paracrine effects that promote PCa cell migration, through increasing stability of enolase 1, a critical enzyme for cellular energy metabolism, and promoting its secretion to the extracellular matrix (Yu et al., 2012).

Other mechanisms underlying the action of estrogens in carcinogenesis include the increase in the proliferation of epithelial cells; the up-regulation of growth factors-dependent signaling pathways that promote aberrant cell growth; the increase of prolactin-receptor signaling; the mitogen-activated protein kinase (MAPK) activation; the increased cell-survival potential through the overexpression of anti-apoptotic mediators; the elevation in oxidative stress-induced DNA damage; changes in gene-expression profiles related to cell proliferation, DNA damage, activation of proto-oncogenes and transforming factors; the breakdown of epithelial basement membrane, stromal extracellular matrix, the stimulation of inflammation and overexpression of anti-apoptotic mediators (Pandini et al., 2007;Ho et al., 2011). Other important question related to the estrogens is that they can be chemicals carcinogens, because they can be converted to reactive estrogens intermediates and cause damage to DNA and lipids causing mutations that may be involved in PCa development (Bosland, 2012). Moreover, catechol estrogens showed the ability of induces proliferation and malignant transformation in prostate epithelial cells (Mosli et al., 2013).

The endocrine disruptors (EDs) are a great threat to reproductive physiology and have been linked with the carcinogenesis of reproductive tissues. The majority of the well-studied EDs are estrogen agonists, which mimic the estrogen activity of endogenous hormones. EDs include certain pesticide residues on food, chemicals used in plastics production and phytoestrogens in dietary plant products (Prins, 2008;Hu et al., 2012b). The exposure to some EDs seems to contribute to prostate carcinogenesis (Hardell et al., 2006;Mahajan et al., 2006). The sensitivity of the prostate to ED seems to be higher during the fetal and neonatal development as well as during puberty (Prins et al., 2007;Lobaccaro and Trousson, 2014). However, neither all the EDs have a predisposing role for development of PCa, for example,

some phytoestrogens have showed a potential anti-carcinogenic effect with interest in PCa (Stephens, 1997).

On the other hand, there are evidences of the protective role of estrogens in prostate physiology. In rodents, low doses of estrogens have been shown to enhance prostatic growth, but high doses are generally growth inhibitory (vom Saal et al., 1997). Moreover, pharmacologic levels of estrogens inhibited the prostatic development, resulting in impaired growth and decreased responsiveness to androgens in adulthood (Naslund and Coffey, 1986;Prins, 1992). Other evidences have suggested that estrogens directly inhibit growth of PCa when administered *in vitro* in the absence of circulating hormones (Robertson et al., 1996). E_2 showed to be able to suppress recurrent PCa growth and to delay mortality in multiple castration resistant xenograft models *in vivo* (Corey et al., 2002). Accordingly, E_2 caused inhibition of PCa growth in an animal model by mechanisms that are independent of androgen action (Corey et al., 2002). Other studies are in accordance with the anti-proliferative role of estrogens, showing that E_2 inhibits growth of hormone-nonresponsive PC3 cells (Carruba et al., 1994;Kanagaraj et al., 2007). It is also the case of DU145 cells that showed decreased proliferation with the treatment with estrogens (Pravettoni et al., 2007).

Estrogenic actions are mediated by the classic intracellular receptor proteins (estrogen receptors alpha (ERα) and beta (ERβ), which act as transcription factors regulating the expression of target genes (Gibson and Saunders, 2012). Alternatively, estrogens may elicit signaling events by interaction with the G-protein-coupled receptor-30 (GPR30/GPER) (Prossnitz et al., 2008), but the expression and functionality of this receptor in prostate remains less characterized. ERα and ERβ are expressed in rodent and human prostate during development and into adulthood (Enmark et al., 1997;Prins et al., 1998). The classical ER, ERα, has been detected almost exclusively on the stroma and in subsets of basal cells, and ERβ seems to be expressed in the luminal and basal epithelial cells, with lower or none expression in stromal cells (Leav et al., 2001).

The ability of estrogens to suppress tumor growth and proliferation of prostatic cells may include receptor dependent mechanisms through inhibition of ERβ (Pravettoni et al., 2007), or receptor independent mechanisms such as induction of immune surveillance and metabolism of E_2 to cytotoxic estrogens such as 2-methoxyestradiol (Robertson et al., 1996;Qadan et al., 2001). Other reported mechanism was the inhibition of matrix metalloproteinases and increased levels of IGF binding proteins IGFBP-3 and IGFBP-4 associated with apoptosis, suggesting that estrogens may inhibit the proliferation of PCa cells by inducing apoptosis (Kanagaraj et al., 2007). The tumor suppressive role of E_2 may also be explained by the suppression of androgen levels by E_2 (Montgomery et al., 2010), due to its negative feedback at the hypothalamus/pituitary, and for this reason estrogens have been

used as therapeutic options in PCa (Oh, 2002;Gomella, 2009). However, the application of estrogen based therapies is a theme with strong discussion associated (Oh, 2002).

More recently it was reported that the anti-tumor effects may involve an anti-angiogenesis role of E_2, explained by its ability to decrease the microvessel number in the tumor tissues (Wen et al., 2013).

Currently, it is accepted that the opposite roles of estrogens are related with differential responses driven by ERα and ERβ (Risbridger et al., 2007;Ellem and Risbridger, 2009). Indeed, it has been shown that ERα and ERβ have opposite roles in PCa development. A recent study reported that ERα stimulates the genesis and progression of adenocarcinoma in the rat ventral prostate, while ERβ inhibits the onset of precancerous PIN lesions (Attia and Ederveen, 2012). Other evidences support the adverse effects of ERα and the protective role of ERβ, in respect to inflammation, proliferation and other mechanisms leading to prostate carcinogenesis (Pravettoni et al., 2007;Ellem and Risbridger, 2010;McPherson et al., 2010;Piccolella et al., 2014).

As AR, ERs also may undergo modifications, like genetic polymorphisms that may be responsible for different responses in PCa development (Tanaka et al., 2003). Both ERα and ERβ present splice variants. ERα displays, at least, five distinct mRNA isoforms, ERα-A-E (Ye et al., 2000). All these variants present deletions at the C-terminal ligand-binding domain, which is essential for receptor dimerization (Ye et al., 2000). 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 (Ye et al., 2000). ERβ also presents five different mRNA isoforms (ERβ1-5). In this case the isoforms result from truncation or insertion in the ligand-binding domain (Lewandowski et al., 2002). These modifications are responsible for different roles of each isoform, for example, ERβ1 and ERβ2 showed opposite roles in regulating proliferation and bone metastasis genes, while the first has tumor-suppressing effects, the second has oncogenic capabilities (Dey et al., 2012). Moreover, several polymorphisms of ERα and ERβ were reported to be related with increased PCa risk (Tanaka et al., 2003;Thellenberg-Karlsson et al., 2006).

4. The Stem Cell Factor (SCF)/c-KIT System

The c-KIT and its ligand, the SCF, are expressed in a broad range of tissues, including brain, breast, testis, skin and prostate (Lammie et al., 1994)

The SCF/c-KIT system has been shown to play an important function in melanogenesis, hematopoiesis and gametogenesis (Nishikawa et al., 1991;Ratajczak et al., 1992;Sato et al., 2012) through the regulation of several biological processes, such as cell proliferation, differentiation, migration and apoptosis (Ronnstrand, 2004;Farini et al., 2007). The importance of SCF/c-KIT system in prostate also has been suggested (Leong et al., 2008).

Despite the expression and the important functions of SCF and c-KIT in normal tissues, they are also expressed in several types of cancer (Natali et al., 1992;Simak et al., 2000). Furthermore, they have been described as having important roles in cancer development and progression in many tissues, including lung, breast, pancreas and prostate (Krystal et al., 1996;Di Lorenzo et al., 2004;Ulivi et al., 2004;Wiesner et al., 2008;Zhang et al., 2011).

Moreover, given the close relationship of SCF/c-KIT system with cancer, the development of specific inhibitors interfering with c-KIT signal transduction pathways emerged as an exciting field in cancer treatment (Lennartsson and Ronnstrand, 2006;Ashman and Griffith, 2013).

4.1. The c-KIT Receptor: Molecular Biology and Signaling

The c-KIT receptor, also known as CD117, stem cell factor receptor or KIT receptor, was firstly described in 1986 as the transforming gene of the Hardy-Zuckerman 4 feline sarcoma virus, being identified as the proto-oncogene *v-KIT* (Yarden et al., 1987). The mouse *c-KIT* gene is located in the *dominant white spotted* locus *(W)* (Chabot et al., 1988;Geissler et al., 1988) while the human gene is located on chromosome 4q11-q12 with a total length of 90 kb (d'Auriol et al., 1988). The main product of this gene is a single 5 kb transcript (Yarden et al., 1987) encoding a transmembrane glycoprotein with approximately 145-160 kDa that belongs to the type III receptor tyrosine kinase family (Ullrich and Schlessinger, 1990;Blume-Jensen and Hunter, 2001). This class of receptors shares a common structure encompassing three main functional regions (Fig. I-3): an intracellular kinase domain, a transmembrane region, and an extracellular ligand-binding domain (Lemmon and Ferguson, 2007). The cytoplasmic region of c-KIT, responsible for the signaling transduction, contains proximal and distal kinase domains, separated by an interkinase domain, and binding sites for ATP and magnesium ions (Mol et al., 2003;Roskoski, 2005). The transmembrane region is constituted for a short hydrophobic chain of amino acids, allowing the receptor to fix in the plasmatic membrane. The extracellular region is organized in five immunoglobulin-like domains and

have the function of recognize the ligand, but it also participates in receptor dimerization (Yuzawa et al., 2007;Paulhe et al., 2009).

Mechanisms of alternative splicing and others have been identified to originate c-KIT protein variants. One example is the c-KIT isoforms that differ in the presence or absence of the tetrapeptide Gly-Asn-Asn-Lys (GNNK) sequence in the juxtamembrane region of the extracellular domain of the receptor (Caruana et al., 1999;Voytyuk et al., 2003). Both isoforms of c-KIT bind the ligand with identical affinity (Caruana et al., 1999), but the effect of stimulation is faster and more pronounced in GNNK-negative c-KIT (Montero et al., 2008). Recently, it was demonstrated that the GNNK in the extracellular juxtamembrane domain of c-KIT (Fig. I-3) plays a relevant role regulating receptor activation and signaling (Phung et al., 2013).

A mechanism of alternative promoter usage and RNA transcription from a cryptic exon produces a truncated form of c-KIT protein (tr-KIT) with approximately 30-50 kDa which do not have the extracellular domain and the transmembrane region, and is located at cytoplasm (Rossi et al., 1992;Toyota et al., 1994;Albanesi et al., 1996;Takaoka et al., 1997;Muciaccia et al., 2010). tr-KIT do not have the first part of the kinase domain, lacking the kinase activity (Rossi et al., 1992), but retains the capability to induce signaling transduction (Sette et al., 1998). In fact, this tr-KIT can interact with other tyrosine kinase receptors, or with other receptor types (Sette et al., 1998), as a scaffold protein regulating multiple signaling pathways. This isoform of c-KIT has been detected in germ cells and human cancer cells (Rossi et al., 1992;Toyota et al., 1994).

c-KIT also can be proteolytically cleaved and released from the cell membrane originating a soluble isoform (Broudy et al., 1994;Turner et al., 1995), which could be detected in human serum (Wypych et al., 1995). It has only the extracellular domain and binds the ligand with the same affinity as the full-length c-KIT, suggesting a role in controlling ligand bioavailability (Wypych et al., 1995;Dahlen et al., 2001). More recently, serum levels of soluble c-KIT have been associated with the hematopoietic disorders, mobilization of hematopoietic stem cells to peripheral blood, asthma severity, and clinical outcome of patients with gastrointestinal stromal tumors (Kawakita et al., 1995;Nakamura et al., 2004;Deprimo et al., 2009;Makowska et al., 2009).

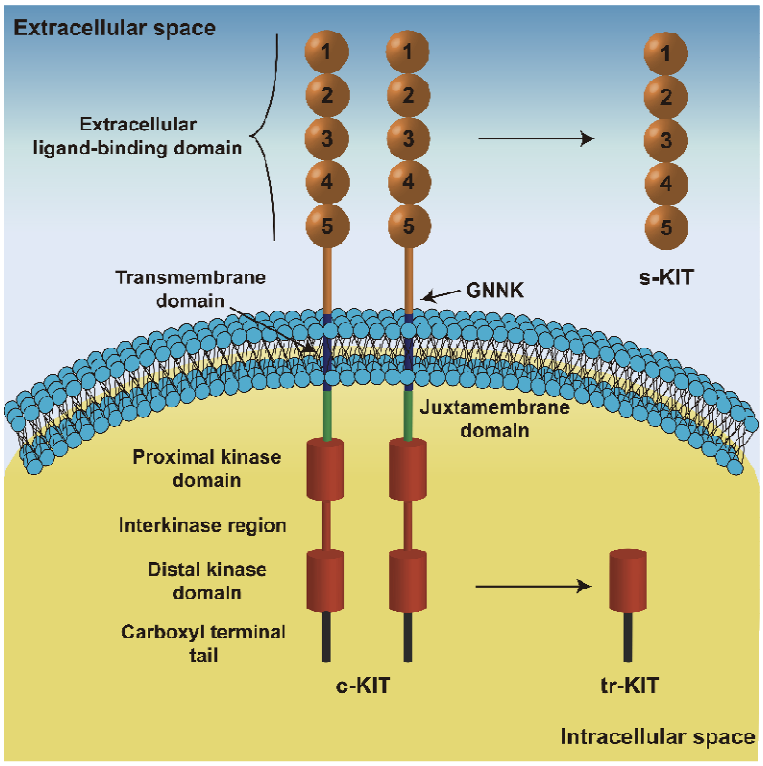


Figure I-3. Schematic representation of c-KIT structure. The five immunoglobin-like domains of the extracellular domain are involved in ligand-binding and receptor dimerization. The transmembrane domain anchors c-KIT in the cytoplasmic membrane. The intracellular region, responsible for signaling transduction, contains proximal and distal kinase domains separated by an interkinase region, and a carboxyl terminal tail. Some alternatively spliced forms of c-KIT are characterized by the presence of the tetrapeptide GNNK in the extracellular juxtamembrane domain. The receptor can be cleaved and released from cell membrane originating a soluble c-KIT (s-KIT) only constituted by the extracellular domain. A truncated form of c-KIT (tr-KIT) originated by mechanisms of alternative promoter usage and RNA transcription from a cryptic exon lacks the extracellular and transmembrane domains retaining part of the kinase domain (Cardoso et al., 2014).

c-KIT signaling mechanisms depend of SCF binding to c-KIT, which results in receptor dimerization and activation of intrinsic tyrosine kinase activity (Blume-Jensen et al., 1991;Philo et al., 1996). The interaction of SCF with c-KIT leads to dimerization of receptor, activation of its tyrosine kinase activity and initiation of downstream signal transduction pathways (Blume-Jensen et al., 1991). The pathways activated by c-KIT signaling include the PI3-K, the Src, the Janus kinase/signal transducers and activators of transcription (JAK/STAT), the phospholipase-Cγ (PLC-γ) and the MAPK pathways.

Through the activation of the several signaling pathways, c-KIT is responsible for many effects at cellular level, including, control of cell proliferation differentiation, survival and apoptosis regulation (Ronnstrand, 2004). Therefore, it is highly understandable that deregulated actions of SCF/c-KIT system have been associated with carcinogenesis.

4.2. Molecular and Functional Aspects of SCF, the c-KIT Ligand

The SCF, also known as steel factor, c-KIT ligand or mast cell growth factor is a growth factor that binds to c-KIT, firstly identified in 1990 (Nocka et al., 1990;Williams et al.,

1990;Zsebo et al., 1990). It is codified on the *Steel locus* (*Sl*) of chromosomes 12 and 10, respectively, in humans and mouse (Zsebo et al., 1990;Geissler et al., 1991). The human, mouse and rat *SCF* genes consist of 9 exons (Martin et al., 1990) and encode a 45 kDa glycoprotein, which is located at the plasma membrane of different cell types (Gagari et al., 2006;Wiesner et al., 2008;Mansuroglu et al., 2009).

The SCF protein is constituted by three distinct regions (Fig. I-4): an intracellular domain (Langley et al., 1994;Zhang et al., 2000), an hydrophobic transmembrane domain, and an extracellular domain responsible for recognizing and binding to c-KIT (Langley et al., 1994). Besides the full-length membrane bound form of SCF (mSCF), with approximately, 45 kDa, soluble forms of SCF also have been identified. The soluble SCF (sSCF) is generated by the specific proteolytic cleavage of an alternative spliced form of SCF. A primary cleavage site is encoded by an alternative exon 6 and produces a 165 amino acid soluble protein (31 kDa) still containing the domain that recognizes c-KIT (Flanagan et al., 1991;Pandiella et al., 1992;Majumdar et al., 1994). Thus, both mSCF and sSCF proteins bind and activate the c-KIT receptor. However, it was verified that mSCF induces persistent activation and longer life span of c-KIT, while sSCF leads to a transient activation and faster degradation (Miyazawa et al., 1995). Miyazawa et al. (1995) proposed that sSCF can down-regulate c-KIT expression by triggering its degradation. The ratio of the two isoforms varies in different tissues, suggesting a tissue specific regulation of the SCF expression (Huang et al., 1992). Moreover, differences exist in the downstream pathways activated by c-KIT upon ligation of soluble or membrane isoforms of SCF (Kapur et al., 2002).

