UNIVERSIDADE DA BEIRA INTERIOR
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The SCF/c-KIT system and imatinib actions in prostate cancer: a cross-talk with RGN?

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«Sei que tenho um cancro e que um dia me vai vencer.
Mas esse dia não chegou e até lá
Tenciono continuar a aproveitar cada momento.
Tive várias derrotas na vida,
Mas de todas as vezes caí de pé.
É preciso nunca deixar de viver.

A vida continua e continuarei a vivê-la,
Todos os dias, sem nunca me distrair

Eu provavelmente morrerei da doença
Mas o que nunca acontecerá é a doença matar-me»

Manuel Forjaz

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Resumo

A progressão do cancro de próstata, de uma fase inicial com o tumor confinado à próstata para formas mais agressivas e invasivas, está associada à perda de resposta a androgénios. Neste estadio da patologia, as células malignas da próstata proliferaram independentemente da ação dos androgénios (o chamado cancro da próstata hormono-resistente), o que leva à falha das terapias clássicas de ablação de androgénios e restringe em muito as opções terapêuticas disponíveis para esta forma, normalmente letal, da doença. O *Imatinib mesylate* é uma droga quimioterapêutica que inibe a atividade de recetores tirosina cinase, como por exemplo o c-KIT, a qual tem vindo a ser usada com sucesso no tratamento de leucemias e tumores gastrointestinais. No entanto, a aplicação com imatinib no tratamento do cancro da próstata não tem sido totalmente eficaz, apresentando eficácia divergente em modelos pré-clínicos e testes *in vivo*. Por outro lado, a regucalcina (RGN), é uma proteína de ligação ao cálcio (Ca^{2+}), que regula a homeostase de Ca^{2+} intracelular e a atividade de várias proteínas envolvidas em vias de sinalização intracelular, como cinases e fosfatases, para a qual têm vindo a ser demonstrados os seus efeitos na supressão da proliferação celular na próstata. Isto levanta a questão de a RGN poder estar associada à regulação da expressão do c-KIT, e do seu ligando, o *stem cell factor* (SCF) em células da próstata. Assim, o objetivo principal da presente dissertação foi analisar os efeitos citotóxicos do imatinib em dois modelos celulares de cancro da próstata hormono-resistente, as linhas celulares DU145 e PC3. Para além disso, avaliou-se o efeito da RGN na expressão do sistema SCF/c-KIT na próstata de ratos através da utilização de modelo animal transgénico que sobre-expressa a RGN (Tg-RGN). Por último, foi estudada a localização sub-celular da RGN em células de cancro da próstata hormono-resistente, e a sua associação com a α-tubulina. As linhas celulares DU145 e PC3 foram incubadas com 20 μM imatinib durante 48 e 72 horas. O ensaio MTS foi utilizado para avaliar a viabilidade celular em resposta ao imatinib e a medição colorimétrica da atividade enzimática da caspase-3 foi incluída como um ponto final da apoptose. A expressão de reguladores do ciclo celular/apoptose em resposta ao imatinib, e a expressão do sistema SCF/c-KIT em ratos Tg-RGN vs. *wild-type* foi determinada através de PCR em tempo real e Western Blot. A expressão da RGN nas células DU145 e PC3 e a co-localização da RGN com α-tubulina foram avaliadas através de imunocitoquímica com marcação por fluorescência. O tratamento com imatinib diminuiu a viabilidade celular das células DU145 em ambos os tempos experimentais, 48 e 72 horas. No caso das células PC3, embora o imatinib tenha diminuído a sua viabilidade 6 horas após o tratamento, de seguida a viabilidade das células aumentou significativamente em relação ao controlo. O tratamento com imatinib aumentou a atividade enzimática da caspase-3 nas células DU145 enquanto nas células PC3 esta diminuiu significativamente, às 48 e 72 horas. Para além disso, as células DU145 exibiram um perfil de expressão reduzida da proteína anti-apoptótica Bcl-2 e níveis aumentados de proteínas

apoptóticas como a caspase-8 e caspase-9. Porém, não foram observadas diferenças nos níveis de expressão destas proteínas nas células PC3. A expressão do mRNA do p21, um reconhecido inibidor do ciclo celular, aumentou em ambas as linhas celulares em resposta ao tratamento com imatinib. Relativamente aos níveis de mRNA do fator de crescimento vascular endotelial (VEGF), estes encontraram-se diminuídos nas células DU145 em resposta ao imatinib, mas foi observado um efeito oposto nas células PC3. Com o intuito de tentar explicar a resposta diferencial das células DU145 e PC3 ao imatinib, caracterizou-se a expressão do c-KIT, e das suas isoformas, nestas linhas celulares. Análises por imunocitoquímica de fluorescência e Western blot mostraram que a expressão da forma membranar ativa do c-KIT se encontra diminuída nas células PC3 relativamente às células DU145, e que as células PC3 apresentam maior expressão das isoformas truncadas do c-KIT. Relativamente aos resultados da expressão do sistema SCF/c-KIT nas próstatas dos ratos Tg-RGN, verificou-se que esta se encontra diminuída. Confirmou-se ainda a expressão da RGN nas linhas celulares de cancro da próstata hormono-resistente e a sua co-localização com α-tubulina, um componente fundamental dos microtúbulos. Os resultados da presente tese demonstraram o efeito apoptótico do Imatinib nas células DU145, provavelmente através da inativação do c-KIT. Por outro lado, os efeitos paradoxais do imatinib observados nas células PC3 poderão estar associados à presença das isoformas truncadas do c-KIT, para as quais não está definitivamente estabelecido o seu papel. Estes resultados contribuíram igualmente para a compreensão da ineficácia do imatinib como opção terapêutica no cancro da próstata. Mais ainda, reforçam o papel da RGN como molécula antiproliferativa e reguladora do ciclo celular, o que é suportado pela diminuição da expressão do sistema SCF/c-KIT em resposta à sobre-expressão da RGN, assim como, pela associação da RGN com os componentes da maquinaria de divisão celular.

Palavras-chave

Cancro da Próstata, DU145, Imatinib, PC3, Regucalcina, Sistema SCF/c-KIT

Resumo Alargado

A progressão do cancro de próstata, de uma fase inicial com o tumor confinado à próstata para formas mais agressivas e invasivas, está associada à perda de resposta aos androgénios. O cancro da próstata é uma doença cuja etiologia apresenta diversas causas. A presença de mutações em proteínas reguladoras da apoptose e proliferação é um acontecimento frequente nesta doença. Além disso, danos no epitélio prostático na sequência de inflamações recorrentes, infeções e/ou exposição a carcinogénios, pode despoletar o aparecimento do cancro. Nos estadios mais avançados da patologia, as células malignas da próstata proliferaram independentemente da ação dos androgénios (o chamado cancro da próstata hormono-resistente), sendo diversos os mecanismos que podem conduzir ao crescimento independente de androgénios. Entre muitos mecanismos, alguns dos quais ainda por esclarecer, destacam-se a amplificação da expressão do recetor de androgénios (AR), as mutações no gene do AR, as modificações em vias de sinalização celular que modulam a função do AR, as alterações na expressão de coreguladores do AR e ainda alterações no metabolismo. Todas estas alterações culminam no crescimento do tumor, mesmo com níveis reduzidos de androgénios, o que leva à falha das terapias clássicas de ablação de androgénios e restringe em muito as opções terapêuticas disponíveis para esta forma, normalmente letal, da doença. Portanto, revelar os mecanismos moleculares que desencadeiam o aparecimento de cancro da próstata resistente ao tratamento hormonal e o desenvolvimento de terapias eficazes para esta fase da doença é de crucial importância. O c-KIT é um recetor que pertence à família de recetores tirosina cinase do tipo III que é ativado pelo seu ligando específico, o *stem cell factor* (SCF). As vias de sinalização do c-KIT têm um papel essencial na decisão do "destino" celular controlando a proliferação, diferenciação e apoptose. Além disso, foi demonstrado que o c-KIT se encontra aumentado em pacientes com cancro da próstata hormono-resistente. O *Imatinib mesylate* é uma droga quimioterapêutica que inibe a atividade de recetores tirosina cinase, como por exemplo o c-KIT, competindo com o ATP no sítio de ligação ao ATP e parando a fosforilação e consequentemente parando as vias de sinalização celular. Tem vindo a ser usado com sucesso no tratamento de leucemias e tumores gastrointestinais. No entanto, a aplicação do imatinib no tratamento do cancro da próstata não tem sido totalmente eficaz, apresentando eficácia divergente em modelos pré-clínicos e testes *in vivo*. Por outro lado, a regucalcina (RGN), é uma proteína de ligação ao cálcio (Ca^{2+}), que regula a homeostase de Ca^{2+} intracelular e a atividade de várias proteínas envolvidas em vias de sinalização intracelular, como cinases e fosfatases. Esta proteína tem vindo a ser detetada em diversos tecidos do trato reprodutor masculino e a sua expressão encontra-se alterada em diversas patologias. Recentemente, foi demonstrado que a RGN tem efeitos supressores na proliferação celular na próstata, diminuindo o peso das próstatas e o índex de proliferação. Além disso, também foi demonstrado que a sobreexpressão da RGN

tem um papel protetor na carcinogénese no cancro da mama. Isto levanta a questão de a RGN poder estar associada à regulação da expressão do c-KIT, e do seu ligando, o SCF em células da próstata. Assim, o objetivo principal da presente dissertação foi analisar os efeitos citotóxicos do imatinib em dois modelos celulares de cancro da próstata hormono-resistente, as linhas celulares DU145 e PC3. Para além disso, avaliou-se o efeito da RGN na expressão do sistema SCF/c-KIT na próstata de ratos através da utilização de modelo animal transgénico que sobre-expressa a RGN (Tg-RGN). Por último, foi estudada a localização sub-celular da RGN em células de cancro da próstata hormono-resistente, e a sua associação com a α-tubulina. As linhas celulares DU145 e PC3 foram incubadas com 20 μM imatinib durante 48 e 72 horas. O ensaio MTS foi utilizado para avaliar a viabilidade celular em resposta ao imatinib e a medição colorimétrica da atividade enzimática da caspase-3 foi incluída como um ponto final da apoptose. A expressão de reguladores do ciclo celular/apoptose em resposta ao imatinib, e a expressão do sistema SCF/c-KIT em ratos Tg-RGN vs. *wild-type* foi determinada através de PCR em tempo real e Western Blot. A expressão de RGN nas células DU145 e PC3 e a co-localização da RGN com α-tubulina foram avaliadas através de imunocitoquímica com marcação por fluorescência. O tratamento com imatinib diminuiu a viabilidade celular das células DU145 em ambos os tempos experimentais, 48 e 72 horas. No caso das células PC3, embora o imatinib tenha diminuído a sua viabilidade 6 horas após o tratamento, de seguida a viabilidade das células aumentou significativamente em relação ao controlo. O tratamento com imatinib aumentou a atividade enzimática da caspase-3 nas células DU145 enquanto nas células PC3 esta diminuiu significativamente, às 48 e 72 horas. Para além disso, as células DU145 exibiram um perfil de expressão reduzida da proteína anti-apoptótica Bcl-2 e níveis aumentados de proteínas apoptóticas como a caspase-8 e caspase-9. Porém, não foram observadas diferenças nos níveis de expressão destas proteínas nas células PC3. A expressão do mRNA do p21, um reconhecido inibidor do ciclo celular, aumentou em ambas as linhas celulares em resposta ao tratamento com imatinib. Relativamente aos níveis de mRNA do fator de crescimento vascular endotelial (VEGF), estes encontraram-se diminuídos nas células DU145 em resposta ao imatinib, mas foi observado um efeito oposto nas células PC3. No intuito de tentar explicar a resposta diferencial das células DU145 e PC3 ao imatinib, caracterizou-se a expressão do c-KIT, e das suas isoformas, nestas linhas celulares. Análises por imunocitoquímica de fluorescência e Western blot mostraram que a expressão da forma membranar ativa do c-KIT se encontra diminuída nas células PC3 relativamente às células DU145, e que as células PC3 apresentam maior expressão das isoformas truncadas do c-KIT. Relativamente aos resultados da expressão do sistema SCF/c-KIT nas próstatas dos ratos Tg-RGN, verificou-se que esta se encontra diminuída. Confirmou-se ainda a expressão da RGN nas linhas celulares de cancro da próstata hormono-resistente e a sua co-localização com α-tubulina, um componente fundamental dos microtúbulos. Os resultados da presente dissertaçâo demonstraram o efeito apoptótico do Imatinib nas células DU145, provavelmente através da inativação do c-KIT. Por outro lado, os efeitos paradoxais do imatinib observados nas células PC3 poderão estar associados à presença das isoformas truncadas do c-KIT, para as

quais não está definitivamente estabelecido o seu papel. Estes resultados contribuíram igualmente para a compreensão da ineficácia do imatinib como opção terapêutica no cancro da próstata. Mais ainda, reforçam o papel da RGN como molécula antiproliferativa e reguladora do ciclo celular, o que é suportado pela diminuição da expressão do sistema SCF/c-KIT em resposta à sobre-expressão da RGN, assim como, pela associação da RGN com os componentes da maquinaria de divisão celular.

Palavras-chave

Cancro da Próstata, DU145, Imatinib, PC3, Regucalcina, Sistema SCF/c-KIT

Abstract

The progression of prostate cancer (PCa), from an early stage confined to prostate to a more aggressive form, is associated with loss of androgen responsiveness. At this stage, PCa cells proliferate independently of androgens actions (the so-called hormone refractory prostate cancer, HRPC), which cause the failure of classical androgen ablation therapies and restricts the therapeutic options for this usually lethal form of disease. Imatinib mesylate is a chemotherapeutic drug that inhibits the tyrosine kinase activity of c-KIT receptors among others, and has been successfully used to treat leukemias and gastrointestinal stromal tumors. However, its application for treatment of PCa has not been totally effective with preclinical models and clinical experimentation producing discordant results. On the other hand, regucalcin (RGN), a calcium (Ca^{2+})-binding protein that regulates intracellular Ca^{2+} homeostasis and the activity of several proteins involved in intracellular signaling pathways, namely, kinases and phosphatases, has been associated with suppression of cell proliferation in rat prostate. These raised the question whether RGN may regulate the expression of c-KIT and its ligand, the stem cell factor (SCF). Therefore, the present dissertation firstly aimed to analyze the cytotoxic effects of imatinib in two cell line models of HRPC, DU145 and PC3 cells. Moreover, the effect of RGN on the expression of SCF/c-KIT in rat prostate was evaluated by means of a transgenic animal model overexpressing RGN (Tg-RGN). Finally, the subcellular localization of RGN in HRPC cell lines and its association with α-tubulin was investigated. DU145 and PC3 cells were incubated with 20 μM imatinib for 48 and 72 hours. The MTS assay was used to assess cell viability in response to imatinib and the colorimetric measurement of the enzymatic activity of caspase-3 was included as an end-point of apoptosis. The expression of cell-cycle and apoptosis regulators in response to imatinib, and the expression of SCF/c-KIT in Tg-RGN vs. wild-type rats were determined by real-time PCR and Western Blot. The expression of RGN in HRPC cells lines in its association with α-tubulin were evaluated through fluorescent immunocytochemistry. Treatment with imatinib decreased the viability of DU145 cells at 48 and 72 hours. Although imatinib decreased the viability of PC3 cells upon 6 hours of treatment, thereafter cell viability significantly increased in relation to control. Accordingly, the enzymatic activity of caspase-3 was increased in DU145 cells whereas diminished activity of caspase-3 was observed in PC3 cells treated with imatinib for 48 and 72 hours. Moreover, DU145 cells displayed reduced expression of anti-apoptotic protein Bcl-2 and increased levels of the executioners of apoptosis caspase-8 and caspase-9. No differences were observed on the expression levels of these apoptosis related proteins in PC3 cells. The mRNA expression of cell cycle inhibitor p21 was increased in both DU145 and PC3 cells. Also, the mRNA levels of VEGF were decreased in DU145 cells in response to Imatinib but the opposite effect was seen in PC3 cells. To start explaining the differential response of DU145 and PC3 cells to imatinib, the expression of c-KIT receptor in these cell lines was characterized. Fluorescent immunocytochemistry and

Western Blot analysis showed that the expression of the active membrane-bound c-KIT is decreased in PC3 cells relatively to DU145. In addition, PC3 cells presented increased expression of truncated isoforms of c-KIT. Relatively to RGN the results obtained showed that the expression of SCF/c-KIT system is diminished in the prostate of Tg-RGN animals, which is in accordance with the antiproliferative effects of RGN, and indicates that regulation of SCF/c-KIT system may be a mechanism by which RGN restricts proliferation. Moreover, it was confirmed the expression of RGN in HRPC cells and its co-localization with α-tubulin, a fundamental component of microtubules. The results presented in this dissertation indicated that Imatinib was effective inducing apoptosis of DU145 cells likely through the inactivation of c-KIT. On the other hand, the paradoxical effects of imatinib in PC3 cells may be associated with the presence of truncated isoforms of c-KIT for which no definitive role has been established. These findings also contributed to understand the inefficacy of imatinib as therapeutic option in PCa. Moreover, the role of RGN as an antiproliferative molecule controlling cell cycle was further highlighted by the observed decreased expression of SCF/c-KIT system with overexpression of RGN, as well as, by the association of RGN with components of the cell division machinery.

Keywords

Imatinib, DU145, PC3, Prostate cancer, Regucalcin, SCF/c-KIT system

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List of Abbreviations

ABL	Abelson tyrosine kinase
ADT	Androgen-deprivation therapy
Apaf-1	Apoptotic protease activating factor-1
AR	Androgen receptor
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BPH	Benign prostatic hyperplasia
Ca^{2+}	Calcium
CZ	Central zone
DHT	5-α-Dihydrotestosterone
DTT	DiThioThreitol
EGF	Epidermal growth factor
FDA	Food and Drug Administration
GIST	Gastrointestinal stromal tumors
GNNK	Gly-Asn-Asn-Lys
GTPase Ran	Ras-related nuclear protein
HGPIN	High-grade PIN
HRPC	Hormone-refractory prostate cancer
IGF-1	Insulin-like growth factor 1
IL	Interleukin
KGF	Keratinocyte growth factor
LHRH	Luteinizing hormone-releasing hormone
MAPK	Mitogen-activated protein kinase
mSCF	Membrane isoform of SCF
NF-kB	Factor nuclear-kappa ß
PAP	Prostatic acid phospathase

PBS	Phosphate Buffer Saline
PCa	Prostate cancer
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PFA	ParaFormAldehyde
PI3K	Phosphoinositide-3-kinase
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PMSF	PhenylMethylSulfonyl Fluoride
pNA	p-Nitro-Aniline
PSA	Prostate-specific antigen
PTEN	Tumor-suppressor phosphatase and tensin homolog
PVDF	PolyVinylidene DiFluoride
PZ	Peripheral zone
qPCR	Real-time Quantitative Polymerase Chain Reaction
Rb	Retinoblastoma protein
RGN	Regucalcin
RIPA	Radioimmunoprecipitation
ROS	Reactive oxygen species
RTKs	Tyrosine kinase receptors
SCF	Stem cell factor
SDS-PAGE	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
Sl	*Steel locus*
S-KIT	Soluble c-KIT protein
SMP30	Senescence marker protein-30
sSCF	Soluble isoform of SCF
TGF-ß	Transforming Growth factor-ß
Tg-RGN	Transgenic overexpressing the RGN protein

Tr-kit	Truncated c-KIT protein
TZ	Transition zone
VEGF	Vascular endothelial growth factor
W	*Spotted locus*
Wb	Western Blot

I. Introduction

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Both authors

1. Brief overview of anatomy and physiology of prostate

The prostate is considered the largest accessory gland organ of male reproductive tract, being, approximately, 4 cm long and 2 cm wide. It is located posterior to the symphysis pubis, anterior to the rectum, and inferior to the bladder. Prostate is constituted by a fibrous capsule containing smooth muscle cells and innumerous veins and nerves (Ali et al., 2004;Lee et al., 2011). Several pioneer studies of Mcneal and colleagues characterized prostate anatomy and divided it into 3 glandular zones: the central zone (CZ), transition zone (TZ), and peripheral zone (PZ) (Fig. I.1). These zones have different embryologic origins and can be distinguished by histology, anatomic landmarks, biological functions, and susceptibility to pathologic disorders (McNeal, 1972; 1981; 1984;Lee et al., 2011). TZ is situated in bilateral regions in the middle to the base of the gland that surrounds the first part of the urethra. CZ is surrounded for PZ and is constituted for a conical structure with ducts diverging from mid-prostate to the prostatic base. PZ, that is a major component of the prostate, containing the ducts of urethra, in the mild-prostate, to the prostatic apex (McNeal, 1981). The PZ represents 70 % of prostate and is recognized as the zone most susceptible for development of prostate cancer (PCa) (Al-Ahmadie et al., 2008;Lee et al., 2011;Young et al., 2014). The prostate gland also includes a fourth non-glandular region that is constituted by fibromuscular stroma (Fig. I.1), which covers the urethra in the anteromedial prostate (Farnsworth, 1999;Young et al., 2014). The supporting stroma is a mixture of collagenous fibrous tissue and smooth muscle fibers (Farnsworth, 1999).

An alternative anatomical classification divides the prostate in an anterior lobe, a dorsal lobe, a lateral lobe and a ventral lobe (Young et al., 2014).

The normal function of prostate gland is fundamental to ensure sperm motility because this accessory gland produces a set of constituents of semen that provide the good environment for spermatozoa. These secretions empty into the prostatic urethra and make up about 30 % of the volume of semen (Frick and Aulitzky, 1991;Hayward and Cunha, 2000;VanPutte et al., 2014). The milky secretions of the prostate have a basic pH that possibly neutralizes the acidic environment of duct deferent and female vagina (VanPutte et al., 2014). Prostate epithelial cells produce other factors essential for proper sperm function, which includes citric acid and proteolytic enzymes (VanPutte et al., 2014).

The glandular prostatic epithelium is composed by the secretory epithelial cells, basal cells, stem cells and neuroendrocrine cells. The majority of epithelial cells, are columnar luminal cells, which are responsible for production of prostatic secretions including the prostate-specific antigen (PSA), the well-recognized indicator of PCa (Young et al., 2014). It is normally secreted into the ductal laminal and is removed by ejaculation. In normal situations PSA does not cross the epithelial basement membrane, however, in pathological conditions the histological organization of prostate is disrupted and PSA can be detected in the bloodstream (Cohen et al., 1992). The population of basal cells is present on the base of prostate gland in contact with basement membrane (Young et al., 2014). Prostate stem cells are confined to the basal compartment and represents a quiescent reserve that can divide

originating basal or luminal epithelial-like stem cells, uncommitted stem cells that differentiate to form semi-committed progenitors, or multiple populations of stem cells (Blum et al., 2010;Chen et al., 2013). The last cell type in the prostatic epithelium are the neuroendocrine cells that can secret neurosecretory products that promote prostate growth (Bonkhoff, 1998;Huang et al., 2006).

The human prostate is small in childhood, but in puberty rises in size to approximately 20-25 g in response to the increasing levels of serum testosterone. The prostate weight stabilizes until the individual has about 30 years and then prostatic weight turns to rise slowly, and this can mean the onset of Benign prostatic hyperplasia (BPH). The BPH nodules formed approximately upon 50 years can be transformed into PCa (Berry et al., 1984;Wong et al., 2003;Timms, 2008).

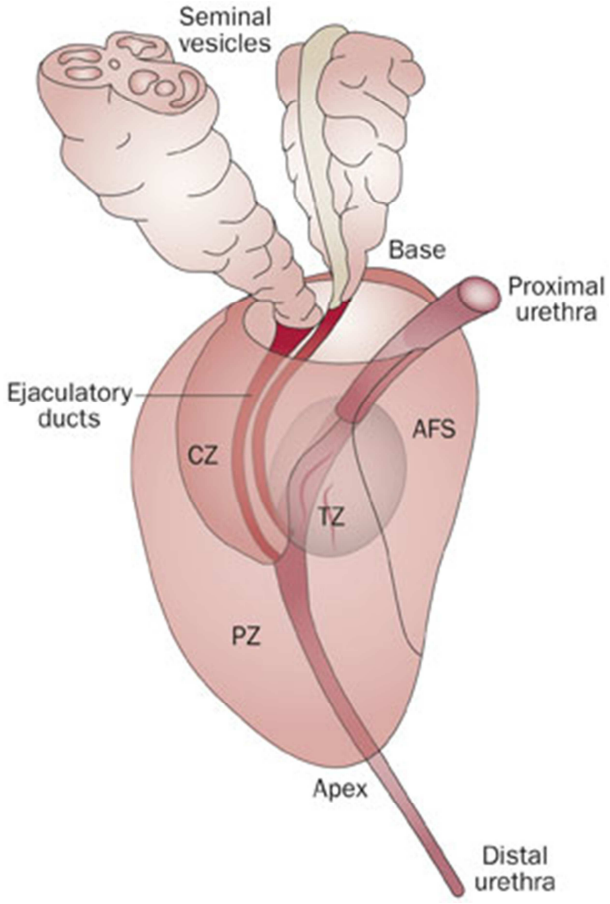

Figure I.1. Anatomy of Prostate gland. The prostate gland surrounds the bladder neck and also the first part of urethra. The anterior and apical surfaces are bounded by anterior fibromuscular stroma (AFS), shaped by collagenous stroma and muscle fibers. Also the prostate is divided in four zones, transition (TZ) zone that surrounds proximal prostatic urethra, central zone (CZ) that surrounds the ejaculatory ducts, peripheral zone (PZ) that consists in approximately 70 % of prostate (Wadhera, 2013).

2. Prostate cancer

2.1. Etiology and development of prostate cancer

PCa is the most common cancer in men and represents the second leading cause of cancer deaths. In 2014, 233,00 new cases are expected to occur accounting for 27 % of total cancer cases diagnosed, which is estimated to represent 29,480 of deaths in the USA (Siegel et al., 2014). In Portugal, statistics indicate that the mortality by PCa has been growing, and that, only in 2011, 1764 men's lost their life to this disease (Miranda, 2013).