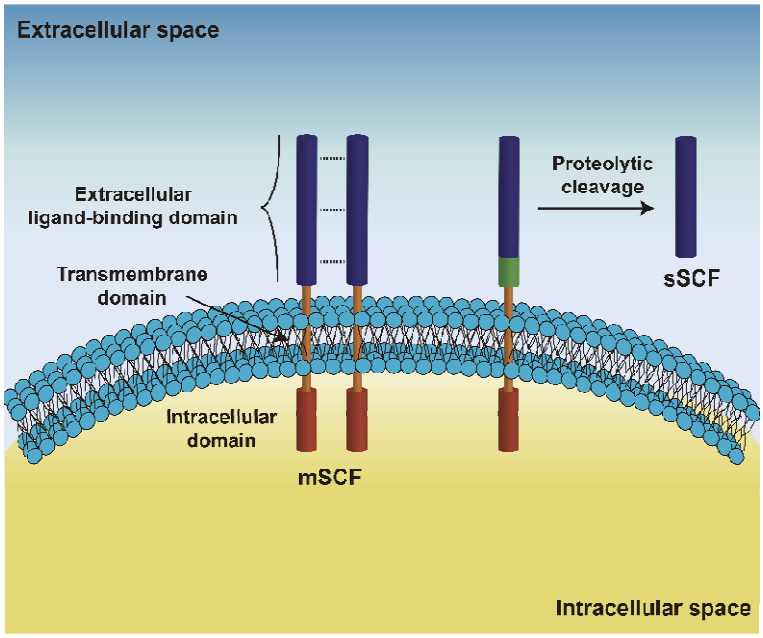


Figure I-4. Schematic representation of SCF structure. The SCF display an extracellular domain, responsible for recognizing and binding to c-KIT, a transmembrane domain and an intracellular domain. The SCF exists as a membrane-bound homodimer (mSCF) or as a soluble protein (sSCF). The sSCF is originated by the proteolytic cleavage of an alternatively spliced variant of SCF that contains the alternative exon 6 (green) (Cardoso et al., 2014).

4.3. The Role of SCF/c-KIT System in Prostate and PCa

SCF is expressed in fibromuscular stroma cells (Lammie et al., 1994;Ceder et al., 2008) and in glandular and ductal epithelial cells, localized in the basal layer of non-malignant prostate (Imura et al., 2012). c-KIT is expressed in basal epithelial cells of ducts and acini, stroma, interstitial and stem cells (Simak et al., 2000;Leong et al., 2008;Imura et al., 2012), and also detected in mast cells (Simak et al., 2000).

SCF/c-KIT system seems to have a preponderant role in PCa development. Expression of c-KIT and SCF was detected in PCa cases (Di Lorenzo et al., 2004;Wiesner et al., 2008). It was also shown that c-KIT was expressed in 5% of BPH cases, 14% of primary PCa, and 40% of bone metastasis (Wiesner et al., 2008). Moreover, it was found a significantly higher expression of SCF and c-KIT in BPH than in normal prostate (Imura et al., 2012), suggesting the involvement of the SCF/c-KIT system in the pathophysiology of prostate. It was observed that SCF increases the proliferation of PrSCs while administration of c-KIT inhibitors blocks SCF-induced proliferative activity (Imura et al., 2012). These SCF/c-KIT effects seem to be mediated by the JAK-STAT signaling pathway and might thus underpin the overgrowth of prostate (Imura et al., 2012).

Also, the expression of tr-KIT, the cytoplasmic variant of c-KIT, appears to be correlated with the more advanced stages of prostatic tumors. It was not expressed in cases of BPH but was expressed in 28% of patients with low Gleason grade tumors and in 66% with more advanced tumors (Paronetto et al., 2004). tr-Kit expression was restricted to the cytoplasm of the epithelial cells of neoplastic prostatic glands (Paronetto et al., 2004). Given the positive correlation between tr-Kit expression and tumor progression, the aberrant expression of nuclear factors able to bind to the human tr-Kit promoter may be an early event in prostate cell transformation (Paronetto et al., 2004).

c-KIT mRNA seems to be significantly increased during androgen deprivation in DuCaP cells compared to non-castrated clinical metastatic disease, although the protein was not detected in these cells (Pfeiffer et al., 2011). As c-KIT play an important role in cell proliferation, this may be one more mechanism involved in cancer reappearance after androgen deprivation (Simmons and Klein, 2009). Considering the apparent role of SCF/c-KIT system in the pathophysiology of prostate, it seems relevant to evaluate the effect of c-KIT inhibitors in the disease progression.

4.4. Hormonal Regulation of the SCF/c-KIT System

The c-KIT receptor and its ligand, SCF, seem to be regulated by hormonal factors, in particular by steroid hormones, and some studies reported this regulation in several tissues.

One evidence of this regulation is the significant correlation found between the expression of c-KIT and the concentrations of testosterone and androstenedione in the follicular fluid (Tanikawa et al., 1998). DHT treatment showed a great induction of SCF expression in the ovary tissue (Shiina et al., 2006). The stimulatory effect of DHT on the expression of SCF was further confirmed in human granulosa-like tumor cells in culture (Shiina et al., 2006). Moreover, testosterone (1 ng/ml) significantly increased the expression of splice variants of SCF in the mouse ovary (Kundu et al., 2012).

In men, it was reported that *in vitro* culture of rat seminiferous tubules with testosterone (10^{-6} M) did not change the mRNA expression of SCF (Yan et al., 1999). Also, no significant changes were found on the expression of SCF in rat Sertoli cells at different developmental ages after treatment with testosterone (10^{-7} M) for 24 h (Bhattacharya et al., 2012). An identical response was described in Sertoli cells of rhesus monkey (*Macaca mulatta*) using the same hormonal doses and time-frame (Majumdar et al., 2012). However, a strong evidence of the c-KIT hormonal regulation is the relationship between c-KIT expression, and the phase of reproductive cycle and the serum levels of androgens. In some seasonal species, the expression of c-KIT throughout reproductive cycle revealed a seasonal pattern linked with the plasma and testicular levels of testosterone. The maximum expression of c-KIT arose during the reproductive period when the testis exhibited the maximum concentration of testosterone (Roelants et al., 2002;Raucci and Di Fiore, 2007; 2009).

Other interesting question is that c-KIT showed to be significantly increased in recurrent PCa when compared to non-castrated clinical metastatic disease, suggesting that cancer initiating cells can proliferate, mediated by c-KIT, even in adaptation to a androgen deprived environment (Pfeiffer et al., 2011).

Relatively to estrogens, E_2 treatment increased the expression of sSCF by gonadal somatic cells, probably by interaction of ERα with an AP-1 response element in the *Steel* gene encoding the SCF protein (Moe-Behrens et al., 2003). Moreover, estrogens seem to increase the proliferation/survival of primordial germ cells, which was underpinned by the enhanced phosphorylation of c-KIT (La Sala et al., 2010). Accordingly, *in vitro* stimulation of primordial germ cells with E_2 lead to the phosphorylation of c-KIT and activation of downstream molecular targets (La Sala et al., 2010). Since ERs do not have intrinsic kinase activity, the E_2-induced phosphorylation occurs indirectly after receptor association with other proteins. The phosphorylation of c-KIT in primordial germ cells in response to E_2 is dependent on the

activity of SRC tyrosine kinases likely through the action of an adaptor protein that mediates the interaction of ERs with SRC proteins (Wong et al., 2002;La Sala et al., 2010).

In the ovary, the first evidence of a relationship between the expression levels of SCF/c-KIT and E_2 appeared with the report of Tanikawa et al. (1998), which positively correlated the expression of c-KIT in human oocyte with the concentration of E_2 in follicular fluid (Tanikawa et al., 1998). Both *in vitro* and *in vivo* approaches demonstrated that E_2 is able to regulate SCF expression in the ovary of new-born mice. The mRNA expression of SCF decreased in mouse ovaries cultured *in vitro* with 10^{-4} M of E_2 (Huansheng et al., 2011). Also, in the ovary of new-born mice injected with E_2 (5 mg/kg/day) a decreased expression of SCF mRNA was observed (Huansheng et al., 2011). However, treatment with a 10^{-8} M dose of E_2 for 4 days seems to induce an increase in the mRNA expression of SCF in mice ovary cultured *in vitro* (Huansheng et al., 2011), which indicates that estrogenic effects on the activity of SCF/c-KIT system may vary with the hormonal dose.

In cultured breast epithelial cells the treatment with E_2 also increased the c-KIT expression (Calaf and Roy, 2007).

Also, in the testis, estrogens seem to regulate c-KIT and SCF. Although, a previous study failed to report the estrogenic regulation of c-KIT and SCF in rat seminiferous tubules cultured *in vitro*, using a 10^{-8} M dose of E_2 (Yan et al., 1999), very recently it was showed that estrogens down-regulate the expression of c-KIT in seminiferous tubules while increased the expression of SCF (Correia et al., 2014). This was concomitant with diminished proliferation and enhanced apoptosis of germ cells (Correia et al., 2014).

Substances mimicking endogenous estrogens influenced the c-KIT expression. Male rats fed daily for 3 months with a standard diet supplemented with soya isoflavones (20 mg/kg/day) displayed a diminished expression of c-KIT in spermatogonia, spermatocytes and spermatids, with important alterations in the morphology of seminiferous tubules (Misiakiewicz et al., 2013). However, in cultured breast epithelial cells the treatment with parathion, an organophosphorous pesticide used in agriculture to control mosquito plagues, increased c-KIT expression alone or in combination with E_2 (Calaf and Roy, 2007).

In rat prostate there is only one study evaluating the expression of c-KIT in response to estrogens. Rats implanted with silastic capsules containing 20 mg of E_2 displayed higher expression of c-KIT than the sham-operated control group which was given an empty capsule (Kusljic and Exintaris, 2010). However, the augmented expression of c-KIT resulted from the increase in the population of interstitial cells expressing c-KIT and not from the increase of expression in cells (Kusljic and Exintaris, 2010).

A resume of the hormonal regulation of SCF/c-KIT system in some reproductive tissues is systematized in Fig. I-5.

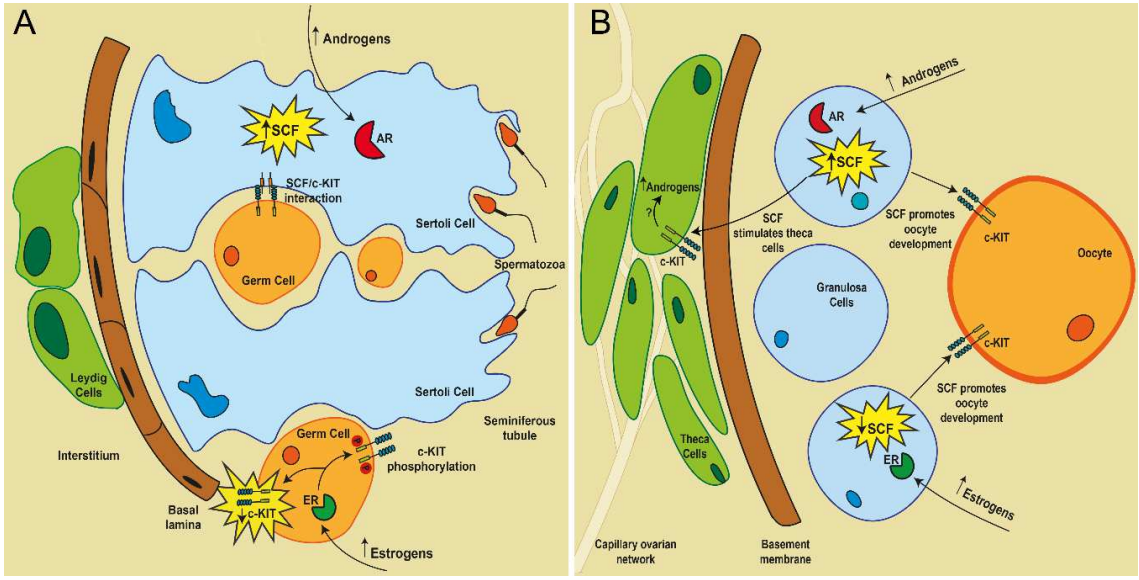


Figure I-5. Hormonal regulation of SCF/c-KIT system in the testis (A) and ovary (B) (adapted from Figueira et al. (2014)).

II. Objectives

It is widely established that the prostate gland is a hormone target organ, highly dependent on androgens actions, which are crucial for its normal function and development. Androgens are also well-known factors in the onset and progression of PCa. More recently, a substantial body of evidence has implicated estrogens in the carcinogenesis of prostate. However, a duality for the possible role of estrogens in prostate physiology has been gaining consistency over the last years. If some studies defend that estrogens are potential causative agents of PCa other strong evidences indicate that these steroids may be protective against PCa.

The SCF/c-KIT system plays a crucial role in the control of cell proliferation and apoptosis, and its expression was described both in normal and malignant prostate tissues. Moreover, SCF and c-KIT have been related with the development and progression of PCa.

The estrogenic regulation of SCF and c-KIT was described in several tissues, but the effect of estrogens controlling the expression of SCF/c-KIT in prostate is unknown.

The present dissertation aimed to evaluate the role of estrogens regulating the SCF/c-KIT system expression in prostate, by means of *in vivo* and *in vitro* experimental approaches. For this purpose, neoplastic and non-neoplastic prostate cell lines were cultured in presence or absence of E_2, and 3-month-old male rats were daily injected with E_2 for 5 days. The *in vivo* effect of estrogens on the proliferation and apoptosis of prostatic cells also was investigated.

III. Material and Methods

1. Cells Lines and Animals

1.1. Neoplastic and Non-neoplastic Human Prostate Cell Lines

The human normal prostate epithelial cell line, PNT1A (*human post-pubertal prostate normal cell line*), and the human PCa cell lines LNCaP (*lymphe node carcinoma of the prostate*), DU145 and PC3 were acquired from the European Collection of Cell Cultures (ECACC, Salisbury, UK). LNCaP, DU145 and PC3 cell lines are widely recognized models of metastatic stages of PCa representing different stages of aggressiveness. LNCaP cells were derived from lymph nodules metastasis and present low metastatic potential (Horoszewicz et al., 1980;Horoszewicz et al., 1983), DU145 cells were derived from brain metastasis and have moderate metastatic potential (Mickey et al., 1977;Stone et al., 1978), and PC3 cells were derived from bone metastasis and have high metastatic potential (Kaighn et al., 1979).

1.2. Animals

Thirty-two 3-month-old Wistar (*Rattus norvegicus*) male rats weighing 250-300 g were housed under constant temperature and humidity, in a 12 hours light–dark cycle, with food and water available *ad libitum*. Animals were handled in compliance with the National and European Union guidelines for handling and care of laboratory animals (Directive-2010/63/EU).

2. Human Prostate Cell Culture and In Vitro Hormonal Treatment

Both neoplastic and non-neoplastic cell lines were maintained in RPMI 1640 phenol red culture medium (Gibco, Invitrogen, Paisley, Scotland), supplemented with 10 % fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany) and 1 % penicillin/streptomycin (Invitrogen, Paisley, Scotland), at 37 ºC in a humid atmosphere equilibrated with 5 % CO_2 .

After reached the required cell number, each cell line was seeded in 40 t-flasks at a density of $1x10^6$ cells per t-flask in 5 mL of culture medium. Cells were also grown up in 12 well plates with glass culture slides at a density of 150×10^3 (PNT1A and PC3) or 100×10^3 (LNCaP and DU145) per well in 1 mL of cell culture medium for immunocitochemistry purposes. When cells reached 60 % of confluence, the medium was replace by phenol red-free RPMI 1640 medium containing 5 % charcoal-stripped FBS (CS-FBS) (Gibco, Invitrogen, Paisley, Scotland), which is devoid, among other components, of steroid hormones. Cells were maintained in this culture medium for 24 h to return at steroid hormone basal levels. Then, for stimulation with E_2 (Sigma, Saint Louis, MO, USA), culture medium was replaced by CS-FBS

containing 0 nM (control group) or 100 nM (treated group) E_2, and cells were exposed for 0, 12, 24, and 48 hours. The 100 nM dose of E_2 is considered a supraphysiological dose (Ropero et al., 2012).

At the end of hormonal treatment, cells were trypsinized and harvested for posterior analysis.

3. *In vivo* Hormonal Treatment

Rats were randomly divided into two distinct groups, each containing 16 animals, and daily injected with vehicle (control) or with E_2 (250 mg/day/Kg) for 5 days. The E_2 dose, as well as duration of treatment were chosen on the basis of our and others previous studies evaluating gene regulation by sex hormones (Harris et al., 2000;Gomes et al., 2013). After treatment, animals were euthanized by cardiac puncture, under anesthesia with 100 µL of a mixture containing ketamine and xilazine (2:1) per 100 g of animal weight. Then, prostrates were removed, weighted and either fixed in 4% paraformaldehyde (PFA) or snap frozen in liquid nitrogen and stored at -80ºC until RNA or protein extraction. In each group of 16 animals, 8 whole prostates were used for protein/RNA extraction and 8 prostates were used for fixation in 4 % PFA.