PCa is mainly diagnosed upon 55 years and the prognostic of survival decreases in advanced ages (Whelan et al., 2013). Race and family history seem to contribute for development of PCa and, in fact, higher prevalence of disease is found in African descents. Also, in men whose first-degree family members are affected by disease the risk of PCa doubles (Ferlay et al., 2010).

Historically the human prostatic acid phosphatase (PAP) was the first serum biomarker for PCa, but it showed insufficient sensitivity, and later on, PSA was considered the ideal biomarker for screening of PCa, which still is the first line diagnosis marker (Ercole et al., 1987;Neal, 2010;Phillips, 2014). Nevertheless, it is also known that 15 % of men with normal or low levels of PSA had PCa (Mulders et al., 1990;Thompson et al., 2004). Therefore, a biopsy material is a fundamental piece for diagnosis, eliminating the presence of false positives and false negatives in PSA screening. The PCa is a heterogeneous and multifocal disease and the mechanisms driven its progression remain to be fully-elucidated. Normally, PCa is an asymptomatic disease that only manifest in latent stages of disease (Whelan et al., 2013). The prostatic epithelium can be damaged due to inflammation, infection and/or exposition to carcinogens, which can lead to the formation of proliferative inflammatory atrophy (PIA) (De Marzo et al., 1999). Alterations in this stage can conduct to the formation of histological lesions so-called prostatic intraepithelial neoplasia (PIN) (Putzi and De Marzo, 2000). PIN is characterized by the appearance of dysplasia of prostate luminal epithelial cells and a loss of distinct basal and secretory layers (Brawer, 2005;Wang et al., 2009;Davidsson et al., 2011). High-grade PIN (HGPIN) can be considered as the precursor of PCa (Brawer, 2005;Wang et al., 2009;Adamczyk et al., 2014).

Genomic lesions in PCa are common and can result in genomic rearrangement including amplification, alteration, deletion or translocation of segments of chromosomes (Saramaki and Visakorpi, 2007;Berger et al., 2011;Mao et al., 2011;Reid et al., 2012). The gain-of-function mutations in oncogenes and the deletion of tumor suppressor genes also are linked with carcinogenesis of prostate (Iurlaro et al., 2014). An uncontrolled cancer presents a combination of exacerbated proliferation, disrupted apoptosis and altered metabolic profiles (Lorenzo et al., 2007;Clarke et al., 2009;Vaz et al., 2012).

The phosphoinositide-3-kinase (PI3K) pathway is activated by several tyrosine kinases receptors and is the best characterized pathway in PCa. This pathway triggers cell proliferation through the activation of Akt and the tumor-suppressor phosphatase and tensin

homolog (PTEN) switches off the PI3K activity (Bitting and Armstrong, 2013). The overactivation of PI3K signaling in PCa is accompanied by PTEN deletion (Wang et al., 2006;Sun et al., 2009;Lonigro et al., 2011;Choucair et al., 2012). Also, alterations on the mitogen-activated protein kinase (MAPK) pathway seem contribute to the severity of PCa (Bakin et al., 2003;Mukherjee et al., 2011;Wang et al., 2012;Pavese et al., 2014).

Several other proteins involved in the control of cell cycle and cell survival are frequently mutated in PCa. Among other, this includes the widely recognized p53 and retinoblastoma protein (Rb). P53 is tumor-suppressor and a proapoptotic factor, and deletions and mutations on the p53 gene have been detected in approximately 40 % of PCa cases (Beltran et al., 2013). Also, the Rb protein, a tumor suppressor regulating cell cycle progression, is inactivated in advanced stages of this disease (Bookstein et al., 1990;Maddison et al., 2004;Thangavel et al., 2014). Other protein, the Homeobox protein Nkx-3.1, an important tumor suppressor in prostate, is frequently lost in PCa, by mechanisms of DNA-methylation and allelic deletion (Lei et al., 2006;Song et al., 2009;Akamatsu et al., 2010;Erbaykent-Tepedelen et al., 2011).

As described above, the inflammatory process may play a fundamental role triggering carcinogenesis. Leucocytes and macrophages migrate to inflammatory area and release reactive oxygen species (ROS). If high concentrations of ROS were produced can damage several cell structures including DNA and lipid-membranes (Ray et al., 2012). This event can contribute to carcinogenesis destructing cell-cell adhesion and cell-membrane functions and also contribute to mutations in DNA (Ishikawa et al., 2008;Kumar et al., 2008;Wong et al., 2009;Debelec-Butuner et al., 2014). Also, it has been shown that growth factors and cytokines released by macrophages in inflammatory response can contribute to suppress essential functions of androgen receptor (AR) in prostate (Debelec-Butuner et al., 2014). Factor nuclear-kappa ß (NF-kB) seems to link inflammation to PCa. Despite its actions promoting apoptosis, NF-kB also can inhibit autophagy, promoting survival of cancer cells. In addition, NF-kB seems contribute to the progression of PCa progression increasing the expression of PSA (Chen and Sawyers, 2002;Ray et al., 2012). Moreover, it has been reported that several microorganism (Poutahidis et al., 2013) and chronic inflammation caused by sexually transmitted infection may promote the appearance of PCa (Sutcliffe et al., 2009;Chung et al., 2013).

The onset of PCa also depends on the activity of molecules produced and secreted by stromal cells, and the cross-talk between cancer cells and stroma has been identified as crucial aspect in carcinogenesis. This includes the secretion of a myriad of factor, such as, growth factors and cytokines. The stroma may sustain invasiveness by releasing products capable of digest the extracellular matrix (Bhowmick and Moses, 2005). Indeed, the key cell–cell binding regulator is the cadherin–catenin complex, whereas cell–matrix binding is largely mediated by integrins. Several evidences indicate that reduced expression of E-cadherin (Pontes et al., 2010;Liu et al., 2014) is related with advanced stages of PCa and similarly with aberrant expression of ß-catenin (Cheng et al., 1996;Jung et al., 2013). In turn, other

important factors like vascular endothelial growth factor (VEGF), transforming growth factor-ß (TGF-ß) and platelet-derived growth factor (PDGF) can be secreted by cancer cells and bind to its tyrosine kinase receptors on endothelial cells promoting angiogenesis (Russo et al., 2012;Roberts et al., 2013).

2.2. From androgen-responsive to castration-resistant prostate cancer (HRPC)

As previously mentioned, pubertal development of the prostate is strictly linked with the circulating levels of androgens. In the adult prostate, androgenic actions, mediated by its cognized receptor, the AR, are primordial for its differentiation and function. The AR is expressed in all luminal cells, as well as, in stromal cells (Prins et al., 1996;Mirosevich et al., 1999), and AR signaling regulates the proliferation and inhibits apoptosis of prostate cells (Cunha, 1973;Wilson, 2011). The testosterone circulating in the blood stream is metabolized in prostate to 5 α-dihydrotestosterone (DHT), by the enzyme 5α-reductase. DHT displays higher activity and binds AR with more affinity than testosterone. The binding of DHT to the AR induces its dissociation from heat-shock proteins, and receptor phosphorylation and dimerization. AR dimers bind to the androgen-response elements in the promoter regions of target genes, which upon recruitment of co-regulatory proteins (co-activators or co-repressors) form the AR-transcriptional complex regulating the gene transcription (Socorro, 2014).

The actions of AR in the initial phases of PCa are similar to those in non-pathological prostate, namely, the control of PSA synthesis, lipid metabolism , growth and apoptosis (Cleutjens et al., 1996;Xu et al., 2006;Arnoldussen et al., 2011;Tennakoon et al., 2013). Because PCa is highly dependent on androgens, androgen-deprivation therapy (ADT) has been used as treatment for metastatic disease. ADT can be achieved surgically by orchiectomy or chemically with the use of anti-androgens, luteinizing hormone-releasing hormone (LHRH) agonists or LHRH antagonists (Schroder et al., 2012). Although the majority of tumors initially respond to these treatments, their effectiveness is limited and a complex process of resistance is developed (Karantanos et al., 2013). In fact, advanced-stages of metastatic PCa acquire several mechanisms that allow survival and invasiveness of cancer cells even in absence of androgens. At this stage patients develop the so-called hormone-refractory prostate cancer (HRPC). The mechanisms supporting the acquisition of this phenotype include amplification of AR expression, point mutations in the AR gene altering the AR activity, changes in cell signaling pathways that modulate the AR function, changes in the expression of coregulator proteins, and changes in steroid metabolism within tumor cells.

Hypersensitive Pathway. One possible mechanism by which PCa cells acquire resistance to ADT relies on the increasing sensitivity to low levels of androgens by increasing the AR synthesis and/or increasing AR sensitivity (Fig. I.2a). Both mRNA and protein levels of AR are augmented in PCa, and AR overexpression has been implicated in HRPC. Upon ADT therapy,

approximately 30% of HRPC cases display amplifications of the AR gene, resulting in increased AR expression, whereas no amplification were found before the ADT (Visakorpi et al., 1995;Koivisto et al., 1997). Complementing this information, it demonstrated that 80% of prostate tumors with AR amplification also displayed higher levels of AR protein (Edwards et al., 2003) (Fig. I.2a). Moreover, cancer cells survive continuing to proliferate even at low androgens levels, which may represent a second mechanism for development of HRPC (Waltering et al., 2009). Chen et al. (2004) verified that androgen-independent cells require 80% lower concentrations of androgens to grow than androgen-dependent cells. In another study, also was verified that the concentration of DHT stimulating growth of androgen-independent cells is four orders of magnitude lower than that required by androgen-dependent LNCaP cells (Gregory et al., 2001). Moreover, the increased stability of AR protein and the enhanced nuclear localization of AR found in androgen-independent cells complete the hypersensitive pathway in the acquisition of HRPC (Gregory et al., 2001). Finally, cancer cells might upregulate the expression of 5α-reductase enzymes increasing the production of DHT and maintaining AR signaling with lower levels of circulating androgens (Makridakis et al., 1997;Thomas et al., 2005;Das et al., 2010;Godoy et al., 2011). Supporting this mechanism are the data showing that ADT suppress testosterone levels in 95 %, while the concentration of DHT is reduced only in 60 % (Labrie et al., 1986), and the fact that ethnic groups with higher levels of activity this enzyme have a higher risk of PCa development (Makridakis et al., 1997). Moreover, castration resistant metastasis express higher levels of many enzymes responsible for the synthesis of adrenal androgens indicating genes involved in steroid biosynthesis are overexpressed (Holzbeierlein et al., 2004;Montgomery et al., 2008).

Promiscuous pathway. The specificity of AR in PCa has been questioned due to mutations present in ligand-binding domain. The frequency of genetic mutations in the AR loci are more frequent in advanced stages of disease (Taplin et al., 1995;Tilley et al., 1996;Ceraline et al., 2004;Sun et al., 2006). These mutations allow the AR to be activated by several non-androgenic molecules (Fig. I.2b), which maintains cell proliferation dependent on AR but stimulated by other ligands. Estrogens or anti-androgens have been pointed as the molecules that can bind to the mutant AR (Veldscholte et al., 1992;Zhao et al., 2000;Thin et al., 2003) (Fig. I.2b). The T7877A substitution is a good example of how a single mutation can alter the AR specificity, since in LNCaP cells, it resulted in cell growth in response to androgens and also to non-androgenic steroids (Suzuki et al., 1996). Again, this mechanism can be a defense of cancer cells, and Marcelli et al. (2000) described AR mutations in patients with lymph node metastasis that undergone ADT, whereas no mutations were found in the prostate glands of patients that received hormonal therapy. Also, other proteins that act as co-ativators and co-repressors of AR-induced transcription can be modified in HRPC. AR co-activators recruit other transcription factors to initiate transactivation of AR-regulated genes. Some of these co-activators, like ARA70, Tip60, TIF2 or SCR1 were increased in HRPC samples (Halkidou et al., 2003;Peng et al., 2008;Culig and Santer, 2012). On the other hand, co-

repressors inhibit the transcription of AR-regulated genes, and the alteration of co-repressors like SMRT can be involved in progression of advanced stages of PCa (Godoy et al., 2012).

Outlaw Pathway. AR can be also activated by nonsteroid molecules, like growth factors and cytokines. The growth factors, insulin-like growth factor 1 (IGF-1), keratinocyte growth factor (KGF) and epidermal growth factor (EGF), and cytokines, such as, interleukin (IL)-6, are capable of activating AR via signal transduction pathways (Culig et al., 1994;Hobisch et al., 1998). IGF-1, EGF and IL-6 are reported to be upregulated in HRPC (Di Lorenzo et al., 2002;Krueckl et al., 2004;George et al., 2005). Moreover, tyrosine kinase receptors (RTKs) also are connected with the activity of AR (Fig. I.2c). HER-2/neu, a member of the EGF receptor family of RTKs is overexpressed in androgen-independent cell lines generated from xenografts implanted in castrated mice (Craft et al., 1999), and this overexpression can activate AR-target genes independently androgens but not in the absence of AR (Craft et al., 1999;Yeh et al., 1999;Nishio et al., 2006;Ricciardelli et al., 2008). Moreover, this pathway can activate AR through the MAPK pathway. In fact, MAPK can phosphorylate the AR and leads to its activation (Yeh et al., 1999) Other pathway involved in cell survival is the Akt pathways and Akt activity is increased in androgen-independent cell lines compared with androgen-dependent cells (Graff et al., 2000;Morgan et al., 2009). Akt signaling can also alter cell cycle regulation by decreasing protein expression of p27, a known cell cycle inhibitor (Graff et al., 2000) (Fig. I.2c).

Bypass Pathway. It is also possible that alternative pathways to AR are capable to bypass and render cells with the ability to survive independently of AR activation. B-cell lymphoma 2) (Bcl-2) can be considered the candidate gene that can block apoptosis and which is not normally expressed in prostate (Colombel et al., 1993). In fact, several studies verified that Bcl-2 is overexpressed in cases of HRPC (Furuya et al., 1996;Fuzio et al., 2011). The blockage of Bcl-2 actions induces apoptosis in LNCaP cells (Yamanaka et al., 2006). Inactivation of the PTEN and subsequent activation of Akt is other bypass mechanism that cancer cells acquire in HRPC (Wang et al., 2006).

Lurker pathway. Other evidences point that HRPC develops because some cells that growth independently androgens were present before ADT. In fact, a minority sub-population of cells in prostate tumors that not express AR, has been identified as prostate cancer stem or progenitor cells (Collins et al., 2005). The cells can continue to proliferate and with ADT can exist a cancer cell selection. These cells are capable of self-renewal and are drug-resistant (Collins et al., 2005;Leong et al., 2008).

It is astonishing the number of different mechanisms underlying HRPC and conducting to more aggressive and lethal forms of PCa, which has hindered the development of specific and effective therapies for this stage of disease.

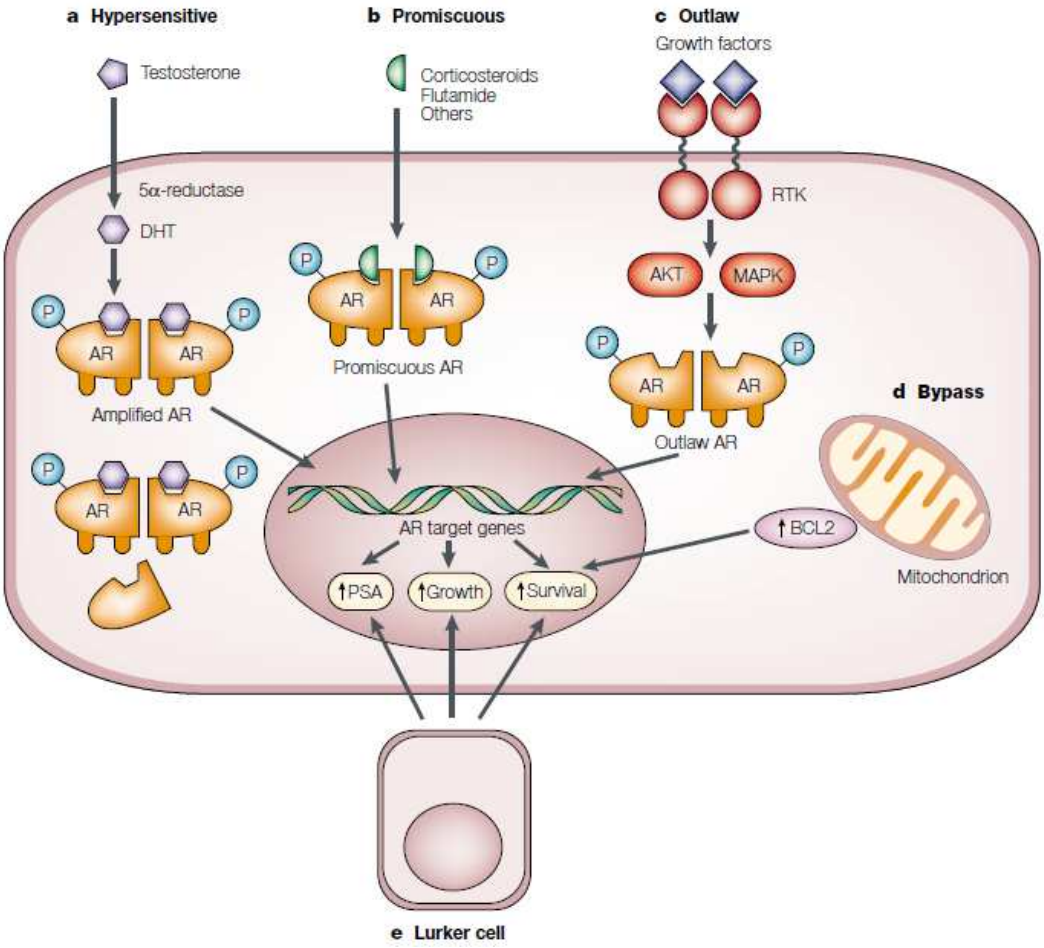

Figure I.2. Putative pathways in development of hormone refractory prostate cancer. a. In the hypersensitive pathway, androgen receptor (AR) gene can be amplificated (more common) and/or enhanced sensitivity to androgens can occur. Also, augmented production of 5 α-dihydrotestosterone (DHT) due to increased activity of 5α-reductase has been indicated. **b.** In the promiscuous pathway, other molecules (like estrogens, corticosteroids, anti-androgens) can activate the AR pathway. **c.** In the outlaw pathway, others receptors, like receptor tyrosine kinases (RTKs) can be overactivated and, through Akt and/or the mitogen-activated protein kinase (MAPK) pathway, can phosphorylate AR (P) and activate it. **d.** In the bypass pathway other molecules involved in cell survival can control AR target genes, such as the Bcl-2 protein. **e.** In the lurker pathway, a minority sub-population of cells in prostate tumors that not express AR might be selected by the androgen deprivation therapy (ADT) (Feldman and Feldman, 2001).

3. The c-KIT receptor and its ligand stem cell factor (SCF) in prostate pathophysiology

The c-KIT is a tyrosine kinase receptor belonging to type III RTKs family, which also includes the PDGFR and the macrophage colony stimulating factor receptor (Liu et al., 2007). It is also kwon like CD117, stem cell factor (SCF) receptor or KIT receptor. Firstly is described in 1986 as the transforming gene of the Hardy-Zuckerman 4 feline sarcoma virus, and 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 (d'Auriol et al., 1988). The main product of this gene is a single 5 kb transcript (Yarden et al., 1987), which encodes a transmembrane glycoprotein with approximately 145-160 kDa. Structurally, three main functional regions are

considered (Fig. I.3): an extracellular ligand-binding domain, a transmembrane region, and an intracellular kinase domain (Qiu et al., 1988;Blechman et al., 1993;Blechman et al., 1995). The extracellular region, which comprises five immunoglobulin-like domains, recognizes the c-KIT ligand and also participates in receptor dimerization (Blechman et al., 1995;Yuzawa et al., 2007;Paulhe et al., 2009). In addition, the presence of the tetrapeptide Gly-Asn-Asn-Lys (GNNK) in the extracellular juxtamembrane domain of c-KIT (Fig. I.3) plays a relevant role regulating receptor activation and downstream signaling (Phung et al., 2013). A short chain of hydrophobic amino acids constitutes the transmembrane region, which allows fixation of c-KIT at the plasmatic membrane. The cytoplasmic region of c-KIT, containing proximal and distal kinase domains separated by an interkinase domain (Fig. I.3), is responsible for transduction of SCF/c-KIT signaling (Mol et al., 2003;Roskoski, 2005). Several c-KIT variants originated by distinct mechanisms have been identified in several cell types. Alternative promoter usage and transcription of a cryptic exon produces a truncated c-KIT protein (tr-KIT), with 30-50 kDa, which contains only the distal kinase domain and the carboxyl-terminal tail (Fig. I.3), and is located at cytoplasm (Rossi et al., 1992;Toyota et al., 1994). Moreover, the c-KIT protein can be proteolytically cleaved originating a soluble isoform (s-KIT, Fig. I.3) (Turner et al., 1995;Broudy et al., 1999).

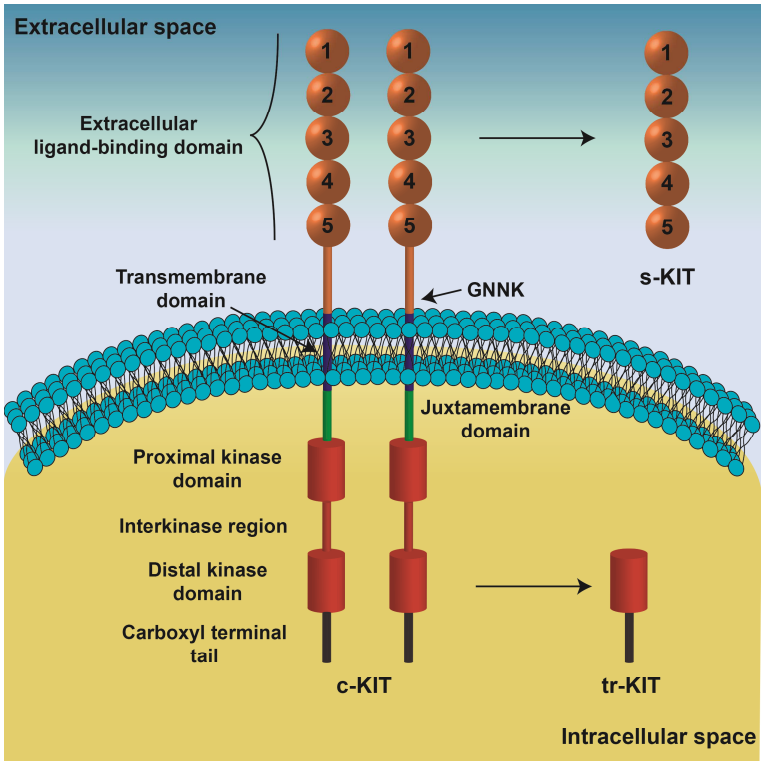

Figure I.3. Schematic representation of c-KIT structure. The five immunoglobin-like domains of the extracellular domain are involved in ligand-binding and receptor dimerization. The transmembrane domain anchors c-KIT in the cytoplasmic membrane. The intracellular region, responsible for signaling transduction, 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 Gly-Asn-Asn-Lys (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 alternative promoter usage and lacks the extracellular and transmembrane domains retaining part of the kinase domain (Cardoso et al., 2014).

SCF is a growth factor/cytokine firstly identified in 1990 (Nocka et al., 1990;Williams et al., 1990;Zsebo et al., 1990). It is also known as c-KIT ligand or mast cell growth factor and it is codified on the *Steel locus* (Sl) of Chromosomes 12 and 10, respectively, in humans and mice (Zsebo et al., 1990;Geissler et al., 1991). The SCF protein, a 45 kDa glycoprotein is constituted by three distinct regions (Fig. I.4), the extracellular domain responsible for recognizing and binding of c-KIT (Langley et al., 1994), the hydrophobic transmembrane domain, and the intracellular domain (Langley et al., 1994;Zhang et al., 2000). The SCF is present at cell membrane as a noncovalent homodimer (mSCF) (Lu et al., 1991;Matous et al., 1996;Zhang et al., 2000), and the proteolytic cleavage of an alternatively spliced variant originates its soluble isoform (sSCF, Fig. I.4) (Flanagan et al., 1991;Pandiella et al., 1992;Majumdar et al., 1994) (Fig. 4). mSCF binds simultaneously two molecules of c-KIT in the membrane of receptor cell, which induces a conformational change that exposes a key dimerization site located in the fourth immunoglobulin-like domain of c-KIT (Fig. I.3) (Blechman et al., 1995;Lemmon et al., 1997;Paulhe et al., 2009). Receptor dimerization allows its autophosphorylation (Paulhe et al., 2009), which creates docking sites for several signal transduction molecules triggering the initiation of multiple signal transduction pathways (Mol et al., 2003;Roskoski, 2005).

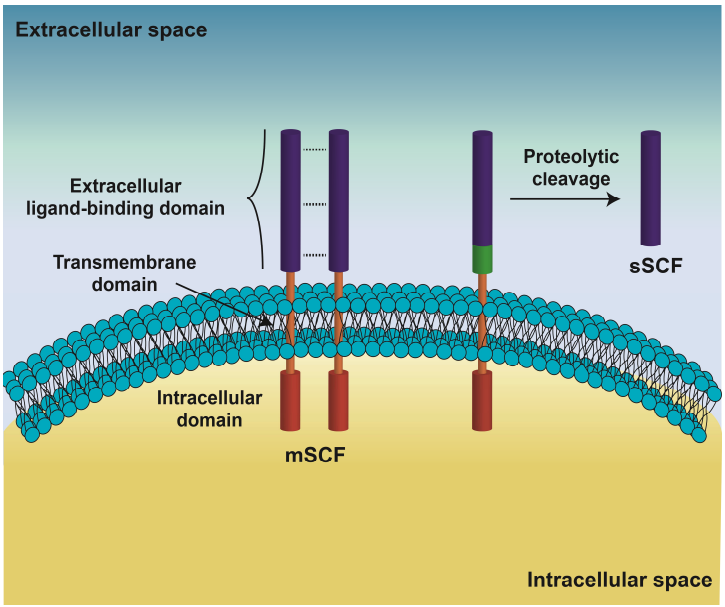

Figure I.4. Schematic representation of stem cell factor (SCF) structure. The SCF display an extracellular domain, responsible for recognizing and binding to c-KIT, a transmembrane domain and an intracellular domain. The SCF exists as a membrane-bound homodimer (mSCF) or as a soluble protein (sSCF). 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).