4. RNA Extraction

Human prostate cells and rat prostate tissues were homogenized in the adequate volume of TRI reagent (5 Prime, Hilden, Deutschland) by pipetting up and down or using an Ultra-turax homogenizer (T25 basic, IKA), respectively. Samples were allowed to stand at room temperature for 5 minutes to ensure complete dissociation of nucleoprotein complexes. Then, 200 µL of chloroform per mL of TRI were added for phase separation and samples were mixed for 15 seconds and allowed to stand at room temperature for 10 minutes. A centrifugation step at 14000 rpm, 4 ºC for 15 minutes separated the mixture into 3 phases: a red organic phase (containing proteins), an interphase (containing DNA) and a colorless upper aqueous phase (containing total RNA), which was collected to fresh tubes. After that, 500 µL of 2-propanol per mL of TRI were added to precipitated RNA and the samples were mixed and allowed to stand at room temperature for 10 minutes, followed by centrifugation at 14000 rpm for 10 minutes at 4 ºC. The supernatant was rejected and the RNA pellet was washed with 500 µL of 75 % cold-EtOH by centrifugation at 9500 rpm for 5 minutes at 4 ºC twice. The RNA pellet was air-dried for 5 minutes and ressuspended in 10 µL of Diethylpyrocarbonate treated-water (DEPC-H_2O).

The quantity of total RNA, was estimated by measurement of optical density at 260 and 280 nm (NanoPhotometer, Implen). The integrity of RNA was evaluated by 1 % agarose gel

electrophoresis with 2 µL of Greensafe Premium per 100 mL gel, and also by amplification of the 18S cDNA (see below). In both cases, agarose gels were visualized using the Molecular Imager FX Pro Plus MultiImager software (Biorad, Hercules, USA) coupled to an image acquisition system (Vilber Lourmat, Marne-la-Vallée, France).

5. cDNA Synthesis

cDNA was synthetized using 1 µg of total RNA and the NZY First Strand cDNA Synthesis Kit (NZYtech, Lisbon, Portugal) in a reaction mixture containing: 10 µL of NZYRT 2x Master Mix, 2 µL of NZYRT Enzyme Mix and the suitable volume of RNA (for 1 µg) and DEPC-treated H_2O up to a final volume of 20 µL. Total RNA was reverse-transcribed by incubating samples at 25 ºC for 10 minutes, followed by 50 ºC for 30 minutes. The reaction was inactivated by heating at 85 ºC for 5 minutes and chilling on ice. 1 µL of NZY RNase H (*E. Coli*) was added to each sample of reverse-transcribed RNA and the reaction was incubated at 37 ºC for additional 20 minutes. At the end, the reaction was stopped by heating at 85 ºC for 5 minutes. The synthesized cDNA was stored at -20 ºC until use.

6. Polymerase Chain Reaction (PCR)

Polymerase chain reaction was used to confirm the mRNA expression of SCF and c-KIT in prostate cell lines and rat prostate. Also, PCR was applied to amplify the 18S cDNA as a strategy to infer about the quality of the synthesized cDNA. Specific primers for the amplification of the target and housekeeping transcripts were designed using the Primer3 v0.4.0 (Rozen and Skaletsky, 2000) or the Beacon Designer 7 (Premier Biosof International, Palo alto, California, USA) software. Primers characteristics and cycling conditions are indicated in Table III-1. Amplification reactions for each target gene were performed using DreamTaq™ Green PCR Master Mix (2X) Kit (Fermentas, Life Sciences, EU) in a mixture containing: 6.25 µL of Master Mix 2X, 0.1 µL of specific primers, 1 µL of cDNA, and nuclease-free water up to a final volume of 12.5 µL. In each PCR a no-template control was included. Reactions were carried out on a thermocycler (Biometra, TProfessional Basic gradient), under the following conditions: initial denaturation at 95 ºC for 3 minutes, and 35 or 40 cycles of denaturation at 95 ºC for 30 seconds, annealing at 58ºC or 60ºC for 30 seconds and extension at 72 ºC during 10 seconds; reactions ended with 5 minutes incubation at 72 ºC. PCR products were run on 1-2 % agarose gels containing 2-3 µL of Greensafe Premium per 100 mL gel. Agarose gels were visualized using the Molecular Imager FX Pro Plus MultiImager software (Biorad, Hercules, USA) coupled to an image acquisition system (Vilber Lourmat, Marne-la-Vallée, France). The size of the amplificated fragments was compared to a DNA ladder (NZYDNA Ladder VI, Nzytech).

7. Real-time Quantitative Polymerase Chain Reaction (qPCR)

Expression analysis of SCF and c-KIT mRNA in prostate cell lines and rat prostate in different experimental conditions was performed by qPCR. Details on primers sequences and cycling conditions for the amplification of the target and housekeeping transcripts are provided on Table III-1. Gene expression was normalized with internal controls: GAPDH and beta-2-microglobulin in the case of prostate cell lines and β-actin in the case of animal prostate tissues. Reactions were performed in an iQ5 system (Bio-Rad, Hercules, CA, USA), and the efficiency of the amplifications was determined for all primer sets using serial dilutions of cDNA (1, 1:3, and 1:9). The primer concentration and annealing temperature were optimized before the assay, and the specificity of the amplicons was determined by melting curve analysis.

Each reaction consisted of a mixture of 10 µL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 0.8 or 1.2 µL of sense and anti-sense primers (depending of the primer specific gene) and sterile water up to 19 µL. Then, 1 µL of cDNA was added in a final volume of 20 µL. All reactions were performed in duplicate and a no-template control was included in each qPCR. Reactions were carried out on a thermocycler (iCycler iQ5™ system, Biorad), under the following conditions: initial denaturation at 95 ºC for 5 minutes, and 35 or 40 cycles of denaturation at 95 ºC for 10 seconds, annealing at 58 ºC or 60 ºC for 30 seconds and extension at 72 ºC during 10 seconds. The melting curve was constructed by heating reactions from 55 ºC to 95 ºC with 10 seconds hold at each temperature. Normalized expression values were determined using the mathematical model proposed by Pfaffl ($2^{-(\Delta\Delta Ct)}$) (Pfaffl, 2001).

Table III-1. Oligonucleotides sequence, amplicon size and cycling conditions.

Gene	Primer Sequence (5' - 3')	Amplicon Size (bp)	AT (°C)	Cycles
Human c-KIT	sense: CCG TCT CCA CCA TCC ATC antisense: ATT CAT TCT GCT TAT TCT CATT CG	144	58	40
Human SCF	Sense: GTT GCC GATT AGT GAT ATG G Antisense: ACT TCT TCC TGC GAT GAC	174	60	35
Human GAPDH	Sense: CGC CAG CCG AGC CAC ATC Antisense: CGC CCA ATA CGA CCA AAT CCG	75	60	35
Human beta-2 microglobulin	Sense: ATG AGT ATG CCT GCC GTG TG Antisense: CCA ACC TCC ATG ATG CTG CTT AC	93	60	35
Rat c-KIT	Sense: CCG TCT CCA CCA TCC ATC C Antisense: TTC GCT CTG CTT ATT CTC AAT CC	143	60	35
Rat SCF	Sense: ATGGCTTGGGAAATGTCTG Antisense: GCTGATGCTACGGAGTTAC	193	58	35
Rat β-Actin	Sense: TGG TGG GTA TGG GTC AG Antisense: CAA TGC CGT GTT CAA TGG	79	60	35

AT- annealing temperature

8. Total Protein Extraction

Human prostate cells and rat prostate tissues were homogenized in the adequate 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) by pippeting up and down or using an Ultra-turax homogenizer (T25 basic, IKA), respectively. The samples were allowed to stand on ice (20 minutes for cells or 1 hour for

prostate tissues) and occasionally mixed. Samples were then centrifuged at 14,000 rpm for 20 min at 4ºC, and total proteins (supernatant) were recovered to fresh tubes. Quantification of total protein concentration in cell and tissue extracts was assessed using the Bradford method (Bradford, 1976) with Bio-rad protein assay dye reagent concentrate (Bio-Rad, Hercules, CA, USA).

9. Western Blot (WB)

Total proteins of human prostate cells (50 µg) and rat prostate (75 µg) were heat-denatured at 100 ºC and resolved on 12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) at 750 mA for 90 to 150 minutes. Membranes were blocked for 1 hour with a 5 % skimmed dried milk and then incubated overnight at 4 ºC with rabbit anti-SCF (1:500, H-189: sc-9132; Santa Cruz Biotechnology), rabbit anti-c-KIT (1:500, C-19: sc-168; Santa Cruz Biotechnology), rabbit anti-FasR (1:500, A-20:sc-1023; Santa Cruz Biotechnology), rabbit anti-Fas-L (1:500, C-178: sc-6237; Santa Cruz Biotechnology), rabbit anti-Bax (1:500, # 2772; Cell Signaling Technology), rabbit anti-Bcl-2 (1:1000, # 2876; Cell Signaling Technology) or rabbit anti-caspase-9 p35 (H-170:sc-8355; Santa Cruz Biotechnology) primary antibodies. A mouse anti-α-tubulin monoclonal antibody (1:5000, T9026, Sigma - Aldrich) was used for protein loading control in all WB analyses. Goat anti-rabbit IgG-AP (1:5000, NIF1317; GE Healthcare) or goat anti-mouse IgG+IgM-AP (1:5000, NIF1316, GE Healthcare) were used as secondary antibodies. Membranes were exposed to ECF substrate (GE Healthcare) for 5 minutes and scanned with Molecular Imager FX Pro plus MultiImager (Bio-Rad, Hercules, CA, USA). Band densities were obtained according to standard methods using the Quantity One Software (Bio-Rad, Hercules, CA, USA) and normalized by division with the respective α-tubulin band density.

10. Fluorescent Immunocitochemistry

PNT1A, LNCaP, DU145 and PC3 cells cultured in presence or absence of E_2 were washed with phenol red-free RPMI 1640 medium and fixed with 4 % PFA. Cells were then permeabilized with 1% Triton X-100 (Sigma, Saint Louis, MO, USA) for 5 minutes at room temperature, and unspecific staining was blocked by incubation with phosphate buffer saline (PBS) containing 0.1 % (w/v) Tween®-20 (PBS-T) and 20 % FBS (Biochrom AG, Berlin, Germany) for 1 hour. After washing with PBS-T cells were incubated with rabbit anti-SCF (1:50, H-189: sc-9132; Santa Cruz Biotechnology) or rabbit anti-c-KIT (1:50, C-19: sc-168; Santa Cruz Biotechnology) primary antibodies diluted in PBS-T containing 1 % FBS, for 1 hour at room temperature. Cells were washed again and incubated with Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 546 goat anti-rabbit IgG secondary antibodies (Invitrogen, Paisley, Scotland) diluted 1:1000 in PBS-T containing 1 % FBS for 1 hour at room temperature.

Additional washes were performed before cell nuclei were stained with Hoechst 33342 (5 µg/mL, Invitrogen) for 10 minutes. Sections were washed with PBST and mounted in Dako fluorescent mounting medium. Specificity of the staining was assessed by the omission of primary antibody. Images were acquired using an Invert Zeiss axiobserver microscope (Carl Zeiss, Göttingen, Germany).

11. Fluorescent Immunohistochemistry

Formalin-fixed paraffin sections (5 µm) of rat prostate were deparaffinized in xylene (2x5 minutes), rehydrated in ethanol/water graded solutions (2x3 minutes in 100 % ethanol, 3 minutes in 95 % ethanol and 3 minutes in 70 % ethanol), and briefly passed by tap water for 30 seconds. After heat-induced antigen retrieval (citrate buffer bath, 10 mM, pH 6.0) sections were allowed to cool at room temperature and incubated with the cell membrane marker wheat germ agglutinin (5 µg/ml, Invitrogen, Paisley, Scotland) for 10 minutes. After permeabilization with 1 % Triton X-100 (Sigma, Saint Louis, MO, USA) for 15 minutes at room temperature, unspecific staining was blocked by incubation with PBS containing 1 % (w/v) BSA (PBA) and 0.3 M glycine (Fisher Scientific) for 30 minutes at room temperature. Incubation with rabbit anti-SCF (1:50, H-189: sc-9132; Santa Cruz Biotechnology) or rabbit anti-c-KIT (1:50, C-19: sc-168; Santa Cruz Biotechnology) primary antibodies diluted in PBA occurred overnight at 4 ºC, followed by incubation with Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 546 goat anti-rabbit IgG secondary antibodies (Invitrogen, Paisley, Scotland) diluted 1:500 in PBA for 1 hour at room temperature. Sections were washed with PBS and mounted in Dako fluorescent mounting medium. Specificity of the staining was assessed by the omission of primary antibody. Dual labeling images of SCF or c-KIT and cell membrane in prostate sections were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany).

12. Proliferation Index

The proliferation index in the prostates of E_2-treated and un-treated rats was estimated by the fluorescent immunohistochemistry of nuclear proliferation marker Ki67. Formalin-fixed paraffin sections (5 µm) of rat prostate were deparaffinized, rehydrated, antigen retrieved, permeabilized and blocked as described in section 11.

After washing steps, sections were incubated overnight at 4 ºC with rabbit anti-Ki67 (ab16667; Abcam) primary antibody diluted 1:50 in PBA. Then, sections were incubated with Alexa Fluor 546 goat anti-rabbit IgG secondary antibody (Invitrogen, Paisley, Scotland) diluted 1:500 in PBA for 1 hour at room temperature and washed. Cell nuclei were stained with Hoechst 33342 (10 µg/mL, Invitrogen) for 5 minutes. Sections were washed with PBS and mounted in Dako fluorescent mounting medium. Specificity of the staining was assessed by

the omission of primary antibody. Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany).

The proliferation index was determined by the percentage of Ki67-positive cells out of the total number of Hoechst stained nuclei in 20 randomly selected 40 magnification fields in each section.

13. Caspase-3 Activity Assay

To determine the enzymatic activity of caspase-3, 25 µg of total protein extracted from rat prostate were incubated with the appropriated volume of reaction buffer (25 mM HEPES, 0.1% 3-[(3-holamidopropyl) dimethylammonio]-1-propanesulfonate, 10% sucrose, and 10 mM Dithiothreitol (DTT), pH 7.5) and 200 µM of caspase-3 substrate (Ac-DEVD-pNA) in a 96-well pate. A negative control without Ac-DEVD-pNA was performed for each sample. Reactions were allowed to proceed at 37 ºC and upon caspase cleavage of Ac-DEVD-pNA, p-nitro-aniline (pNA) is released producing a yellow color, which is measured spectrophotometrically at 405 nm. The amount of generated product was calculated by extrapolation from a standard curve of free pNA and is directly proportional to the activity of caspase-3.

14. Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL)

Terminal deoxynucleotidyl transferase dUTP nick-end labeling was performed using the In Situ Cell Death Detection Kit, TMR red (Roche, Penzberg, Germany) following the manufacturer's instructions. Rat prostate sections (5 µm) were deparaffinized as described before and permeabilized with 0.25% Triton X-100 (Sigma, Saint Louis, MO, USA), for 30 minutes at room temperature. Afterwards, sections were incubated in a humidified atmosphere with TUNEL reaction mixture (enzyme solution equilibrated in label solution) for 1 hour at 37ºC, protected from light. After additional washes with PBS, cell nuclei were stained with Hoechst 33342 (10 mg/mL; Invitrogen) for 5 minutes. Sections were then washed with PBS for 10 minutes and mounted in Dako fluorescent mounting medium. Images were acquired using an Invert Zeiss axiobserver microscope (Carl Zeiss, Göttingen, Germany).

15. Statistical Analysis

Statistical significance of differences between controls and hormonal treatments in both *in vitro* and *in vivo* experiments was evaluated by unpaired T-test with Welch's correction, using GraphPad Prism v5.00 (GraphPad Software, San Diego,CA). Significant differences were considered when $p<0.05$. All experimental data are shown as mean ± SEM.

IV. Results

1. Characterization of the SCF/c-KIT System Expression in Human Prostate Cell Lines and Rat Prostate

The SCF/c-KIT system has been identified in some PCa cell lines (Stoop et al., 2008) and several tissues (Morii et al., 1992;Chui et al., 1996). However, in the case of the prostate, some of the previous studies are contradictory and unclear (Savarese et al., 1998;Wiesner et al., 2008). Before start evaluating the hormonal regulation of the SCF/c-KIT system in neoplastic and non-neoplastic prostate cell lines, as well as in rat prostate, the expression of c-KIT receptor and its ligand in prostate cells and tissues was confirmed.

1.1. SCF and c-KIT are Expressed in Both Neoplastic and Non-neoplastic Prostate Cell Lines

To evaluate the expression of c-KIT mRNA in human prostate cell lines it was performed a PCR and the amplificated products were visualized in an agarose gel. An amplicon of the expected size 144 bp long was found in both neoplastic and non-neoplastic human prostate cell lines as shown in Fig. IV-1A.

A qPCR was performed to determine the relative expression of c-KIT mRNA in human prostate cells (Fig. IV-1B). The highest expression of c-KIT was observed in DU145 cells (2.08 ± 0.24 fold variation, $P<0.01$) while PC3 displayed the lowest levels (0.45 ± 0.14 fold variation, $P<0.05$). No significant differences were found between c-KIT expression in LNCaP and PNT1A cells (Fig.IV-1B).