The expression of this system in prostate is poorly known, particularly in the case of rodents. 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 human prostate c-KIT protein is detected in mast cells and in the stromal compartment but not in epithelial cells, and c-KIT

positive cells were identified as interstitial cells (Simak et al., 2000;Imura et al., 2012). SCF expression in human prostate is found in epithelial and stromal cells (Simak et al., 2000;Imura et al., 2012). However, c-KIT and SCF transcripts were detected at low levels in basal epithelial cells (Simak et al., 2000).

Considering the pathological conditions of prostate, it was found a significantly higher expression of SCF and c-KIT in cases of BPH comparatively to normal prostate (Imura et al., 2012). Moreover, the expression of c-KIT mRNA is higher during androgen deprivation compared to noncastrated clinical metastatic disease (Pfeiffer et al., 2011). The expression of c-KIT it is also higher in bone metastasis cancers (40%) relatively to primary PCa (14%) (Wiesner et al., 2008), suggesting an important function of c-KIT in metastatic forms of disease. Nevertheless, prostate tumors seem to specifically express the cytoplasmic tr-KIT. tr-KIT is detected in tumoral tissues while the contralateral prostate not invaded by tumor cells, as well as, cases of BPH are negative, and its expression increases pronouncedly (~28% to ~66%) from less to more advanced stages of PCa (Paronetto et al., 2004). Studies in cases of human prostate adenocarcinoma strongly identified c-KIT in the cytoplasm and membrane of epithelial tumor cells (Simak et al., 2000), and in stromal cells (Di Lorenzo et al., 2004), while SCF showed to be frequently expressed in cytoplasm of tumor cells (Imura et al., 2012).

In human PCa cell lines the available literature has been controversial. If some studies revealed that c-KIT mRNA is not expressed in LNCaP, PC3 or DU145 cells, other reports showed its expression (Savarese et al., 1998;Wiesner et al., 2008). At protein level, some studies have reported c-KIT as being expressed in LNCaP and PC3 cells (Savarese et al., 1998), and in opposition others authors described that c-KIT is only present in DU145 cells (Wiesner et al., 2008;Brooks et al., 2012). Considering the tr-KIT, it was detected in LNCaP cells, but not in PC3 (Paronetto et al., 2004). It is liable to assume that, discrepancies verified on the expression of c-KIT and tr-KIT can be due to the use of different antibodies. Antibodies that recognized extracellular domain detected only the full-length c-KIT located at cell membrane, whereas antibodies that recognized the c-terminus of protein are capable of recognize both c-KIT and tr-KIT.

Relatively to SCF, transcripts were detected in all cancer cell lines already studied (Savarese et al., 1998;Simak et al., 2000), with DU145 cells displaying the highest expression comparatively with LNCaP and PC3 (Wiesner et al., 2008). The sSCF was detected at low levels in DU145 and PC3 cells (Savarese et al., 1998), which contradicts the findings of Wiesner et al. (2008) that reported only the expression of mSCF (Wiesner et al., 2008).

In vivo studies also demonstrated that in prostate adenocarcinoma, c-KIT was strongly identified in cytoplasm and membrane of epithelial tumor cells (Simak et al., 2000) and stromal cells (Di Lorenzo et al., 2004). On the other hand, SCF showed to be frequently expressed in cytoplasm of tumor cells (Imura et al., 2012).

Table I.1. Expression of SCF/c-KIT system in prostate cells (Cardoso et al., 2014).

Cell type	c-KIT	SCF	References
Fibromuscular stroma cells	+	+	Lammie et al. (1994), Ceder et al. (2008), Imura et al. (2012)
Basal epithelial cells	+	+	Simak et al. (2000), Imura et al. (2012)
Mast cells	+	-	Natali et al. (1992), Lammie et al. (1994), Simak et al. (2000)
Interstitial cells	+	-	Imura et al. (2012)
Stem cells	+	-	Leong et al. (2008)

+, positive expression; -, negative expression

4. Prostate cancer treatment and the use of tyrosine kinase inhibitor Imatinib

While localized PCa is potentially cured by surgery and radiation therapy. However, frequently this cancer advances to more aggressive stages, for which the most common treatment in locally and metastatic disease is ADT, as cited above. This therapy is effective at initial phases but in long-term, upon some months, resistance to therapy occurs and consequently the stage of HRPC emerges. Historically, treatment for HRPC was mainly palliative, and overall survival rates are in best of the cases approximately 35 months (Suzman and Antonarakis, 2014). In 2004, docetaxel became the first chemotherapy agent to extend survival in men with HRPC (Petrylak et al., 2004;Tannock et al., 2004). In last years, numerous studies evaluated the combination with docetaxel with new formulations but clinical benefit has not been demonstrated. However, in 2010, cabazitaxel, a taxane, was demonstrated to improve survival in patients (Galsky et al., 2010;Abidi, 2013). At present, cabazitaxel remains the only chemotherapy that shown survival benefits as a second-line treatment after docetaxel. But third-line options have been described (Fig. I.5). In fact, taxanes (like doxetaxel and carbizitaxel), that function by stabilizing the dynamic polymerization of microtubules in dividing cancer cells, are the principal drugs having success in treatment of HRPC. Nevertheless, other therapies have been tested in PCa (Fitzpatrick and de Wit, 2014). Is the case of immunotherapeutic approaches aiming to modulate the prostate cancer specific antigen and prolong the cytotoxic lymphocyte effects in tumor regression (Schweizer and Drake, 2014). Several approaches also have aimed to inhibit angiogenesis, more specifically the VEGF pathways involved in this process. However, trials with VEGF inhibitors do not improved survival (Kelly et al., 2012). Also, cell-signaling inhibitors have been tested like tyrosine kinase inhibitors. For example Desatinib, an inhibitor of Src kinases

demonstrate that in combination with abiraterone and in II trials have interesting results (Araujo et al., 2012).

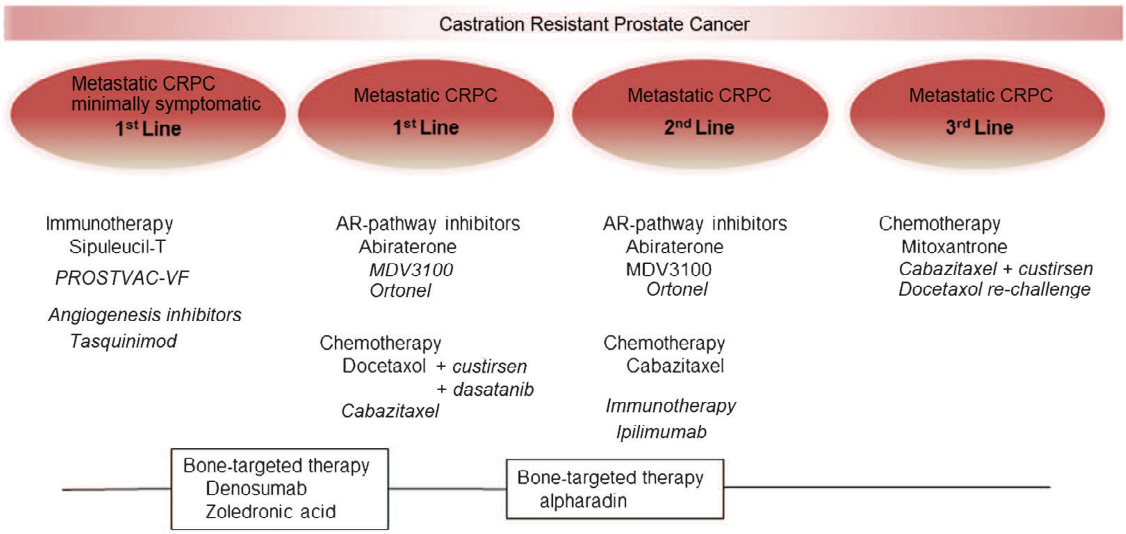

Figure I.5. Evolution of treatment of hormone refractory prostate cancer (HRPC). Therapies currently approved are shown in regular font; those in ongoing phase III trials are shown in italic font. AR, androgen receptor; CRPC, castrate-resistant prostate cancer or HRPC (Toren and Gleave, 2013).

Imatinib mesylate is a selective inhibitor of the tyrosine kinases like Abelson tyrosine kinase (ABL), c-KIT and platelet-derived growth factor receptor (PDGFR). Imatinib was formulated in 1990 and approved by Food and Drug Administration (FDA) in 2001 (FDA, 2001). Since then, imatinib has been shown to be highly effective in treatment of chronic myeloid leukemia and gastrointestinal stromal tumors (GIST) (Druker et al., 1996;Tuveson et al., 2001). The active sites of these RTKs (c-KIT and PDGFR) are dependent of adenosine triphosphate (ATP) that, catalyzes the transfer of the phosphate to tyrosine kinase residues on its substrates. Imatinib competes with ATP for receptor binding sites and stops the phosphorylation and consequently the signaling pathways. Pharmacokinetics studies verified that the imatinib is well absorbed and is highly protein bound (95%) (Peng et al., 2005). Moreover, it is metabolized by P450 (CYP) isoenzymes present in gut and liver and the half-life time for elimination of imatinib is approximately 18 hours, while the elimination half-life of the active metabolite is 40 hours (range, 30-50 hours) (Peng et al., 2005).

The success of imatinib in treatment of chronic myeloid leukemia and GIST encouraged clinicians and the scientific community to explore its effects on other human cancers. Imatinib also has been tested for treatment of HRPC. Nevertheless, the administration of imatinib has shown modest therapeutic effectiveness into the clinical setting (Tiffany et al., 2004;Corcoran and Costello, 2005;Mathew et al., 2007;Lipton et al., 2010). These outcomes are discordant with experimental findings in cell and animal models, where imatinib had cytotoxic effects and improved chemo- and radiosensitivity of PCa cells (Kubler et al., 2005;Kimura et al., 2007;Choudhury et al., 2009).

The biochemical actions of imatinib include the inhibition of both PDGFR and c-KIT (Radford, 2002). Although PDGFR has been considered the potential target for imatinib in prostate (Uehara et al., 2003;Kim et al., 2004), it was shown that only 16 % of metastatic PCa

express the PDGFRβ (Hofer et al., 2004), which is the subunit associated with cell proliferation, migration and angiogenic effects (Claesson-Welsh, 1994). This finding strongly reinforces the importance of c-KIT in PCa and fosters the interest of scientific community to deeply understand the actions of c-KIT and imatinib in HRPC.

5. The regucalcin protein and its relationship with prostate cancer

Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein, that does not contains the typical EF-hand Ca^{2+} -binding motif (Yamaguchi and Yamamoto, 1978;Shimokawa and Yamaguchi, 1993). It was also named senescence marker protein-30 (SMP30) due to its characteristic down-regulated expression with aging in rat kidney and liver (Fujita et al., 1995;Fujita et al., 1996;Kim et al., 2013). The RGN gene is localized on human chromosome Xq11.3-q11.2 and rat chromosome Xq11.1-11.2 (Fujita et al., 1995;Shimokawa et al., 1995). In both cases, the gene consists of seven exons and six introns, encoding a protein with approximately 33 kDa (Fujita et al., 1992;Maia et al., 2008). Alternative splicing of the RGN gene was described, and 3 transcripts were identified: the full-length mRNA with 897 bp, 681 and 549 bp transcripts corresponding to spliced variants without exon 4 (Δ4) and exons 4 and 5 (Δ4,5) respectively (Maia et al., 2009;Laurentino et al., 2012).

RGN was initially described as being highly expressed in the liver (Shimokawa and Yamaguchi, 1993) and kidney (Yamaguchi and Kurota, 1995), but its expression has been reported in several other tissues, namely, brain (Yamaguchi et al., 2000), heart (Yamaguchi and Nakajima, 2002), bone (Yamaguchi et al., 2004) and lung (Mori et al., 2004). RGN is also expressed in a broad range of male and female reproductive tissues, including ovary (Kagami et al., 2013), breast (Maia et al., 2008), testis (Laurentino et al., 2011), seminal vesicles (Laurentino et al., 2011), epididymis (Laurentino et al., 2011;Correia et al., 2013) and prostate (Maia et al., 2008;Maia et al., 2009). RGN protein is localized in cytoplasm, mitochondrial fraction, microsomal membranes, and also in cell nucleus (Mori and Yamaguchi, 1991;Kurota and Yamaguchi, 1997;Takahashi and Yamaguchi, 2000;Tsurusaki et al., 2000;Ishigami et al., 2003;Maia et al., 2008). The gene expression of RGN is regulated by various transcription factors that include the AP-1, NF1-A1, RGPR-p117 and ß-catenin (Yamaguchi, 2011). Other hormonal and non-hormonal factors also regulate RGN tissue expression. Ca^{2+} modulates RGN expression (Nakajima et al., 1999) by a calmodulin or protein kinase C-dependent mechanism (Hamano and Yamaguchi, 1999;Nakajima et al., 1999). Caloric restriction and oxidative stress also influence tissue levels of RGN (Marques et al., 2014a). At hormonal level RGN expression is modulated by thyroid and parathyroid hormones

(Yamaguchi et al., 2008), aldosterone, insulin, calcitonin, and sex steroids (Maia et al., 2008;Maia et al., 2009;Laurentino et al., 2011;Marques et al., 2014a;Vaz et al., 2014).