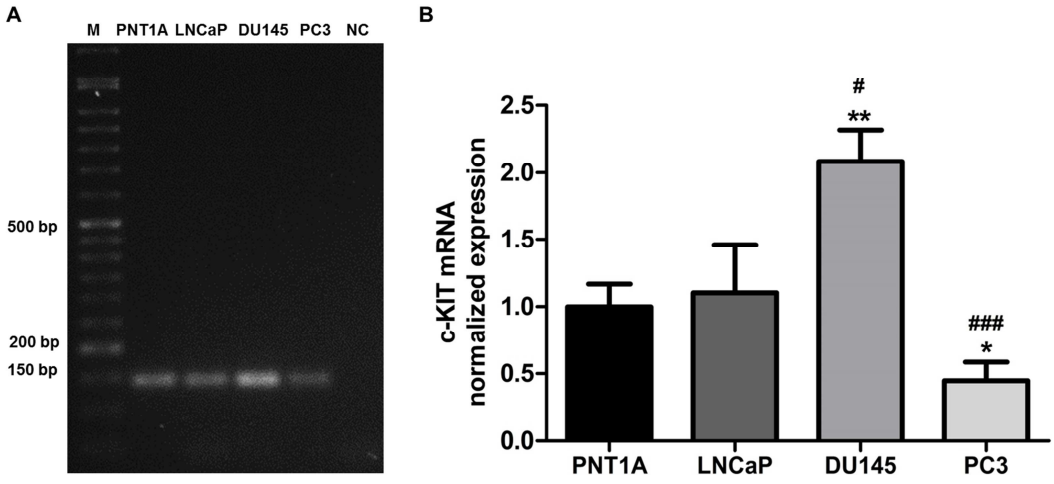


Figure IV-1. c-KIT mRNA expression in non-neoplastic (PNT1A) and neoplastic (LNCap, DU145 and PC3) human prostate cell lines. (A) PCR products run on 2% agarose gel; M, molecular weight marker; NC, negative control. **(B)** Relative expression of c-KIT determined by qPCR, after normalization with GAPDH and beta-2 microglobulin as internal reference genes. Results are expressed as the fold variation relatively to PNT1A. Error bars indicate mean ± S.E.M ($n\geq5$). *$P<0.05$ and **$P<0.01$ compared with PNT1A cell line. #$P<0.05$ compared to LNCaP. ###$P<0.0001$ compared with DU145.

The expression of c-KIT proteins in neoplastic and non-neoplastic human prostate cell lines was confirmed by WB analysis. The full-length c-KIT, the membrane form of the receptor, with 145 kDa, as well as its variants, namely, the truncated isoform of c-KIT, with approximately, 30 kDa and a 50 kDa isoform were detected in all cell lines (Fig. IV-2A). No significant differences were found on the expression of c-KIT in different cell lines. However, the full-length c-KIT and the isoform with 50 kDa seem to be more expressed by DU145 cell. On the other hand, the tr-KIT (30 kDa) shows an higher expression in PC3 cells (Fig. IV-2B).

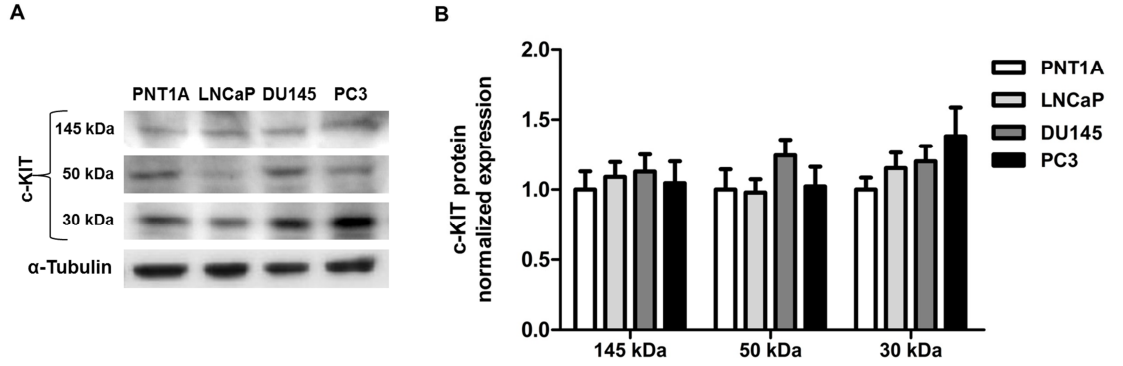


Figure IV-2. Expression of full-length (145 kDa), and 50 and 30 kDa isoforms of c-KIT in non-neoplastic (PNT1A) and neoplastic (LNCap, DU145 and PC3) human prostate cell lines. (A) Representative WB experiments. **(B)** c-KIT protein expression in prostate cells determined by WB analysis after normalization with α-tubulin. Results are expressed as fold-variation relatively to PNT1A cells. Error bars indicate mean ± S.E.M (n ≥ 3).

The expression of SCF mRNA in prostate cell lines was also analyzed by PCR. The amplificated products were visualized in an agarose gel and it was confirmed the presence of an amplicon of the expected size 174 bp long in all cell lines as shown in Fig. IV-3A.

A relative-quantification of the SCF mRNA expression was performed by qPCR. The highest expression of SCF was observed in DU145 cells, although without statistical significance; PC3 cells showed the lowest expression levels of SCF mRNA, which is significantly decreased in comparison with the other cell lines (e.g. 0.44 ± 0.13 fold variation to PNT1A, $P<0.01$, Fig. IV-3B). No significant differences were found between SCF expression in LNCaP, DU145 and PNT1A cells (Fig.IV-3B).

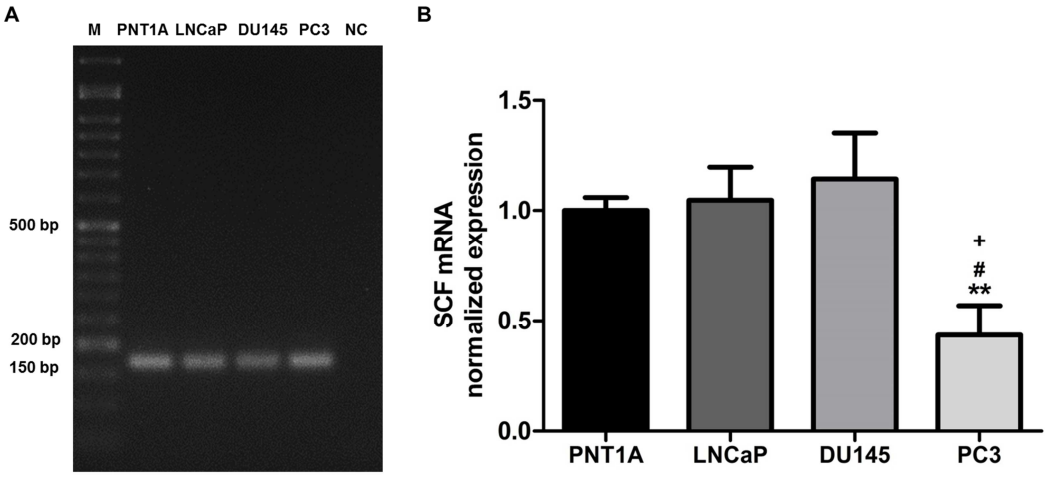


Figure IV-3. SCF mRNA expression in non-neoplastic (PNT1A) and neoplastic (LNCaP, DU145 and PC3) human prostate cell lines. (A) PCR products run in 2% agarose gel; M, molecular weight marker; NC, negative control. **(B)** Relative expression of SCF determined by qPCR after normalization with GAPDH and beta-2 microglobulin as internal reference genes. Results are expressed as the fold variation relative to PNT1A. Error bars indicate mean ± S.E.M (n ≥ 5). **P<0.01 compared with PNT1A cell line. #P<0.05 compared to LNCaP. +P<0.05 compared with DU145.

At protein level, a SCF specific band, with an approximate size of 63 kDa, was detected in both neoplastic and non-neoplastic prostate cells, with only slight variations between cell lines (Fig. IV-4A). Although without statistically significant difference, the expression of SCF seems to be higher in DU145 cells and lower in PC3 (Fig. IV-4B).

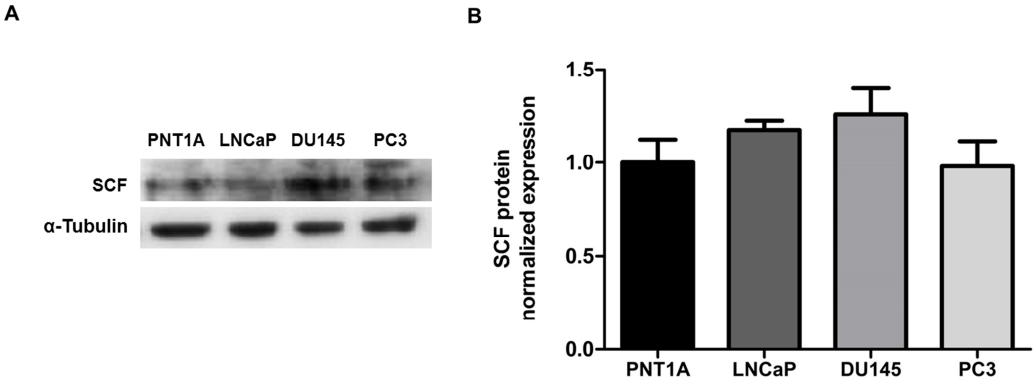


Figure IV-4. SCF protein expression in non-neoplastic (PNT1A) and neoplastic (LNCaP, DU145 and PC3) human prostate cell lines. (A) Representative WB experiments. **(B)** SCF protein in prostate cells determined by WB analysis after normalization with α-tubulin. Results are expressed as fold-variation relatively to PNT1A cells. Error bars indicate mean ± S.E.M (n ≥ 3).

1.2. The SCF/c-KIT System is Expressed in Rat Prostate

c-KIT expression in rat prostate was confirmed both at mRNA and protein level. The run of PCR products in agarose gel revealed an amplicon of, approximately, 144 bp (Fig. IV-5 A). In respect to protein, it was detected different isoforms of c-KIT by WB, namely, the full-length c-KIT, the membrane form of the receptor, with 145 kDa; the truncated isoform of c-KIT, with approximately, 30 kDa and other isoform with a band size of 50 kDa (Fig.IV-5B). The two last ones seem to be weakly expressed.

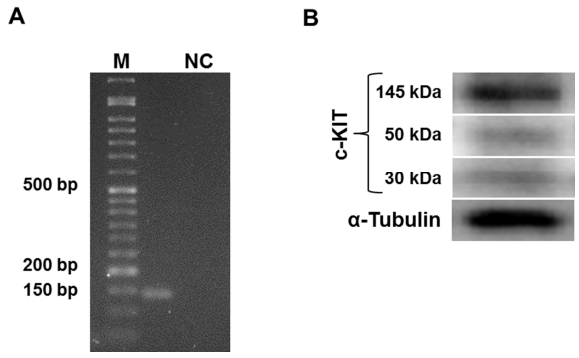


Figure IV-5. c-KIT mRNA (A) and protein (B) expression in rat prostate. (A) PCR products were run in 2% agarose gel; M, molecular weight marker; NC, negative control. **(B)** Protein samples were run in 12.5 % acrylamide gel and detected by WB analysis. The full-length c-KIT (145 kDa), and the 50 and 30 kDa isoforms are indicated.

PCR and WB analysis confirmed the expression of SCF in rat prostate. At mRNA level, an amplicon of, approximately, 174 bp long was identified (Fig. IV-6A). At protein level, although SCF can exist in different forms, only a unique specific band was detected in WB analysis. It was a strong SCF specific band, presenting a molecular weight of, approximately, 63 kDa (Fig. IV-6B).

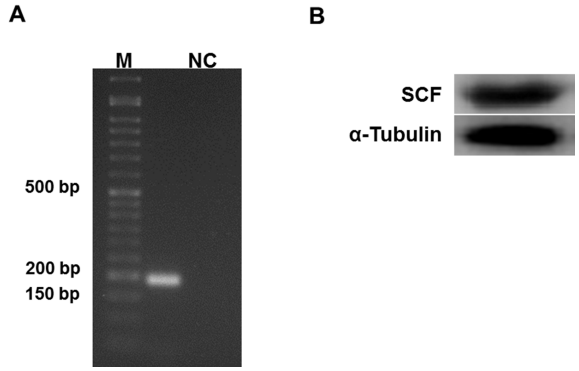


Figure IV-6. SCF mRNA (A) and protein (B) expression in rat prostate. (A) PCR products were run in 2% agarose gel; M, molecular weight marker; NC, negative control. **(B)** Protein samples were run in 12,5% acrylamide gel and detected by WB analysis.

2. Estrogens Regulate the Expression of SCF/c-KIT System in Neoplastic and Non-neoplastic Prostate Cell Lines

The SCF/c-KIT system has been showed to be regulated by estrogens in some cell lines. Considering this, and after confirmed the expression of both c-KIT and SCF in all the prostate cell lines selected to study, it was evaluated the effect of estrogens on the expression of SCF/c-KIT system. PNT1A, LNCaP, DU145 and PC3 cells were exposed to 100 nM of E_2 for 0, 12, 24 and 48 h. Expression of SCF and c-KIT was studied at mRNA and protein level respectively, by qPCR and WB and compared with the untreated control group (0 nM E_2).

c-KIT mRNA expression was evaluated in neoplastic and non-neoplastic prostate cells exposed to 0 nM or 100 nM E_2 for 0 h and 24 h. As expect, no significant differences were observed for c-KIT expression in all cell lines for 0 h of E_2-stimulation. On the other hand, at 24 h of E_2-stimulation, a significant increase of the c-KIT expression was detected in PNT1A, DU145 and PC3 cells (respectively, 1.86 ± 0.31, 2.11 ± 0.34 and 3.19 ± 0.93 fold variation, $P<0.05$, Fig. IV-7A, C and D). In fact, the most accentuated increase was verified in PC3 cells (Fig. IV-7D). Contrarily, in LNCaP cells, c-KIT mRNA expression decreased with E_2 exposure (0.53 ± 0.14 fold variation, $P<0.05$, Fig. IV-7B).

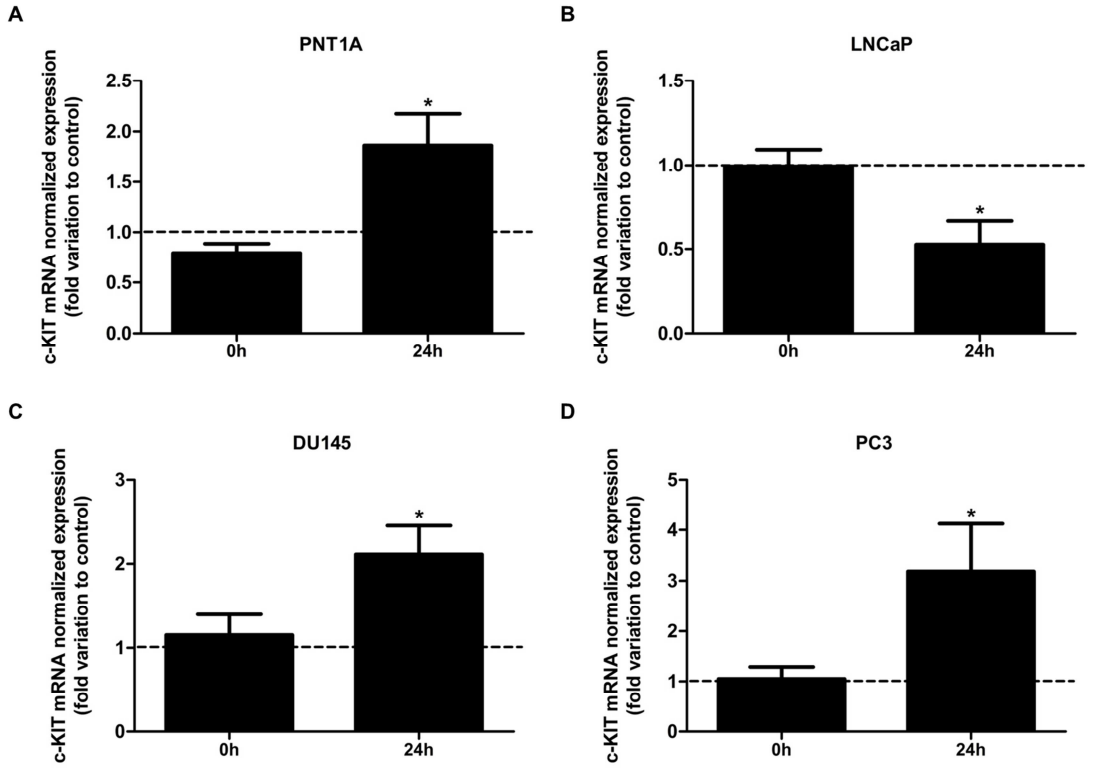


Figure IV-7. Effect of E_2 (100 nM) on c-KIT mRNA expression in human prostate cell lines. The mRNA expression was determined by qPCR after normalization with GAPDH and beta-2 microglobulin housekeeping genes. Results are expressed as the fold variation relative to the control group (0 nM E_2, dashed line). Error bars indicate mean ± S.E.M (n ≥ 5). *P<0.05.

Considering the estrogenic effect on c-KIT proteins it was shown that treatment with 100 nM E_2 for 24 h down-regulated the expression of all c-KIT protein isoforms in PNT1A, (although without statistical significance) and DU145 cells (0.87 ± 0.01 fold variation to control ($P<0.05$) for the full-length; 0.94 ± 0.01 fold variation to control ($P<0.05$) for the 50 kDa and 0.92 ± 0.02 fold variation to control ($P<0.05$) for the 30 kDa) (Fig. IV-8A, B and C). Curiously, for the same time of stimulation, an opposite effect was observed in PC3 cells, which showed increased expression of the full-length and truncated form of c-KIT (1.37 ± 0.16 and 1.32 ± 0.08 fold variation to control, $P<0.05$, respectively, Fig. IV-8D). This opposite effect was also observed in PNT1A at 12 h of E_2-stimulation, but without significance. No changes were verified in the expression of full-length c-KIT in LNCaP cells, but the truncated 50 kDa and 30 kDa proteins were also decreased (0.85 ± 0.02 and 0.83 ± 0.02 fold variation to control, $p<0.01$, respectively). No expression changes were visible for 0, 12 or 48 h of stimulation.

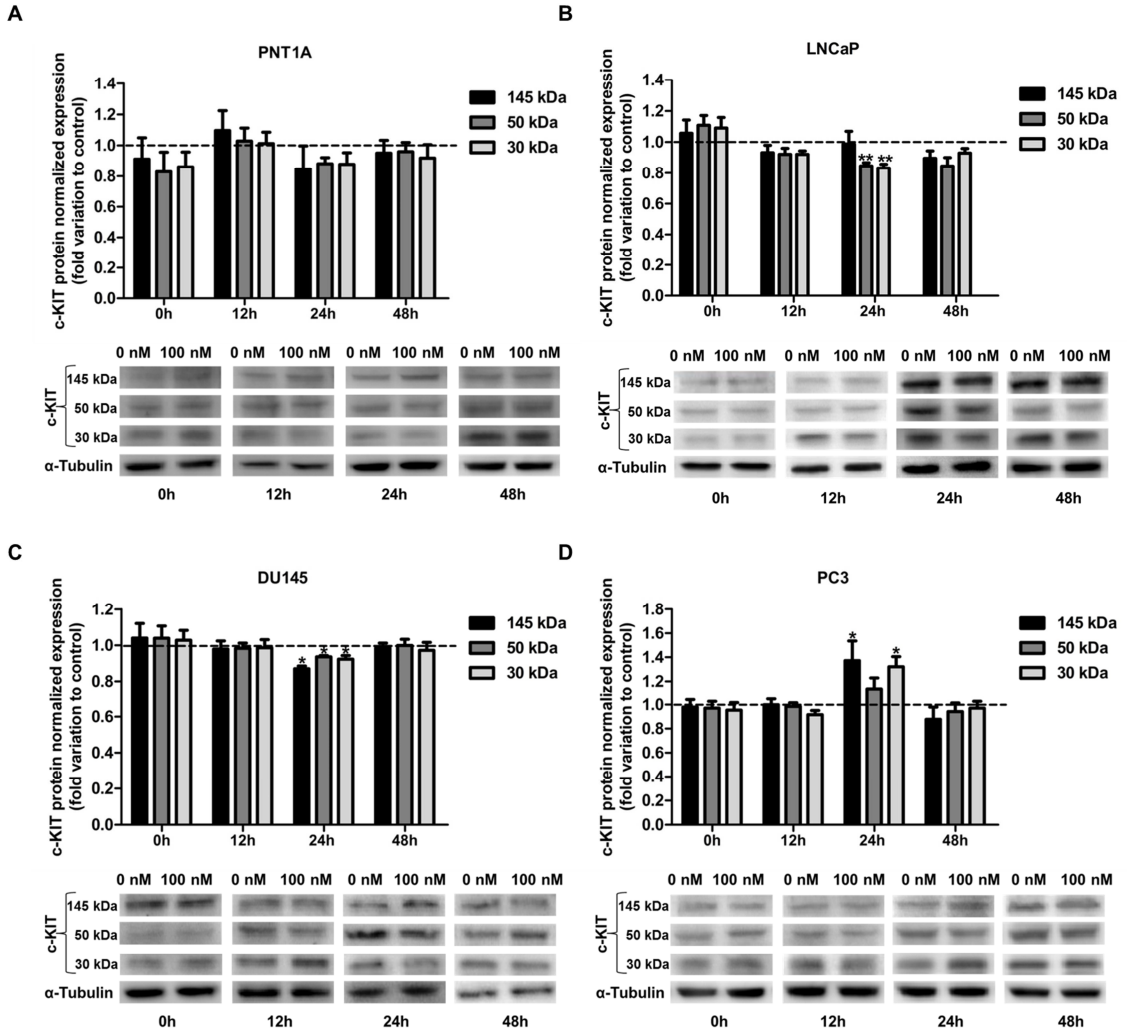
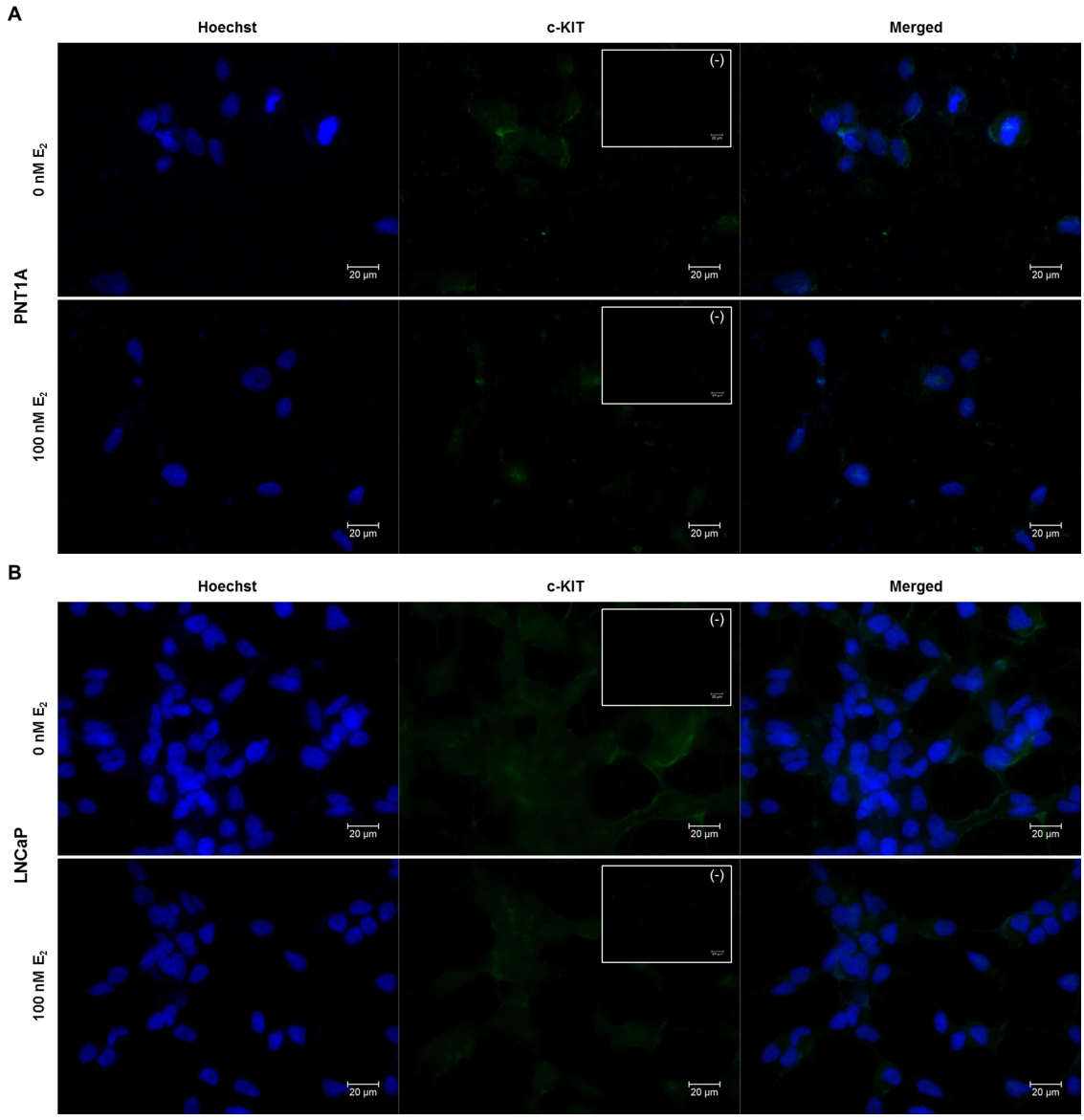


Figure IV-8. Effect of E_2 (100 nM) on c-KIT protein expression (full-length 145 kDa, and 50 and 30 kDa isoforms) in human prostate cell lines. The protein expression was determined by WB analysis after normalization with α-Tubulin. Results are expressed as the fold variation relative to control group (0 nM E_2, dashed line). Error bars indicate mean ± S.E.M ($n \geq 5$). *$P<0.05$, *$P<0.01$.

In order to further confirm these results, the effect of E_2 on c-KIT protein expression was also evaluated by fluorescent immunocytochemistry in PNT1A, LNCaP, DU145 and PC3 cells stimulated with E_2 for 24 h comparatively with the respective control groups. Representative microscopy images are shown in Fig. IV-9.



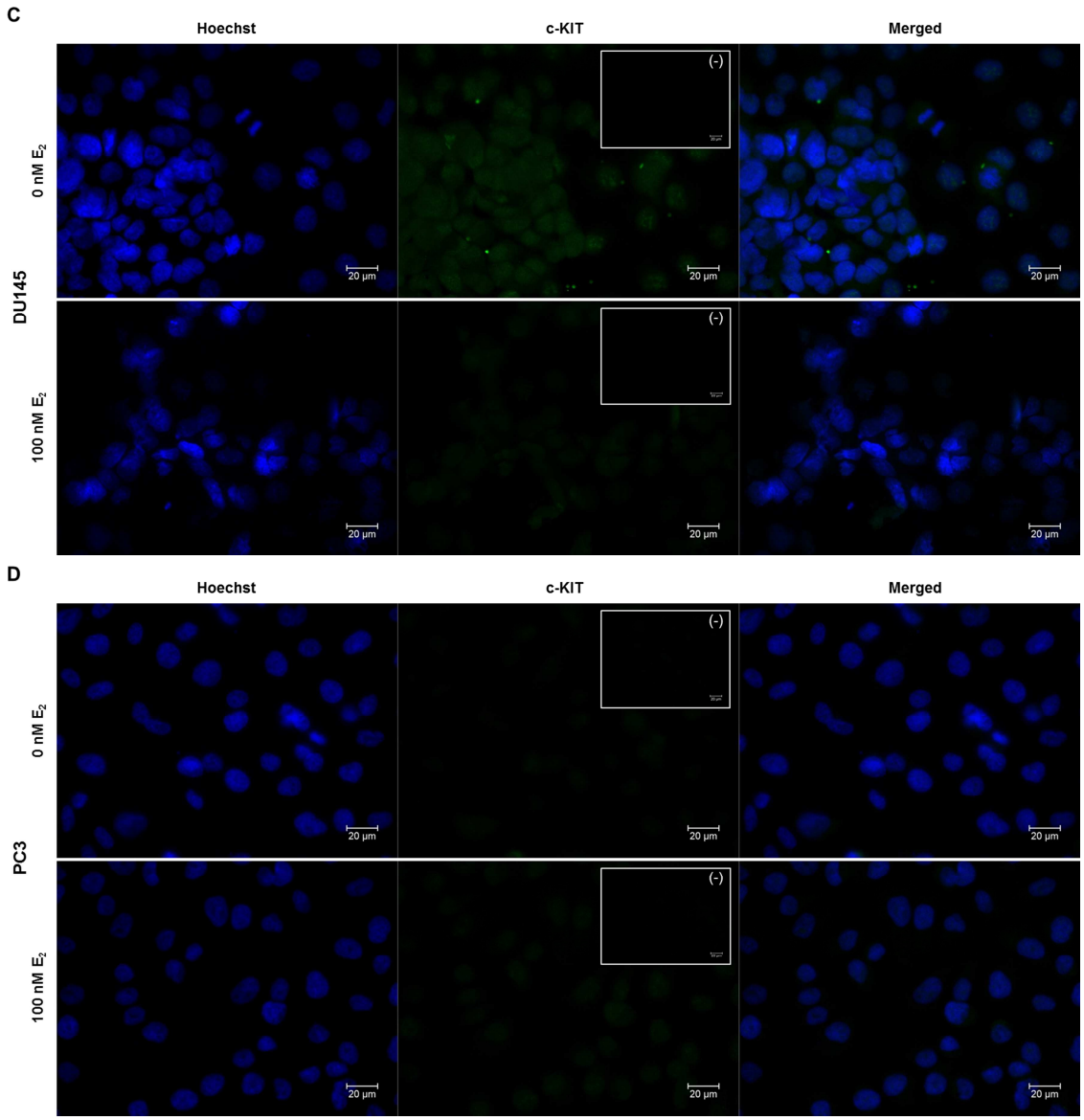


Figure IV-9. Effect of E_2 (100 nM) on c-KIT protein expression in prostate cell lines. Representative fluorescence microscopy images obtained in Invert Zeiss axiobserver microscope are showed. Nuclei are stained with Hoechst 33342 (blue) and fluorescence c-KIT-positive staining is green. Negative controls for c-KIT obtained by omission of the primary antibody are provided as insert panels (-). Images were amplified 63x.

The mRNA expression of c-KIT ligand, SCF, was evaluated by qPCR in cells exposed to 0 nM or 100 nM for 0 h and 24 h. As expected, no variation was verified at 0 h of stimulation for all cell lines. 24 h after E_2 administration a significant increase of SCF expression was observed in all cell lines. However, PC3 cells showed the most pronounced increase, with 2.75 ± 0.49 fold variation relatively to control ($P<0.05$, Fig. 6D), and almost the double of the effect seen in LNCaP cells (1.49 ± 0.13 fold variation, $P<0.05$, Fig. 6 B). Thus, PNT1A and DU145 showed a moderate increase of SCF mRNA expression in response to E_2 (respectively, 2.18 ± 0.32 and 1.67 ± 0.10 fold variation, $P<0.05$, Fig. 6A and C).

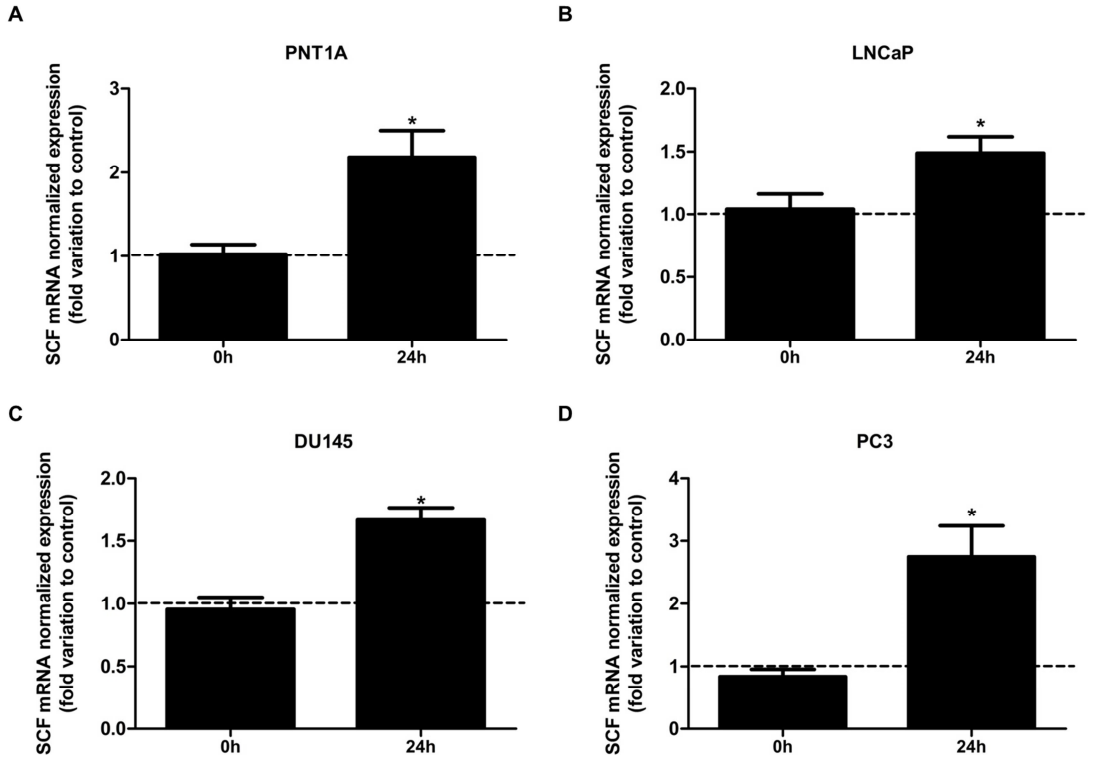


Figure IV-10. Effect of E_2 (100 nM) on SCF mRNA expression in prostate cell lines. The mRNA expression was determined by qPCR after normalization with GAPDH and bate-2 microglobulin housekeeping genes. Results are expressed as the fold variation relative to control group (0 nM E_2, dashed line). Error bars indicate mean ± S.E.M (n ≥ 5). *P<0.05.

The SCF protein expression showed a response to E_2 stimulation similar to c-KIT. At 24 h after stimulation, it was down-regulated in PNT1A (0.82 ± 0.07 fold variation to control, P<0.05), LNCaP (0.85 ± 0.05 fold variation to control, P<0.05) and DU145 cells (0.69 ± 0.09 fold variation to control, P<0.05) (Fig. IV-9 A, B and C), but it was almost two-fold up-regulated in PC3 cells (1.98 ± 0.06 fold variation to control, P<0.001, Fig. IV-9 A). No changes were verified for 0, 12 or 48 h of stimulation.