The biological functions of RGN (Fig. I.6) include a role in Ca^{2+} homeostasis by regulating the activity of Ca^{2+}-pumps at plasma membrane, endoplasmic reticulum and mitochondria (Fujita et al., 1998;Yamaguchi and Nakajima, 2002;Yamaguchi and Daimon, 2005). The RGN's role also has been linked to the control of intracellular signaling pathways and distinct experimental approaches demonstrated that RGN suppresses cell proliferation and apoptosis (Correia et al., 2014a;Marques et al., 2014a;Vaz et al., 2014). Also, the activity of several protein kinases and phosphatases were down-regulated with the overexpression of RGN (Katsumata and Yamaguchi, 1998;Inagaki and Yamaguchi, 2001;Fukaya and Yamaguchi, 2004).

Another feature of RGN protein is its antioxidant activity by reducing the production of ROS and increasing the mechanisms of antioxidant defense (Handa et al., 2009;Kondo et al., 2014). Knockout animals for the RGN gene display higher levels of oxidative stress in the brain than their wild-type counterparts (Son et al., 2006). Accordingly, overexpression of RGN in different cell types and tissues is linked with increased antioxidant potential (Correia et al., 2013;Marques et al., 2014a).

At the nuclear compartment RGN is involved in regulation of protein transport, decreasing the expression of small GTPase Ran (ras-related nuclear protein), which is essential for protein export from the nucleus and protein import into the nucleus (Tsurusaki and Yamaguchi, 2001). When RGN is translocated to the nucleus mediates several nuclear functions decreasing DNA fragmentation, inhibiting endonuclease activity (Yamaguchi and Sakurai, 1991), and reducing DNA and RNA synthesis (Fig. I.6) (Yamaguchi and Kanayama, 1996;Yamaguchi and Ueoka, 1997).

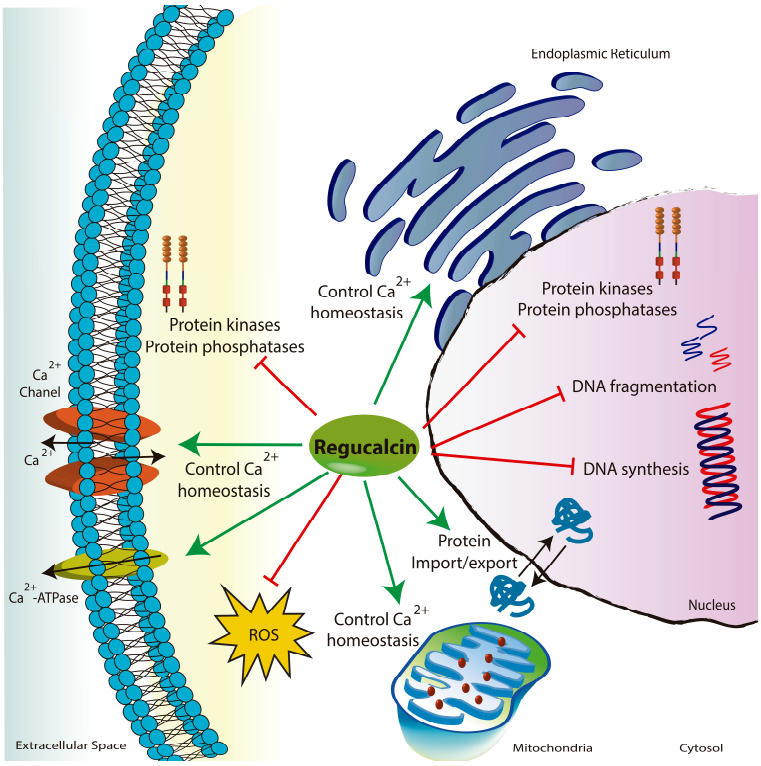

Figure I.6. The role of regucalcin (RGN) in cell biology. RGN regulates the activity of several Ca^{2+}-dependent enzymes and the concentration of intracellular Ca^{2+} by modulating the activity of Ca^{2+}-channels, and Ca^{2+}-ATPase in the plasma membrane, mitochondria and endoplasmic reticulum. It has suppressive effects on the activity of protein kinases and phosphatases and, once translocated to the nucleus also inhibits DNA fragmentation, DNA and RNA synthesis. Moreover, RGN regulates the nuclear protein export and import through the GTPase Ran (ras-related nuclear protein). Another feature of RGN protein is its antioxidant activity by reducing the production of reactive oxygen species (ROS).

Several studies in the last years have characterized the RGN expression in diverse pathological cases, namely, in cases of defective spermatogenesis and acute liver failure, as well as in neurological diseases and cancer (Marques et al., 2014a).

Loss of RGN expression has been described as an important feature in hepatomas, and breast and prostate cancer and all of type cancers it expression is under-expressed (Makino and Yamaguchi, 1996;Maia et al., 2009). Studies of our research group firstly identified RGN in rat prostate in 2008, and characterized its location in the cytosol and nucleus of prostate epithelial cells (Maia et al., 2008). In the case of PCa anterior studies showed that RGN expression is significantly diminished in prostate adenocarcinoma comparatively with non-neoplastic prostate or BPH cases (60 %). In addition, it was also demonstrated that diminution of RGN accompanies the progression of disease (Maia et al., 2009), indicating that loss of RGN may favors tumor development. This is strongly supported by recent findings in my investigation group showing that RGN overexpression protects against carcinogen-induced mammary gland tumor development (Marques et al., 2014a). Very recently, Vaz et al. (2014) also showed that rat prostate overexpressing the RGN protein display inhibited cell proliferation (concomitant with reduced prostate weight) and inhibited apoptotic pathways, which demonstrated its role maintaining the prostate tissue balance. These RGN actions underpinned altered expression of cell-cycle regulators and oncogenes, such as, H-ras and

p21. Also, in hepatoma cells overexpressing RGN suppressed expression of c-myc, H-ras and c-src mRNAs and enhanced expression of p53 and Rb mRNAs was found (Tsurusaki and Yamaguchi, 2003). Interestingly, another study has shown that RGN suppresses protein kinase activity (Inagaki and Yamaguchi, 2001), but it is unknown if this may be related with the antiproliferative role of RGN and protection against carcinogenesis.

II. Aim of the Thesis

The RTK c-KIT and its ligand SCF regulate several biological processes having a determinant role in the control of survival, and apoptotic and proliferation pathways. It has been demonstrated that c-KIT is overexpressed in cases of PCa. Moreover, the expression of c-KIT in clinical samples of HRPC are upregulated indicating the necessity to study of this receptor in this phase of disease. However, the expression of c-KIT and tr-KIT in cell line models of PCa has remained a matter of controversy.

Imatinib mesylate is a tyrosine inhibitor that inhibits the activity of c-KIT and PDGFR, but its clinical efficacy for treatment of HRPC has been questioned, with preclinical models and clinical experimentation producing not always concordant results.

On the other hand, recent findings identified RGN as an under-expressed protein in human PCa, which has a suppressive effect on cell proliferation and regulates several intracellular signaling pathways including the activity of protein kinases. However, nothing is known about the expression of RGN in cell line models of HRPC and the relationship between RGN and c-KIT expression also is unexplored. Moreover, the cellular mechanisms by which RGN may regulate cell division remain largely unknown.

The present dissertation aims to shed light on these questions by:

- Analyzing the cytotoxic effects of imatinib in two cell line models of HRPC; cell viability, and the expression and activity of apoptosis regulators will be determined; the expression of c-KIT isoforms in cells lines also will be studied;

- Studying the effect of RGN on the expression of SCF/c-KIT in rat prostate making use of a transgenic animal model overexpressing RGN;

- Evaluating the subcellular localization of RGN in HRPC cell lines and investigating its association with α-tubulin.

III. Material and Methods

1. Cell lines

Human PCa cell lines (DU145 and PC3) were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Both DU145 and PC3 cell lines are nonresponsive to androgens and models of metastatic (advanced) stages of PCa. The PC3 cell line had origin from bone metastasis of an undifferentiated grade IV adenocarcinoma of the prostate (Kaighn et al., 1979). DU145 cells were derived from brain metastasis and have moderate metastatic potential comparatively with the PC3 cell line (Stone et al., 1978).

2. Animals

Three-month-old male Sprague Dawley rats (*Rattus norvegicus*) wild-type and transgenic overexpressing the RGN protein (Tg-RGN) were obtained, from Charles River (Barcelona, Spain) and Japan SLC (Hamamatsu, Japan) respectively. Tg-RGN animals were originally generated by Yamaguchi (Yamaguchi et al., 2002) by oocyte transgene pronuclear injection and have been used as a good model to explore the roles of RGN *in vivo* (Correia et al., 2014a;Marques et al., 2014b;Vaz et al., 2014). Animals were handled in compliance with the NIH guidelines (NIH Publication No. 85–23, revised 1996) and the European Union rules (Directivenumber2010/63/EU) for the care and handling of laboratory animals during the course of all experiments. They were housed under a 12h light: 12h darkness cycle, with food and water available *ad libitum* during the experiment. All rats were euthanized under anesthesia (Clorketam 1000, Vetoquinol, Lure, France) and then whole prostates were removed. Right or left dorsolateral lobe of the prostate, including ventral, dorsal, and lateral prostate (Shappell et al., 2004) were either frozen in liquid nitrogen for RNA or protein extraction.

3. Cell Culture and Imatinib Treatment

PC3 and DU145 cells were maintained in RPMI 1640 medium (Gibco, Life Technologies, Paisley, Scotland) supplemented with 10 % fetal bovine serum (FBS, Biochrom AG, Berlin, Germany) and 1 % penicillin/ streptomycin (Gibco, Life Technologies) in an air incubator equilibrated with 5 % CO_2 at 37 ^{o}C. In all experiments, cells grow up to 60 % confluence and after additional 24 h were treated with Imatinib mesylate (CAS 220127-57-1, Santa Cruz Biotechnology. Cells were exposed to a cytotoxic concentration of imatinib 20 μM (Kubler et al., 2005) for 6 to 72 h. After treatment, cells were trypsinized and harvested for posterior analysis.

4. Cell Viability assay

Cells (1500/well) were grown in 96-well plates and cell viability was determined by the colorimetric CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) at 6, 12, 24, 48 and 72 h after treatment with imatinib. The conversion of MTS (3-

(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2M)tetrazolium compound to the colored formazan product was detected at 490 nm in a microplate reader (Biochrom, Anthos 2020). The relative number of viable cells in each experimental condition was calculated in triplicate by normalizing the absorbance to that of the corresponding control. Results were represented as percentage of the control.

5. Real-time quantitative Polymerase Chain Reaction (qPCR)

Total RNA was isolated from DU145 and PC3 human PCa cells and from rat prostate (wild type and Tg-RGN) using the TRI reagent (5 Prime, Hilden, Deutschland) according to the manufacturer's instructions. The quantity and quality of total RNA was assessed by spectrophotometry at 260 and 280 nm (Pharmacia Biotech, Ultrospec 3000), and agarose gel electrophoresis (1%), respectively. 1 µg of total RNA was reverse-transcribed using the NZY First Strand cDNA Synthesis Kit (NZYtech, Lisbon, Portugal) in a final volume of 20 µL through sequential incubations: 25 ºC for 10 minutes and 50 ºC for 30 minutes. The reaction was inactivated by heating at 85 ºC for 5 minutes and then chilled 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 (Maia et al., 2009;Vaz et al., 2012). The quality of the synthesized cDNA was also accessed by the PCR amplification of 18S. 1 µL of synthesized DNA was used to determine the expression of p21 and vascular endothelial growth factor (VEGF) in DU145 and PC3 cells in response to imatinib treatment. Also, 1 µl of synthesized DNA from rat prostate total RNA was used to analyze the expression of SCF and c-KIT in wild type and Tg-RGN rats. Information about the specific primers and cycling conditions used for the amplification of each target gene and housekeeping genes is resumed in Table III.2. β-2-microglobulin, β-actin, GAPDH or 18S genes were used as internal controls to normalize gene expression. qPCRs were carried out in an iQ5 system (Bio-Rad, Hercules, CA, USA) and efficiency of the amplification was determined for all primer sets using serial dilutions of cDNA (1; 1:5; 1:25). Primer concentration and annealing temperature for each primer set were optimized and the specificity of the amplicons was determined by melting curve analysis. qPCR reactions were carried out in a 20 µL reaction containing 10 µL Maxima^{TM} SYBR Green/Fluorescein qPCR Master Mix (Bio-Rad) and 300 nm (p21, VEGF, c-KIT and SCF) or 200 nM (housekeeping genes) of sense and antisense primers for each gene. Samples were run in triplicate in each qPCR assay. Normalized expression values were calculated following the mathematical model proposed by Pfaffl using the formula: ($2^{(-\Delta\Delta Ct)}$) (Pfaffl, 2001).