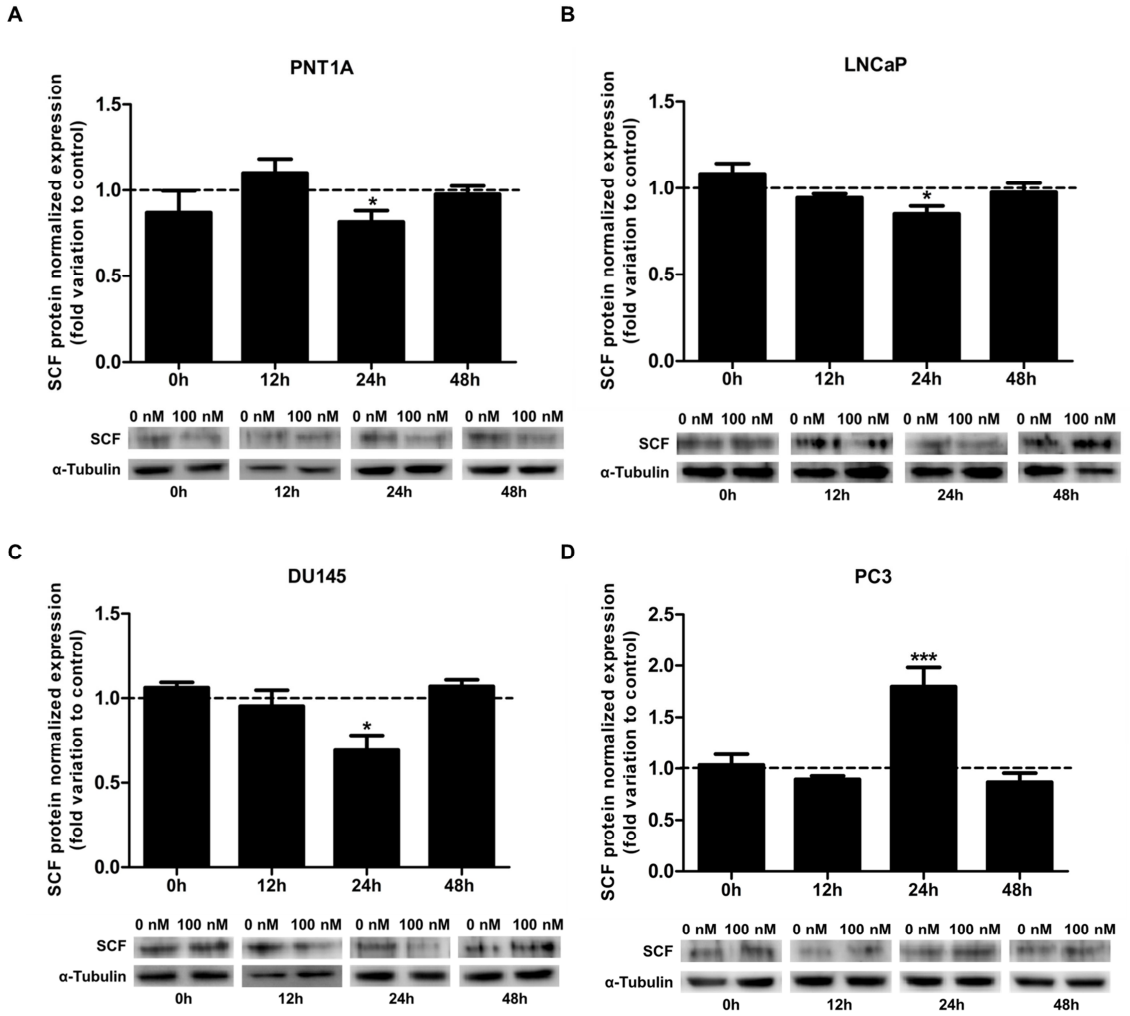
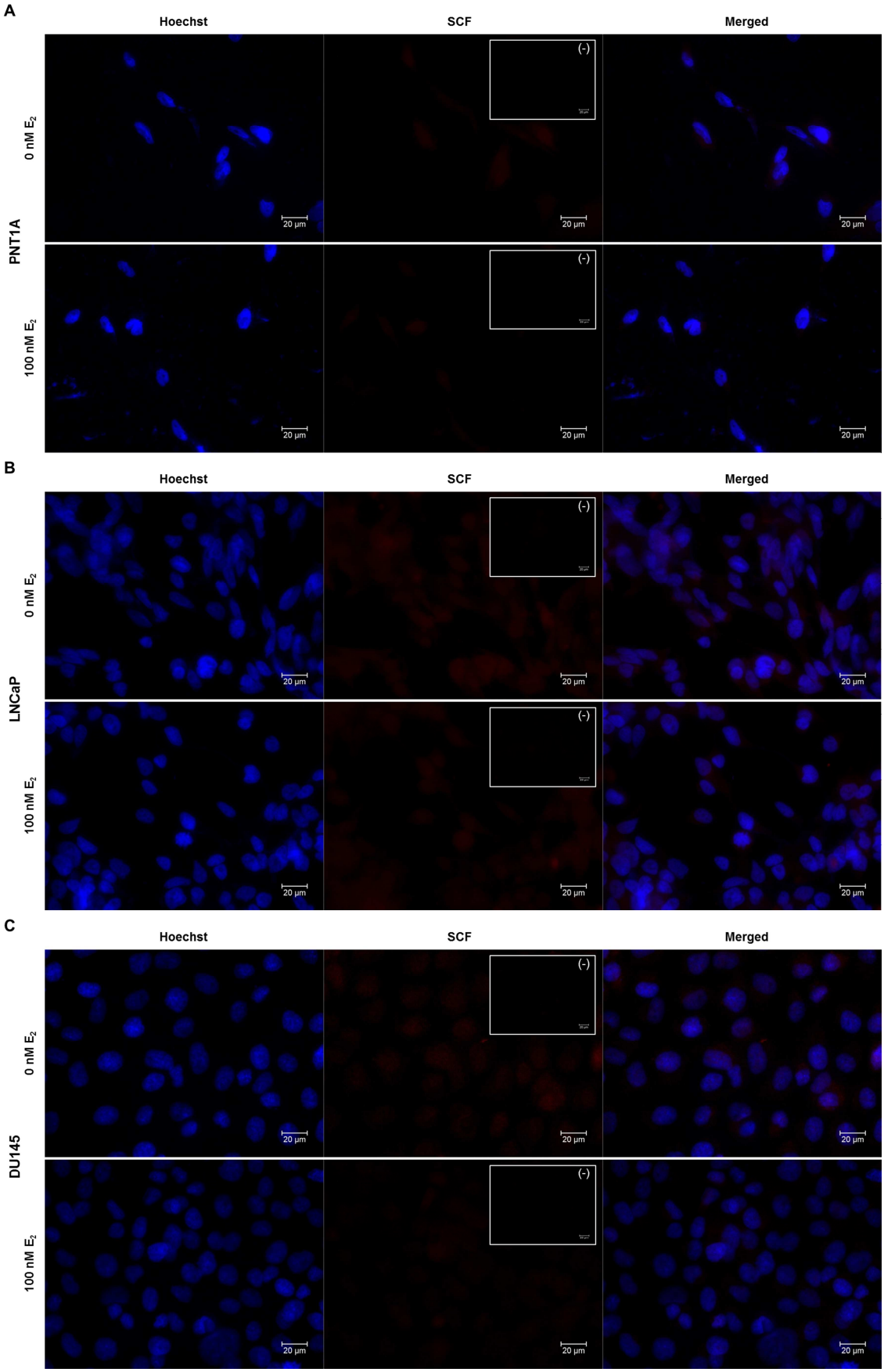


Figure IV-11. Effect of E_2 (100 nM) on SCF protein expression in prostate cell lines. The protein expression was determined by WB analysis after normalization with α-Tubulin. Results are expressed as the fold variation relative to control (0 nM E_2, dashed line). Error bars indicate mean ± S.E.M (n ≥ 5). *P<0.05, *P<0.01.

The protein expression results of SCF were further confirmed by fluorescent immunocytochemistry in PNT1A, LNCaP, DU145 and PC3 cells stimulated with E_2 for 24 h, comparatively with the respective control groups. Representative microscopy images are shown in Fig. IV-12.


A
Hoechst
SCF
Merged
PNT1A
0 nM E2
100 nM E2
(-)
20 µm
B
LNCaP
C
DU145

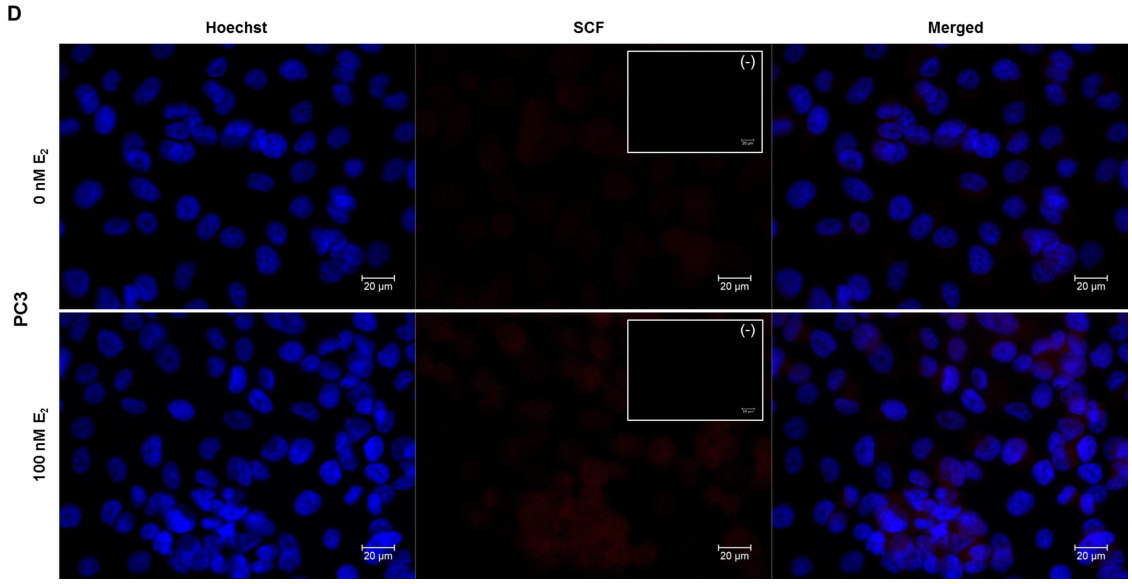


Figure IV-12. Effect of E_2 (100 nM) on SCF protein expression in prostate cell lines. Representative fluorescence microscopy images obtained in Invert Zeiss axiobserver microscope are showed. Nuclei are stained with Hoechst 33342 (blue) and fluorescence SCF-positive staining is red. Negative controls for SCF obtained by omission of the primary antibody are provided as insert panels (-). Images were amplified 63x.

3. Estrogenic Regulation of SCF/c-KIT System Expression in Rat Prostate

SCF and c-KIT have been identified as estrogen-target genes in a broad range of tissues. However, in prostate, nothing is known about the regulation of this system by E_2. To evaluate the effect of E_2 on the expression of SCF and c-KIT in rat prostate, adult animals were daily injected with vehicle (control) or with E_2 (250 µg/day/Kg) for 5 days. The mRNA and protein expression of SCF and c-KIT was analyzed by qPCR and WB, respectively.

3.1. E_2 Down-Regulates the Expression of SCF and c-KIT in vivo

In vivo, E_2 treatment caused a significant increase in the mRNA expression of c-KIT (2.53 ± 0.28 fold variation to control, $P<0.001$, Fig. IV-13A).

However, at protein level, distinct effects were observed for different c-KIT isoforms. E_2 treatment significantly up-regulated the expression of 50 kDa (1.17 ± 0.02 fold variation to control) and 30 kDa isoforms (1.64 ± 0.09 fold variation to control) ($P<0.001$, Fig. IV-13B and C). However, an opposite effect was verified relatively to the full-length c-KIT. WB analysis revealed that the membrane form of c-KIT was down-regulated by E_2 (0.89 ± 0.04 fold variation to control, $P<0.05$, Fig. IV-13B and C).

In order to confirm these results a dual-labeling fluorescent immunohistochemistry was performed. Together with the c-KIT immunodetection, cell membrane was labeled with a cell-marker to distinguish the full-length from the truncated cytoplasmic c-KIT (Fig. IV-13D).

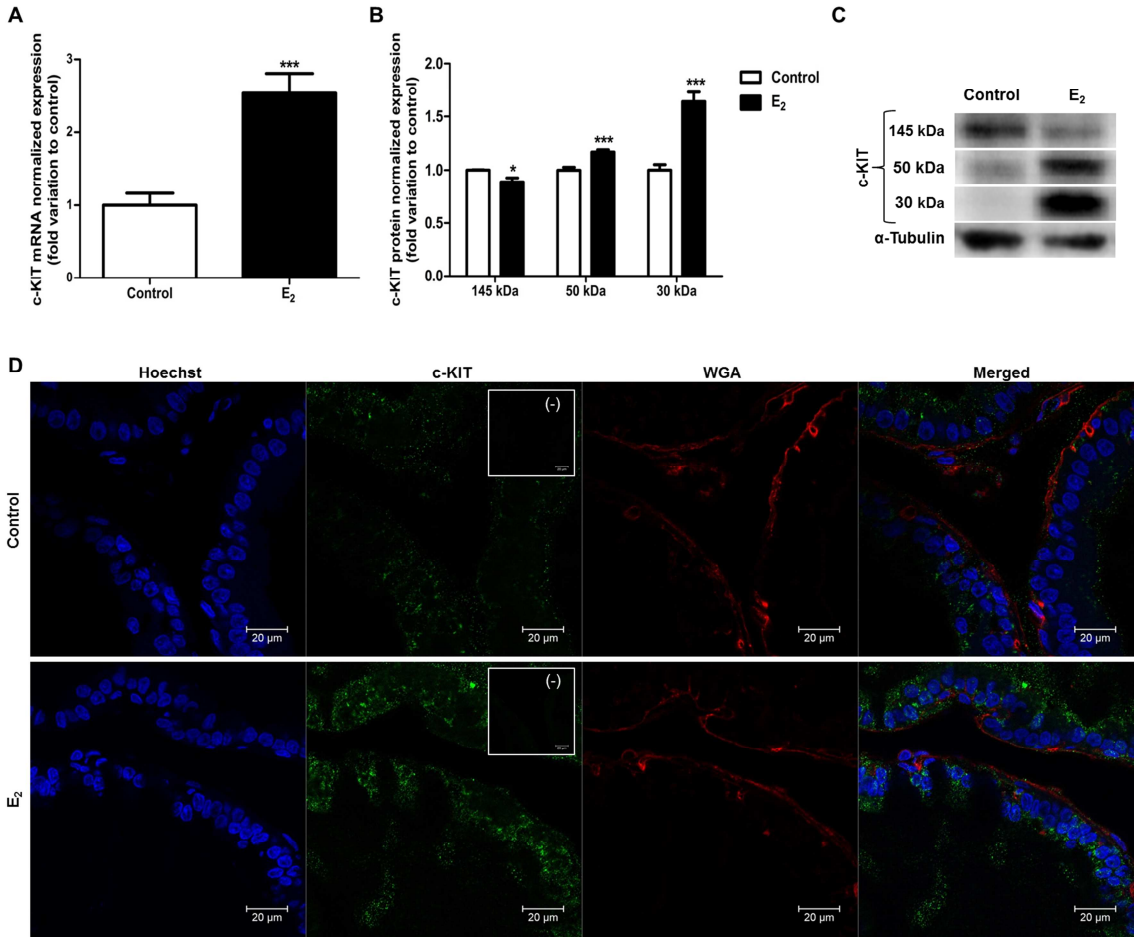


Figure IV-13. Effect of E_2 on the mRNA (A) and protein (B) expression of c-KIT in rat prostate tissue. **(A)** The mRNA expression was determined by qPCR after normalization with β-actin housekeeping gene. **(B)** Protein expression was determined by WB analysis after normalization with α-tubulin. Results are expressed as the fold variation relative to control (0 nM E_2, dashed line). Error bars indicate mean ± S.E.M (n ≥ 5). *P<0.05; ***P<0.001. **(C)** Representative immunoblots and **(D)** confocal microscopy images showing the co-localization of c-KIT with cell membrane in control and E_2-treated groups are provided. Nuclei are stained with Hoechst 33342 (blue), c-KIT-positive staining is green and cell membrane was stained with WGA (red). Negative controls for c-KIT obtained by omission of the primary antibody are provided as insert panels (-).

Considering SCF, its mRNA was up-regulated in E_2-treated group (3.70 ± 0.54 fold variation to control, P<0.001, Fig. IV-14A).

Contrarily, SCF protein was down-regulated by E_2 treatment (0.78 ± 0.01 fold variation to control, P<0.05, Fig. IV-14B and C). Although SCF can exist in different forms, only a unique specific band was detected in WB analysis (Fig. IV-14). Nevertheless, both membrane and soluble forms of SCF were detected in dual labeling fluorescent immunohistochemistry. The immunohistochemistry also revealed that SCF was decreased in the treated group comparatively to the control (Fig. IV-14 D).

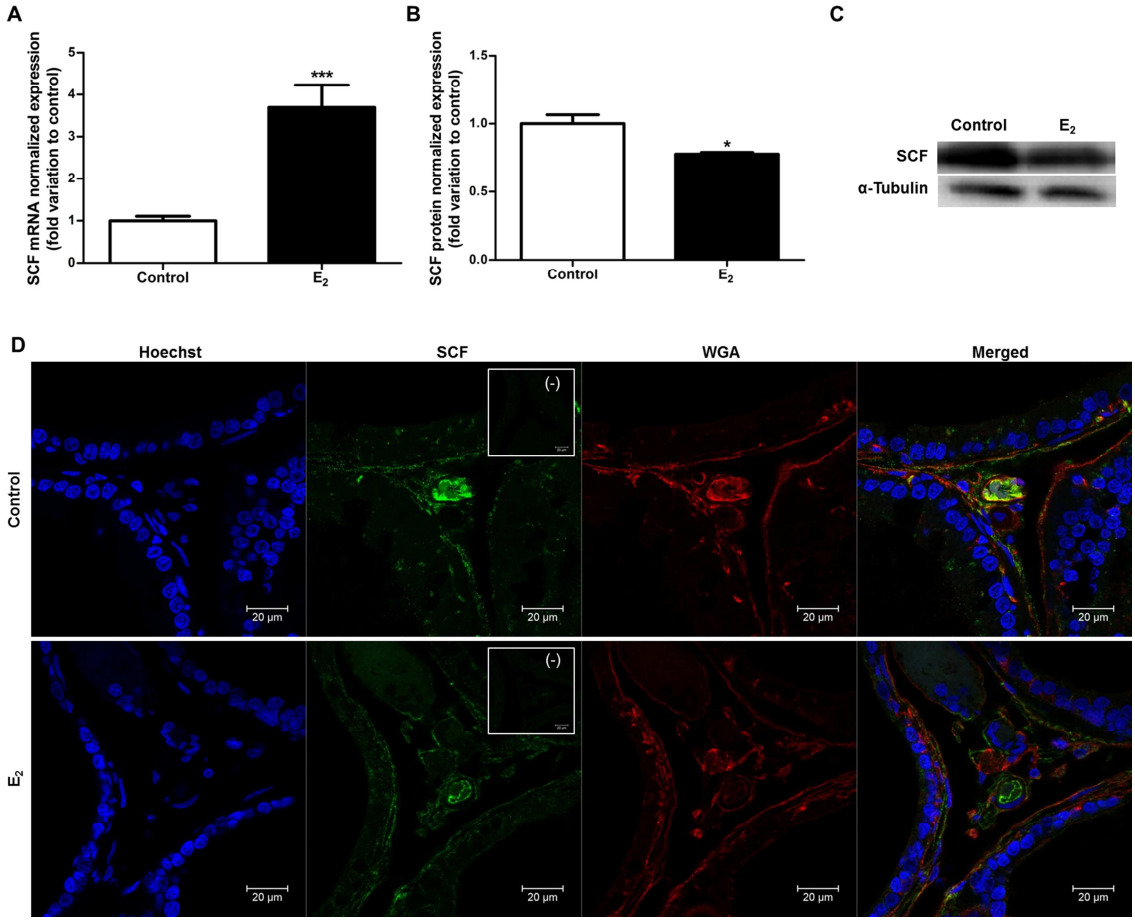


Figure IV-14. Effect of E_2 on the mRNA (A) and protein (B) expression of SCF in rat prostate tissue. **(A)** The mRNA expression was determined by qPCR after normalization with ß-actin housekeeping gene. **(B)** Protein expression was determined by WB analysis after normalization with α-tubulin. Results are expressed as the fold variation relative to control (0 nM E_2, dashed line). Error bars indicate mean ± S.E.M (n ≥ 5). *P<0.05; ***P<0.001. **(C)** Representative immunoblots and **(D)** confocal microscopy images showing the co-localization of SCF with cell membrane in control and E_2-treated groups are provided. Nuclei are stained with Hoechst 33342 (blue), SCF-positive staining is green and cell membrane was stained with WGA (red). Negative controls for SCF obtained by omission of the primary antibody are provided as insert panels (-).

3.2. Diminished Expression of c-KIT in Response to E_2 is Concomitant with Decreased Prostate Weight, Diminished Proliferation and Increased Apoptosis

SCF/c-KIT system activity is strongly related with the promotion of cell proliferation and control of apotosis, and the diminished expression of c-KIT receptor in rat prostate in response to E_2 suggests that proliferation/apoptosis may be affected. Thus, it was compared prostate weight, proliferation index and apoptosis between controls and E_2-treated rats.

E_2-treatment significantly decreased prostate weight in almost an half in comparison with the control group (Fig. IV-15B). On the other hand, no changes were verified in animal weight (Fig. IV-15A).

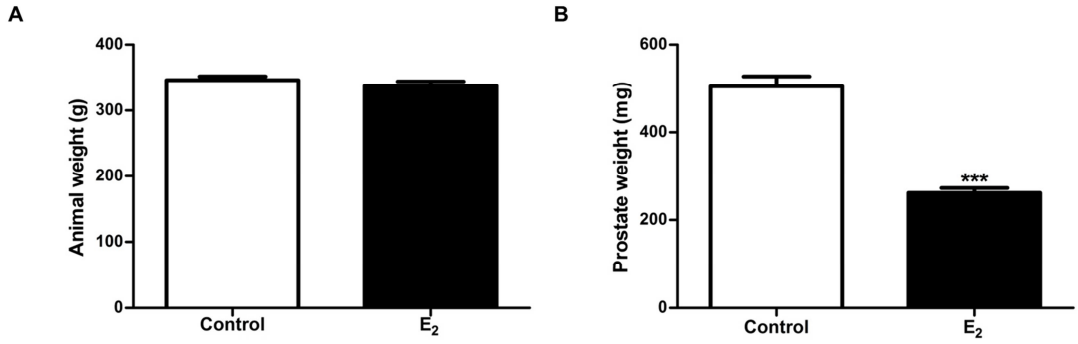


Figure IV-15. Effect of E_2 treatment on animal weight (A) and prostate weight (B). Error bars indicate mean ± S.E.M (n ≥ 5). ***P<0.001.

Ki67 is considered a marker of proliferation, since it is found in the nucleus of proliferating cells in all active phases of the cell division cycle but is absent in nonproliferating cells (Gerdes et al., 1984). Ki67 immunofluorescence was used to determine the cell proliferation index in the prostate of E_2 treated animals. The cell proliferation index, determined by the number of Ki67-positive cells relative to the total cell number, was reduced by approximately half in rats treated with E_2 comparatively to control group (0.50 ± 0.10 fold variation to control, P<0.05, Fig. IV-16).

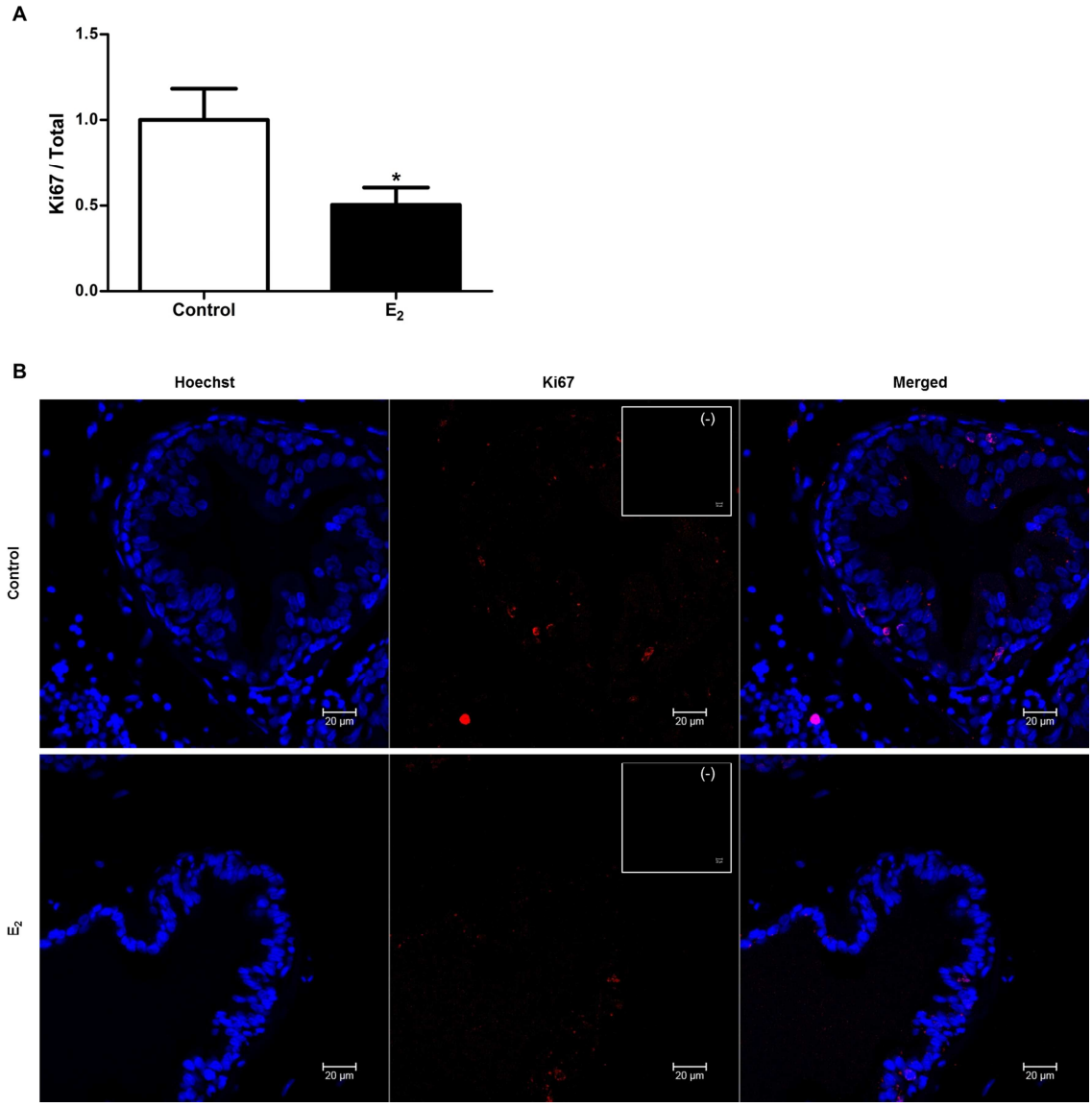


Figure IV-16. Proliferation in the prostate of control and E_2-treated animals. (A) Proliferation index determined by Ki67 immunofluorescence analysis. Percentage of Ki67-positive cells relative to the total cell number. Results are expressed as the fold variation compared with control. Error bars indicate mean ± S.E.M (n ≥ 5). *P<0.05. **(B)** Representative confocal microscopy images showing the Ki67 labelling in control and E2-treated groups. Nuclei are stained with Hoechst 33342 (blue) and Ki67 positive staining is red.

Additionally to the proliferation analysis, it was evaluated the effect of E_2 treatment on apoptosis of rat prostate cells. Independently of the apoptotic pathway, the cascade of cell-death always leads to the activation of caspase-3 (Lawen, 2003). Thus, caspase-3 activity is widely used as a measurement of apoptosis. A huge increase in the activity of caspase-3 was observed in E_2-treated group relatively to control (5.18 ± 0.44 fold variation, P<0.001, Fig. IV-17A). The prostate of animals injected with E_2 also revealed increased DNA fragmentation in comparison with non-treated ones as indicated by TUNEL assay (Fig. IV-17B).

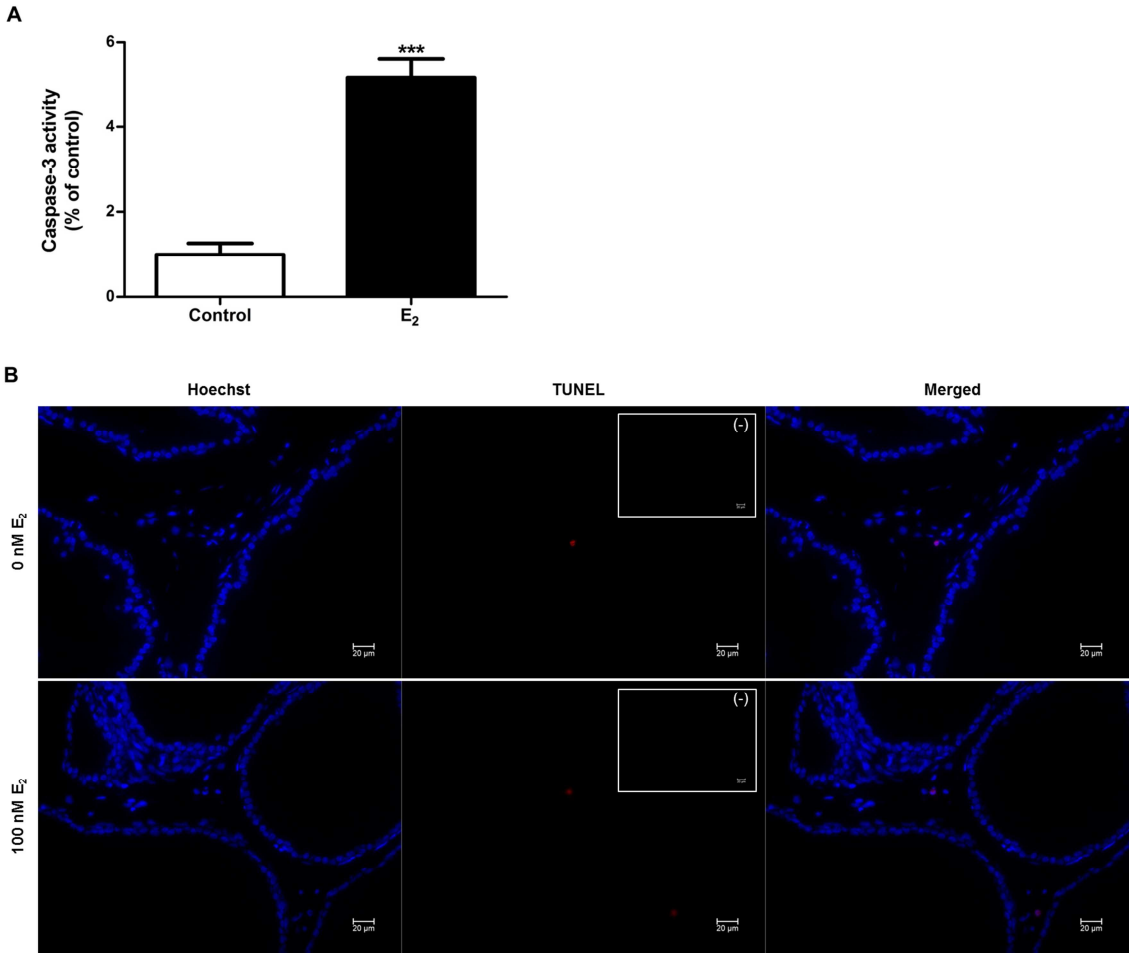


Figure IV-17. Apoptosis in the prostate of control and E_2-treated animals. (A) Caspase-3 activity. Results are expressed as the fold variation compared with control. Error bars indicate mean ± S.E.M (n ≥ 5). ***P<0.001. **(B)** Representative fluorescence microscopy images showing the TUNEL labeling in control and E2-treated groups.

Apoptosis may occur by two distinct but connected pathways: the intrinsic and the extrinsic pathway. There are evidences that estrogens increase apoptosis of different cell types through activation of extrinsic pathway (Zhao et al., 2011;Correia et al., 2014). In order to evaluate the apoptotic pathway activated by E_2 in rat prostate, the expression of some proteins related with apoptosis was assessed by WB analysis.

The Bax and Bcl-2 proteins are, respectively, proapoptotic and antiapoptotic members of the Bcl-2 family of apoptosis regulators (Lawen, 2003) and are mainly involved in the activation of intrinsic pathway of apoptosis (Harris and Thompson, 2000). The Bax/Bcl-2 protein ratio is considered a powerful marker of cell apoptosis (Mackey et al., 1998;Harris and Thompson, 2000). Bax/Bcl-2 protein ratio was decreased in E_2-treated group when compared with the control group (0.72 ± 0.04 fold variation, P<0.001, Fig. IV-18A). This reduction seems to be due to an increase in Bcl-2 expression in treated rats (1.46 ± 0.07 fold variation to control, P<0.001, Fig. IV-18B), since no changes were verified on Bax expression.

Caspase-9, which is involved in the intrinsic pathway of apoptosis, did not showed altered expression between treated animals and the control group (Fig. IV-18B).

The death factors Fas (receptor) and Fas-L (ligand) trigger the activation of the extrinsic pathway (Wajant, 2002). Both receptor and ligand were up-regulated in the prostate of E_2-treated animals comparatively with the control group (respectively, 1.26 ± 0.06 ($P<0.01$) and 1.28 ± 0.11 fold variation, ($P<0.05$), Fig. IV-18C).