Table III.2. Information about Primers used in qPCR.

Gene	Primer Sequence (5' - 3')	Amplicon Size (bp)	Annealing temperature (°C)	Cycles
Human VEGF	***Fwd:*** CGA AAC CAT GAA CTT TCT GC ***Rev:*** CCT CAG TGG GCA CAC ACT CC	301	60	35
Human p21	***Fwd:*** TCC AGC GAC CTT CCT CAT C ***Rev:*** AGC CTC TAC TGC CAC CAT C	102	58	35
Human GAPDH	***Fwd:*** CGC CAG CCG AGC CAC ATC ***Rev:*** CGC CCA ATA CGA CCA AAT CCG	75	60	35
Human ß-2-microglobulin	***Fwd:*** ATG AGT ATG CCT GCC GTG TG ***Rev:*** CAA ACC TCC ATG ATG CTG CTT AC	93	60	35
18S	***Fwd:*** AAG ACG AAC CAG AGC GAA AG ***Rev:*** GGC GGG TCA TGG GAA TAA	152	60	30
Rat c-KIT	***Fwd:*** CCG TCT CCA CCA TCC ATC C ***Rev:*** TTC GCT CTG CTT ATT CTC AAT CC	143	60	35
Rat SCF	**Fwd:** ATG GCT TGG GAA ATG TCT G ***Rev:*** GCT GAT GCT ACG GAG TTA C	193	58	35
Rat GAPDH	**Fwd:** GTT CAA CGG CAC AGT CAA G **Rev:** CTC AGC ACC AGC ATC ACC	177	60	35
Rat- ß-Actin	**Fwd:** ATG GTG GGT ATG GGT CAG **Rev:** CAA TGC CGT GTT CAA TGG	79	60	35

6. Protein Extraction

Total proteins were isolated from DU145 and PC3 human PCa cells and from rat prostates (wild type and Tg-RGN) using RIPA buffer (150 mM NaCl, 1 % Nonidet-P40 substitute, 0.5 % Na-deoxycholate, 0.1 % SDS, 50 mM Tris pH 8.0, and 1 mM EDTA) supplemented with protease inhibitors cocktail (Sigma-Aldrich, St.Louis, MO, USA) and 10 % PMSF (Sigma-Aldrich). Samples were kept on ice (20 minutes), occasionally mixed, and then centrifuged at 14,000 rpm for 20

minutes at 4ºC. Total proteins (supernatant) were collected and protein concentration was determined by the Bradford assay (Bio-Rad).

7. Western Blot (WB)

50 µg of protein extracts from DU145 and PC3 cells and rat prostates were resolved on a 12,5 % gel by SDS–PAGE. Proteins were electrotransferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK) 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 4ºC with rabbit anti-SCF (1:500, H-189: sc-9132; Santa Cruz Biotechnology), rabbit anti-c-kit (1:1000, C-19: sc-168; Santa Cruz Biotechnology), rabbit anti-caspase-9 (1:500, H-170: sc-8355; Santa Cruz Biotechnology), mouse anti-caspase-8 (1:200, D-8: sc-5263; Santa Cruz Biotechnology), rabbit anti-Bax (1:500, no. 2772, Cell Signaling Technology) or rabbit anti-Bcl-2 (1:1000, no. 2876; Cell Signaling Technology) as previously described (Correia et al., 2014a;Correia et al., 2014b). 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 (1:5,000, NIF1317, GE Healthcare) or goat anti-mouse IgG+IgM-AP (1:5000, NIF1316, GE Healthcare) were used as secondary antibodies. Membranes were incubated with ECF substrate (GE Healthcare) for 5 minutes and scanned with Molecular Imager FX Pro plus Multi Imager (Bio-Rad). Band densities were obtained according to standard methods using the Quantity One Software (Bio-Rad) and normalized by division with the respective α-tubulin or β-actin band density.

8. Caspase-3 activity assay

The enzymatic activity of caspase-3 was determined spectrophotometrically at 405 nm by detecting the presence of the yellow product p-nitro-aniline (pNA). In brief, 50 µg of total protein cell extracts were incubated overnight at 37 ºC with reaction buffer (25 mM HEPES, 0.1 % 3CHAPS, 10 % sucrose, and 10 mM DTT, pH 7.5) and 200 µM of caspase-3 substrate (Ac-DEVD-pNA). The amount of generated pNA was calculated by extrapolation with a standard curve.

9. Fluorescent Immunocitochemistry

DU145 and PC3 cells were fixed on glass culture slides with 4 % paraformaldehyde for 10 min, and permeabilized with 0.1 %Triton X-100 for 5 min. Then, a blocking step was performed by incubating cells with 20 % FBS in phosphate buffer saline (PBS) containing 0,1 % tween®-20 (PBST) for 1 h at room temperature. After washing cells were incubated with anti-c-KIT antibody (1:50, C-19: sc-168; Santa Cruz Biotechnology), anti-RGN mouse monoclonal antibody (1:50, HM3018, Cell sciences) or anti-α-tubulin mouse monoclonal antibody (1:500, T9026, Sigma-Aldrich) for 1 h at room temperature. The Alexa fluor 488-conjugated goat anti-rabbit IgG, Alexa Fluor 546-conjugated goat anti-mouse IgG or Alexa Fluor 488-conjugated goat anti-mouse IgG (1:100, Invitrogen, Life Technologies, Karlsruhe LMA, Germany) were

used as secondary antibodies for detection of c-KIT, RGN and α-tubulin respectively. The specificity of the staining was accessed by omission of the primary antibody. Cell nuclei were stained with Hoechst 33342 (10 mg/mL, Invitrogen) for 10 minutes. Lamellae were washed and mounted onto microscope slides with Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany).

10. Statistical Analysis

Statistical significance of differences between experimental groups was evaluated by unpaired t-test with Welch's correction using GraphPad Prism v5.00 (GraphPad Software). *p*-values <0.05 were considered to be statistically significant. All experimental data are shown as mean ± SEM.

IV. Results

1. Viability of DU145 and PC3 cells in response to Imatinib

The effect of imatinib on the viability of DU145 and PC3 cells was determined by the MTS assay. 48 h and 72 h after administration of imatinib the number of DU145 viable cells was significantly decreased to 89,93 ± 2.32 and 83,24% ± 3.71, respectively (Fig. IV.7). Although the viability of PC3 cells markedly decreased upon 6 h of treatment with imatinib (33.26% ± 2.39 reduction *vs*. control, $p<0.001$), thereafter this effect was inverted (Fig. IV.7). At 24, 48 and 72 h the viability of PC3 cells treated with imatinib significantly increased relatively to control (5.83% ± 2.04, 15.25% ± 1.22 and 15.45% ± 1.47, respectively).

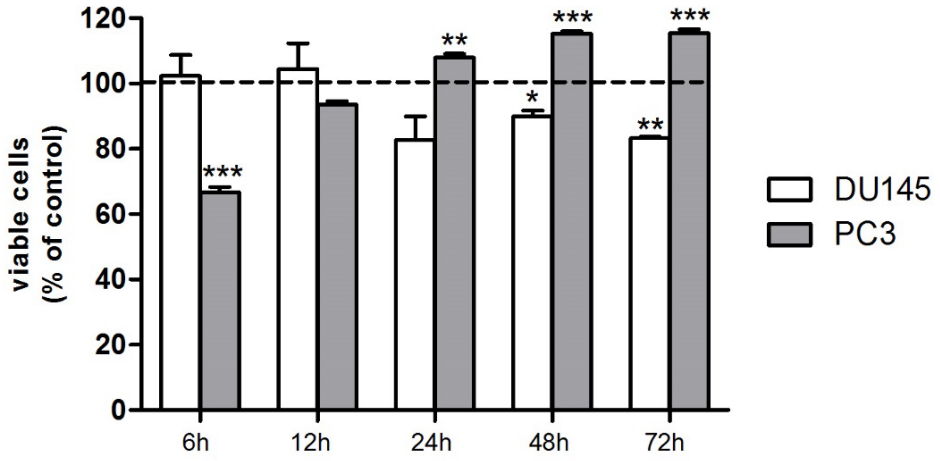

Figure IV.7. Number of DU145 and PC3 viable cells after treatment with imatinib for 6, 12, 24, 48 and 72 h. Results are expressed as fold-variation relatively to control group (dashed line). Error bars indicate mean ± S.E.M (n≥ 5). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared with the control group.

2. Imatinib induces cell-death by apoptosis of DU145 cells whereas increasing the survival of PC3 cells

The induction of apoptosis has been a widely used strategy for treatment of several types of human cancer (Munshi et al., 2002;O'Connor et al., 2006). The process of apoptosis involves two major pathways, the receptor-mediated (or extrinsic) and the mitochondrial (or intrinsic) (McKenzie and Kyprianou, 2006;Hensley et al., 2013). Independently of the activated pathway, the apoptotic process is triggered by the caspases enzymes, and both pathways converge at the activation of caspase-3, which has been considered a remarkable end-point of apoptosis (Ghobrial et al., 2005;Fiandalo and Kyprianou, 2012). Therefore, we used the activity of caspase-3 as a measurement of apoptosis induced by imatinib. In DU145 cells, the enzymatic activity of caspase-3 was significantly increased in response to 20 μM of imatinib for 72 h (50.00 % ± 20.89 fold variation relatively to control, Fig. IV. 8). In contrast, PC3 cells displayed a significantly decreased activity of caspase-3 both at 48 h and 72 h after administration of imatinib (respectively, 37.79 % ± 15.29 and 41.18 ± 10.30 reduction relatively to control, Fig. IV.8).

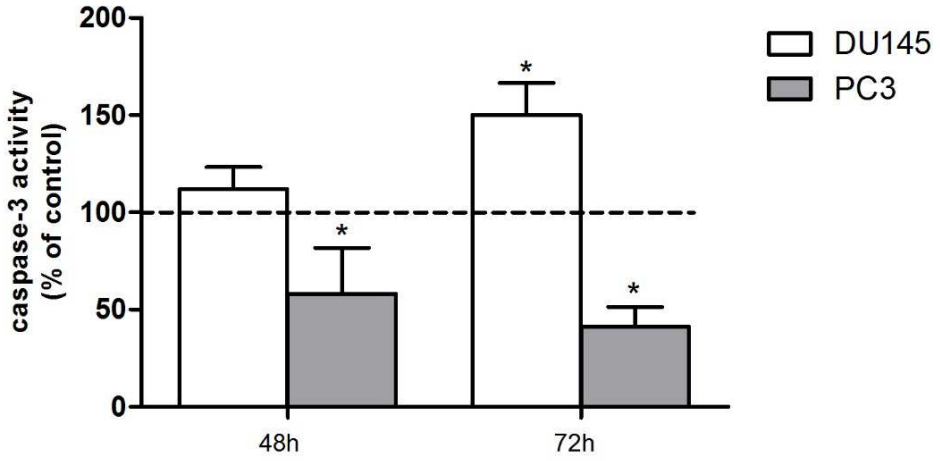

Figure IV.8. Caspase-3 activity in DU145 and PC3 cells after imatinib treatment for 48 and 72h. Results are expressed as fold-variation relatively to control group (dashed line). Error bars indicate mean ± S.E.M (n= 5). *p<0.05

Since imatinib affected the viability and apoptosis of DU145 and PC3 cells at 72 h after beginning of treatment, this experimental time-point was selected for the gene expression analysis of apoptosis regulators.

Caspase-8 is the essential mediator of the extrinsic apoptosis pathway, which interacts with death receptors and activates the downstream effectors of apoptosis, namely, caspase.3 (van Raam and Salvesen, 2012;Stupack, 2013). In DU145 cells, the expression of caspase-8 was significantly increased relatively to control (1.73 ± 0.23 fold variation, Fig. IV.9A). No differences were observed for protein levels of caspase-8 in PC3 cells in response to imatinib (Fig. IV. 9A).

The Bax and Bcl-2 proteins are proapototic and antiapoptotic members of the Bcl-2 protein family, which regulate the apoptotic pathway triggered at the mitochondria (Youle and Strasser, 2008). The Bax protein is involved in permeabilization of mitochondrial pores and release of cytochrome c while Bcl-2 is known to repress this process (Jurgensmeier et al., 1998;Rosse et al., 1998). Treatment with 20 μM of imatinib for 72 h decreased the expression of antiapoptotic Bcl-2 protein in DU145 cells (0.41 ± 0.16 fold variation comparatively with the control, Fig. IV.10A) while no effect was seen in PC3 cells (Fig. IV.10A). DU145 cells were negative for Bax and PC3 cells do not show differences on the expression of this proapoptotic protein in response to imatinib (Fig. IV.10C). Once cytochrome c is released to cytosol it interacts with apoptotic protease activating factor-1 (Apaf-1) leading to the activation of caspase-9, which in turn activates caspase-3 (Li et al., 1997;Srinivasula et al., 1998).