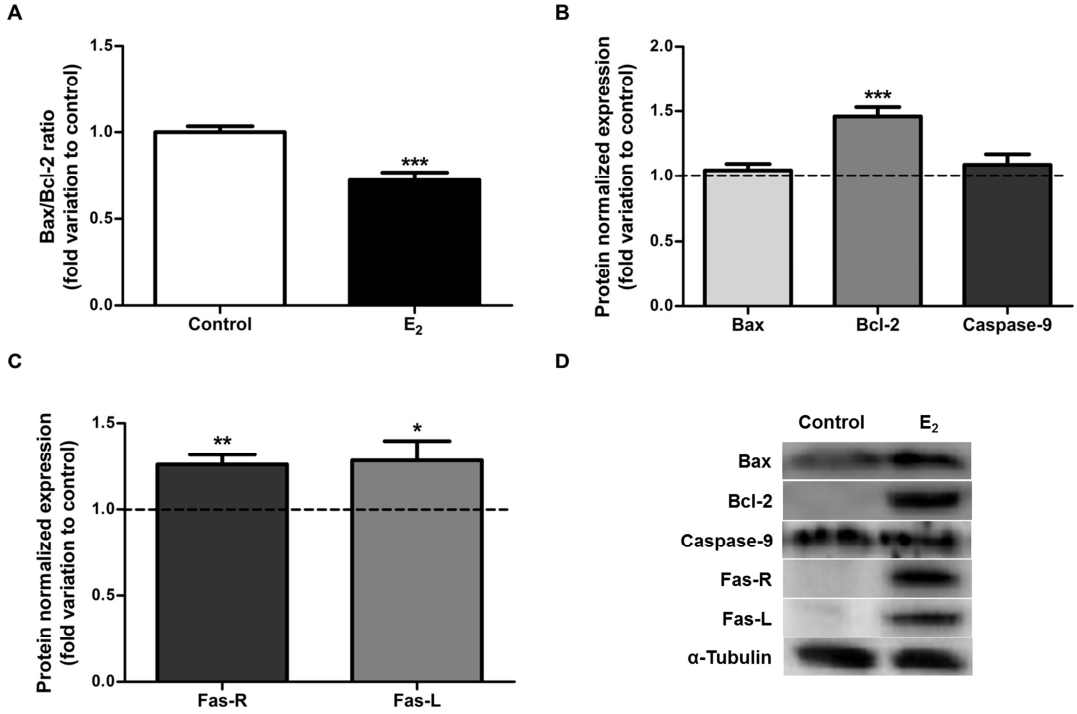


Figure IV-18. Expression of apoptosis regulators in the prostate of control and E_2-treated animals. **(A)** Bax/Bcl- ratio. **(B)** Apoptosis regulators of the intrinsic pathway. **(C)** Death factors involved in the extrinsinc pathway. Results are expressed as the fold variation compared with control. Error bars indicate mean ± S.E.M ($n \geq 5$). *$P<0.05$, **$P<0.01$, ***$P<0.001$. **(D)** Representative immunoblots.

V. Discussion

PCa is the most frequent type of oncological disease in men, with an increasing incidence in last years. The onset of a tumor includes several changes in cell behavior, as a result of alterations in the molecular events underlying cell cycle progression, proliferation, differentiation, development, motility, and apoptosis. The SCF/c-KIT system has been reported as an important regulator of a variety of cell biological processes involved in tumor onset, development and invasion (Liang et al., 2013). In addition, some studies have showed that SCF/c-KIT system is expressed in several human cancers, including PCa, and that it plays a role in the control of cell proliferation and invasion (Natali et al., 1992;Simak et al., 2000). Nevertheless, the expression of this system in prostate is poorly known, particularly in the case of rodents. To date, only one study has described the expression of c-KIT in rat prostate, which was regularly observed in the space between the smooth muscle layer and the glandular layer of the prostatic duct system (Kusljic and Exintaris, 2010).

In the present dissertation, the expression of SCF and c-KIT was confirmed in rat prostate, both at mRNA and protein level. Moreover, it was also confirmed the expression of SCF/c-KIT system in neoplastic (PNT1A) and non-neoplastic prostate cell lines (LNCaP, DU145 and PC3). Different isoforms of c-KIT were found both in prostate *in vivo* and in *in vitro* cell cultures, which included the full-length and the tr-KIT, a cytoplasmic variant devoid of extracellular and transmembrane domains (Rossi et al., 1992;Takaoka et al., 1997). By means of distinct experimental approaches, and at least for our knowledge, this is the first report definitely characterizing, the expression pattern of SCF/c-KIT system in all PNT1A, LNCaP, DU145 and PC3 cells. Previous studies only have described the SCF and c-KIT expression in some of these neoplastic cells and the obtained results were discordant (Savarese et al., 1998;Wiesner et al., 2008). It is also a novelty of this dissertation, the identification of the cytoplasmic tr-KIT in these cell lines models of PCa, which is in accordance with a previous report describing the expression of this truncated variant in cases of PCa (Paronetto et al., 2004). Furthermore, it was also found an isoform of c-KIT with 50 kDa, which may be generated by a covalent interaction of the 30 kDa tr-KIT protein with some other proteins present in prostate cells, as was suggested in other studies (Muciaccia et al., 2010). The differences in the detection of c-KIT among existing studies may be explained by the use of different antibodies recognizing distinct epitopes of the protein. Herein, it was used an antibody that recognizes the C-terminus of c-KIT, so it can detect both full-length and tr-KIT. Other studies have used antibodies against the extracellular domain of c-KIT, detecting only the membrane isoform, which explains the failure in detecting the tr-KIT. Regarding SCF, only one isoform with 63 kDa was detected in WB analysis, which is a molecular weight higher than that of the described isoforms (45 kDa for mSCF and 31 kDa for sSCF). This may be related with the association of SCF with other proteins, due to inefficient denaturation of protein complexes, or simply, due to the WB technique variability.

The expression pattern of SCF and c-KIT in PNT1A, LNCaP, DU145 and PC3 cells, was also firstly described in the present study. Although without statistically significance due to the reduced number of samples, it was suggested that these cell lines present a differential expression of SCF and c-KIT variants. DU145 cells seem to display the highest expression for both SCF and c-KIT, with the exception of tr-KIT, which seems to increase with the aggressiveness stage from LNCaP to DU145 and then to PC3. This is further supported by the relationship that was previous found between the expression of tr-KIT and more advanced stages of PCa: tr-KIT is absent in BPH but is expressed in 28% of patients with low Gleason grade tumors and in 66% of those with more advanced tumors (Paronetto et al., 2004). Nevertheless, the mechanisms that regulate the expression levels of c-KIT and SCF in prostate cells remain to be identified.

Development and progression of PCa is highly dependent on the hormonal action, with androgens classically playing a role on these processes. In the last years, estrogens also have been implicated in the carcinogenesis of prostate (Carruba, 2007;Ho et al., 2011). However, other experimental evidences have been indicating that these steroids may be protective against PCa (Corey et al., 2002;Pravettoni et al., 2007). On the other hand, it has been established that the SCF/c-KIT system is hormonally regulated and a target of estrogens in several tissues except prostate (Figueira et al., 2014). Therefore, the main objective of this dissertation was to evaluate the effect of E_2 on the expression of SCF/c-KIT system in PCa cell lines and in rat prostate *in vivo*. It was verified that E_2 was capable of modulate the levels of SCF and c-KIT in prostate cell lines both at mRNA and protein level. A down-regulated expression of SCF and c-KIT protein was observed in all prostate cell lines, except PC3 cells, which displayed an up-regulated expression of SCF in response to E_2. Noteworthy, the estrogenic effects were highly time-specific with changes in the SCF/c-KIT expression observed only for 24 h of stimulation, while at 0, 12 or 48 h of stimulation no expression changes were visible. Since the estrogens act through the activation of distinct ERs, and SCF/c-KIT system is related with proliferation and apoptosis control, events regulated by ERs, it is likely that the differences in the regulation SCF/c-KIT system across the different cell lines may be explained by a different expression pattern of ERs. Previous studies have described that PNT1A, LNCaP and DU145 cells express ERβ but not ERα, while PC3 cells express both classical ERs (Lau et al., 2000;Pravettoni et al., 2007;Mak et al., 2013). However, ERβ seems to be lost with the progression of PCa to more advanced stages (Horvath et al., 2001) and, since PC3 cells represent the most aggressive stage, it is possible that they express lower levels of ERβ than the other cell lines. Moreover, it has been accepted that ERα is associated with pro-tumorigenic effects while the ERβ have an anti-tumorigenic role (Attia and Ederveen, 2012). In addition, the two isoforms of ERβ, ERβ1 and ERβ2, were reported in PC3 cells, with one of them contributing to adverse effects of E_2 (Dey et al., 2012). Thus, it is reasonable to assume that the up-regulated expression of SCF/c-KIT system in response to E_2 in PC3 cells is an effect mediated by ERα, while in PNT1A, LNCaP and DU145 cells, which only

express the ERβ, the expression of c-KIT and SCF is inhibited. The biological responses to estrogens are also mediated by the G-protein estrogen receptor (GPRE), and it has been indicated that it is involved in inhibition of proliferation and induction of apoptosis (Chan et al., 2010;Chimento et al., 2013), which raises the question whether GPER may play a role in the regulation of SCF and c-KIT.

It is also remarkable that the down-regulated expression of c-KIT in prostate cells occurred for all the variants detected, namely, the 145 kDa full-length c-KIT, the 50 kDa and the 30 kDa (tr-KIT). The exception were the LNCaP cells, which did not showed altered expression of the full-length of c-KIT in response to E_2. Interestingly, very recent studies reported that LNCaP cells express the ERα (Lee et al., 2014), as well as, the ERβ1 and ERβ2 isoforms (Hurtado et al., 2008). Furthermore, these cells express a mutated AR, which binds estrogens acting as pro-tumorigenic agent (Veldscholte et al., 1990;Elo et al., 1995). Altogether, these features of LNCaP cells may underlie the opposite effect of E_2 regulating the expression of the full-length c-KIT. However, it does not explain the decreased expression of the truncated isoforms.

It cannot be excluded from the discussion the fact that E_2-treatment increased the mRNA expression of SCF and c-KIT, which do not follow the results obtained at protein level. Indeed, the increase in mRNA with non-correspondent increase in protein is widely described, and can be explained since innumerable factors are associated with the great complexity of gene expression regulatory mechanisms, namely, mRNA retention in the nucleus, stability and half-life time of mRNA molecule, control of protein translation efficiency, post-translational modifications, and protein degradation (Mata et al., 2005). Nevertheless, the results of fluorescent immunocytochemistry further confirmed the WB analysis and were concordant for both SCF and c-KIT.

The results on the estrogenic regulation of SCF/c-KIT system in rat prostate *in vivo*, generally, reproduced those obtained in human prostate cell lines, with E_2–treatment down-regulating the protein expression of SCF and full-length c-KIT. Relatively to SCF, only one isoform was detected, and the results of fluorescent immunohistochemistry supported the observed diminished expression in response to E_2. Although the expression of the full-length c-KIT was down-regulated by E_2, in the case of 30 and 50 kDa truncated variants their expression was up-regulated in E_2-treated animals. This differentially effect did not occurred in cell lines, and may be explained by the complexity of the *in vivo* systems, which include an intricacy of regulatory processes that there are absent in simple cell systems *in vitro*. It also indicated that the estrogenic regulation of SCF/c-KIT system in rat prostate involve complex mechanisms dependent or independent of ERα and ERβ actions (Prins et al., 1998), or even can cross with the activity of endogenous hormones like androgens.

Dual labeling fluorescent immunohistochemistry using cytoplasmic membrane staining allowed localizing the membrane forms of SCF and c-KIT. Although WB analysis with anti-SCF antibody detected only one immune-reactive protein, results of fluorescent immunohistochemistry confirmed the location of SCF both at cell membrane and cytoplasm of rat prostate cells. This discrepancy may be due to the high sensitivity of fluorescent labeling comparatively with detection method in WB technique.

Considering that the activity of SCF/c-KIT system is strongly related with the promotion of cell proliferation and inhibition of apoptosis, the diminished expression of c-KIT receptor and SCF in rat prostate in response to E_2 suggests that proliferation/apoptosis may be affected. The Ki67 is a recognized marker of cell proliferation, since it is found in the nucleus of proliferating cells in all active phases of the cell division cycle but is absent in non-proliferating cells (Gerdes et al., 1984). The cell proliferation index, estimated thorough Ki67 fluorescence immunohistochemistry, was significantly reduced in the prostate of E_2-treated rats. The pronounced reduction observed in the prostate weight of E_2-treated animals comparatively with the control group, further confirms the anti-proliferative action of E_2 in rat prostate cells. Previous studies in rodents, reported that low doses of estrogens enhanced prostatic growth, while high doses are generally growth inhibitory (vom Saal et al., 1997). In this dissertation, a dose of 250 µg/day/Kg of E_2 was administrated to adult rats, which is a within a supraphysiological range, thus a high dose. Indeed, similar results were obtained with doses of 200 or 300 µg/Kg (vom Saal et al., 1997). However, in this previous study, the exposure to E_2 was during fetal life, while in the present work adult animals were used.

The effect of E_2 stimulation on the apoptosis of rat prostate cells also was investigated. The apoptotic cell death may be triggered by distinct pathways, the receptor-mediated (or extrinsic) and the mitochondrial (or intrinsic) pathway (Elmore, 2007). Independently of the apoptotic pathway activated, the cascade of cell-death always leads to the activation of caspase-3 (Lawen, 2003). Thus, caspase-3 activity is widely used as a measurement of apoptosis. A huge increase in the activity of caspase-3 was observed in prostates of E_2-treated group relatively to control. Also, a TUNEL assay demonstrated increased DNA fragmentation in the prostates of animals exposed to E_2. These results indicate that E_2 treatment increases apoptosis in rat prostate cells, which also has been reported in other tissues of reproductive tract (Correia et al., 2014).

Previous studies also have shown that estrogens trigger apoptosis through the activation of extrinsic pathway (Zhao et al., 2011;Correia et al., 2014). In order to evaluate which was the apoptotic pathway involved in the estrogenic regulation, the expression of several apoptosis related proteins was determined by WB analysis.

The Bax and Bcl-2 proteins, respectively, proapoptotic and antiapoptotic members of the Bcl-2 family of apoptosis regulators (Lawen, 2003) are mainly involved in the activation of

intrinsic pathway of apoptosis, although they can also be regulated in the extrinsic pathway (Harris and Thompson, 2000). The Bax/Bcl-2 protein ratio, considered a powerful marker of cell commitment to apoptosis (Mackey et al., 1998;Harris and Thompson, 2000) was decreased in the rat prostate under E_2 stimulation. This reduction was caused by the increased expression of Bcl-2 expression in treated rats, since no changes were verified on Bax expression. In addition, caspase-9, an initiator caspase involved in the intrinsic pathway of apoptosis, did not showed altered expression with E_2 treatment.

On the other hand, the activation of the extrinsic pathway of apoptosis is triggered by death factors at cell membrane, like the complex Fas (receptor) and Fas-L (ligand) (Wajant, 2002). Both Fas and Fas-L proteins were up-regulated in the prostate of E_2-treated animals comparatively with the control group, which indicates that E_2 stimulates apoptosis in rat prostate cells through enhanced activity of the extrinsic pathway.

VI. Conclusion

The present dissertation showed that E_2 regulates the expression of SCF/c-KIT system in neoplastic and non-neoplastic human prostate cells, as well as, in rat prostate *in vivo*.

E_2 down-regulated the expression of SCF and c-KIT in PNT1A, LNCaP and DU145 cells, but an opposite effect was observed in PC3 cells, which may be consequence of a differential expression pattern of ERs.

The SCF/c-KIT system is also down-regulated by E_2 in rat prostate, which was accompanied by decreased cell proliferation and increased apoptosis. Moreover, it was indicated that E_2 triggers apoptosis of prostate cells through the activation of extrinsic pathway of apoptosis.

In conclusion, the present findings demonstrated the anti-proliferative and apoptosis-inducer effects of estrogens, which may depend on the down-regulation of the SCF/c-KIT system. Furthermore, the results obtained support the protective role of estrogens against PCa. In the future, disclosing the molecular mechanisms underlying the estrogenic effects in prostate will be crucial to identify possible targets for treatment of PCa.

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VIII. Publications and Communications

1. Articles in International Peer-Reviewd Journal

Figueira MI[#], Cardoso HJ[#], Correia S, Maia CJ, Socorro S (2014). Hormonal regulation of c-KIT receptor and its ligand: implications for human infertility? *Progress in Histochemistry and Cytochemistry* (In press). DOI: 10.1016/j.proghi.2014.09.001. IF: 5.909

Cardoso HJ[#], **Figueira MI**[#], Correia S, Vaz CV, Socorro S (2014). The SCF/c-KIT system in the male: survival strategies in fertility and cancer. *Molecular Reproduction and Development* (In press). DOI: 10.1002/mrd.22430. IF: 2.812

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2. Oral Communications

Figueira MI, Correia S, Vaz CV, Cardoso HJ, Gomes IM, Marques R, Maia CJ, Socorro S. Estrogens down-regulate the stem cell factor (SCF)/c-KIT system in rat prostate: evidence of antiproliferative and apoptotic effects. IX ANNUAL CICS SYMPOSIUM 30th June and 1st July 2014, Covilhã, Portugal.

Cardoso HJ, Vaz CV, Correia S, **Figueira MI**, Marques R, Maia CJ, Socorro S. Paradoxical and contradictory effects of imatinib in two cell line models of prostate cancer. IX ANNUAL CICS SYMPOSIUM 30th June and 1 st July 2014, Covilhã, Portugal.

3. Posters

Figueira MI, Correia S, Vaz CV, Cardoso HJ, Gomes IM, Marques R, Maia CJ, Socorro S. The estrogenic regulation of stem cell factor (SCF) and c-KIT in rat prostate supports the protective role of estrogens in prostate cancer. 1st ASPIC INTERNATIONAL CONGRESS 25-26 November 2014, Lisbon, Portugal.

Cardoso HJ, Vaz CV, Correia S, **Figueira MI**, Marques R, Maia CJ, Socorro S. Paradoxical and contradictory effects of imatinib in two cell line models of prostate cancer. 1st ASPIC INTERNATIONAL CONGRESS 25-26 November 2014, Lisbon, Portugal.