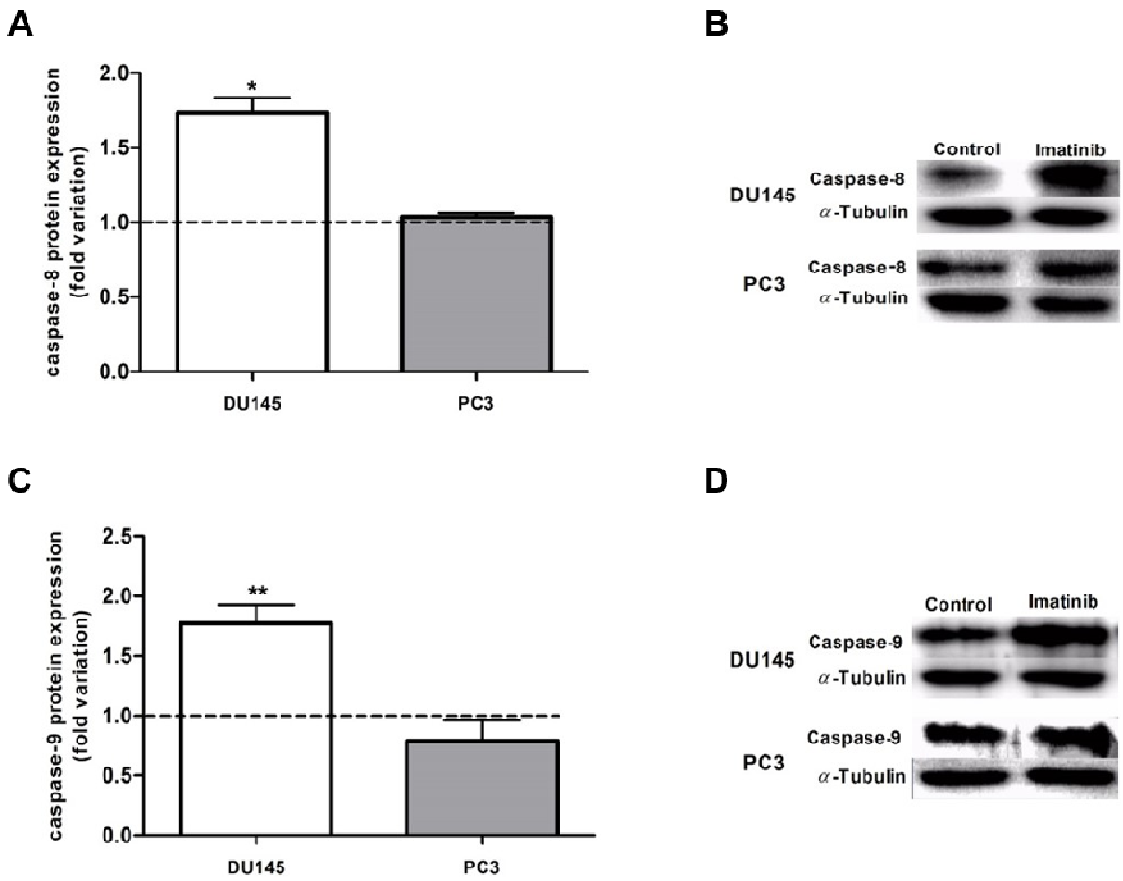

Figure IV.9. **Effect of imatinib on protein levels of caspase-8 (A) and caspase-9 (C) in DU145 and PC3 cells determined by WB analysis.** Representative immunoblots for caspase-8 and caspase-9 are shown, respectively, in panels B and D. Results are expressed as fold-variation relatively to control group (dashed line) after normalization with α-tubulin. Error bars indicate mean ± S.E.M (n= 5). * $p<0.05$; ** $p<0.01$.

WB analysis showed that the protein levels of caspase-9 were significantly increased in DU145 cells treated with imatinib (1.77 ± 0.19 fold variation relatively to control, Fig. IV.9C). The expression of caspase-9 protein in PC3 cells treated with imatinib was not significantly different from control (Fig. IV.9C).

The tumor suppressor protein p53 has a critical role in the regulation of the Bcl-2 family members, and also controls the expression of p21, a cyclin-dependent kinase inhibitor that induces cell cycle arrest (Miyashita et al., 1994;Waldman et al., 1995). PC3 cells were negative for p53 and no differences were observed on the levels of p53 in DU145 cells in response to imatinib (Fig. IV.10E).

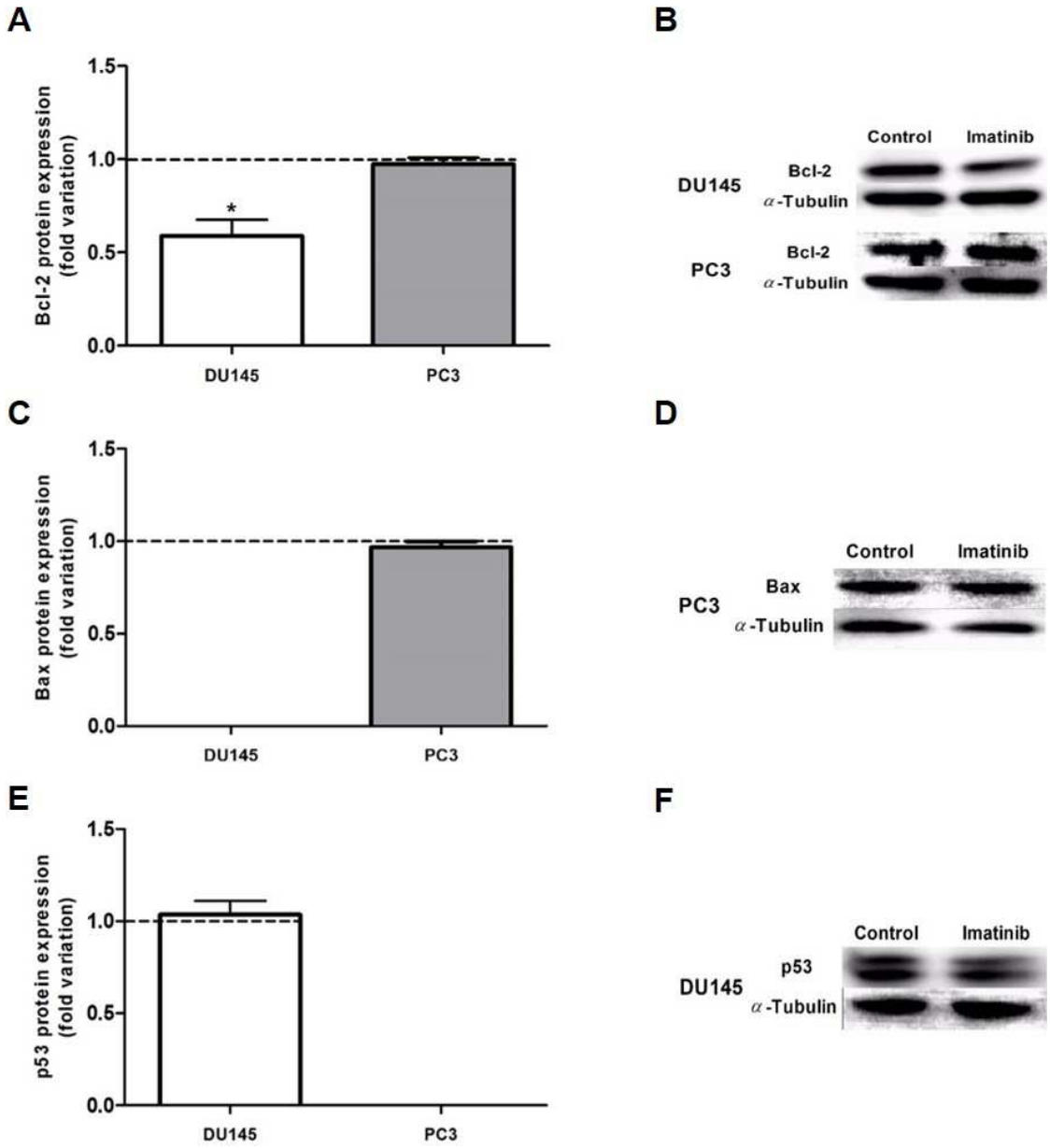

Figure IV.10. Effect of imatinib on protein levels of Bcl-2 (A), Bax (C) and p53 (E) in DU145 and PC3 cells determined by WB analysis. Representative immunoblots for Bcl-2, Bax and p53 are shown, respectively, in panels B, D and F. Results are expressed as fold-variation relatively to control group (dashed line) after normalization with α-tubulin. Error bars indicate mean ± S.E.M (n= 5) * $p<0.05$.

3. Expression of VEGF and cell-cycle inhibitor p21 in DU145 and PC3 cells treated with imatinib

qPCR analysis was used to determine the expression of p21 and VEGF in DU145 and PC3 cells after treatment with imatinib for 72 h. Imatinib significantly increased the mRNA expression of p21 both in DU145 and PC3 cells (respectively, 1.48 ± 0.12 and 1.72 ± 0.28 fold variation in comparison with the control, Fig. IV.11A).

The VEGF is a well-known stimulator of angiogenesis, which is highly expressed in PCa and contributes to the dissemination of prostate tumor cells to skeleton or other tissues (Roberts et al., 2013). The mRNA expression of VEGF was significantly decreased in DU145 cells after treatment with imatinib (0.33 ± 0.13 fold variation relatively to control, Fig. IV.11B). In opposition, PC3 cells treated with imatinib displayed a strong increase in the mRNA levels of VEGF (2.09 ± 0.48 fold variation in comparison with control, *** p<0.001, Fig. IV. 11B).

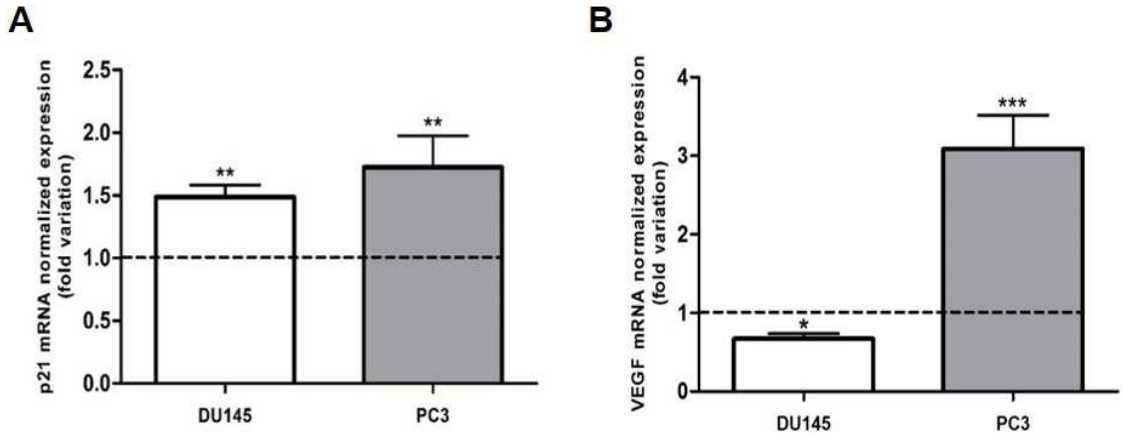

Figure IV.11. Effect of imatinib on mRNA levels of p21 (A) and VEGF (B) in DU145 and PC3 cells determined by qPCR. Results are expressed as fold-variation relatively to control group (dashed line) after normalization with ß-2-microglobulin, GAPDH and/or 18S housekeeping genes. Error bars indicate mean ± S.E.M (n= 5) * p<0.05; ** p<0.01; *** p<0.001.

4. DU145 and PC3 cells differentially express the full-length c-KIT and its truncated isoforms

The RTK c-KIT is one of the putative targets for imatinib (Knight et al., 2008;Imura et al., 2012) and the expression of c-KIT isoforms has been correlated with the onset and progression of PCa (Paronetto et al., 2004). However, the characterization of c-KIT expression in PCa tissues and cell lines has remained underexplored. Therefore, we decided to investigate the expression of the full-length c-KIT and truncated isoforms in DU145 and PC3 cells, also with the aim to understand the differential response of these cell lines to imatinib. WB analysis demonstrated that PC3 cells display reduced levels of the full-length c-KIT (145 kDa) relatively to DU145 cells (0.26 ± 0.11 fold variation, p<0,05, Fig. IV.12A). However, the expression of 50 kDa and 30 kDa isoforms of c-KIT was significantly higher in PC3 cells comparatively with DU145 (respectively 1.78 ± 0.06 and 1.35 ± 0.13 fold variation, Fig. IV.12A). The results of c-KIT immunofluorescent labeling also showed that c-KIT is expressed in both PC3 and DU145 cells and confirmed its higher expression in DU145 cells (Fig. IV.12C).

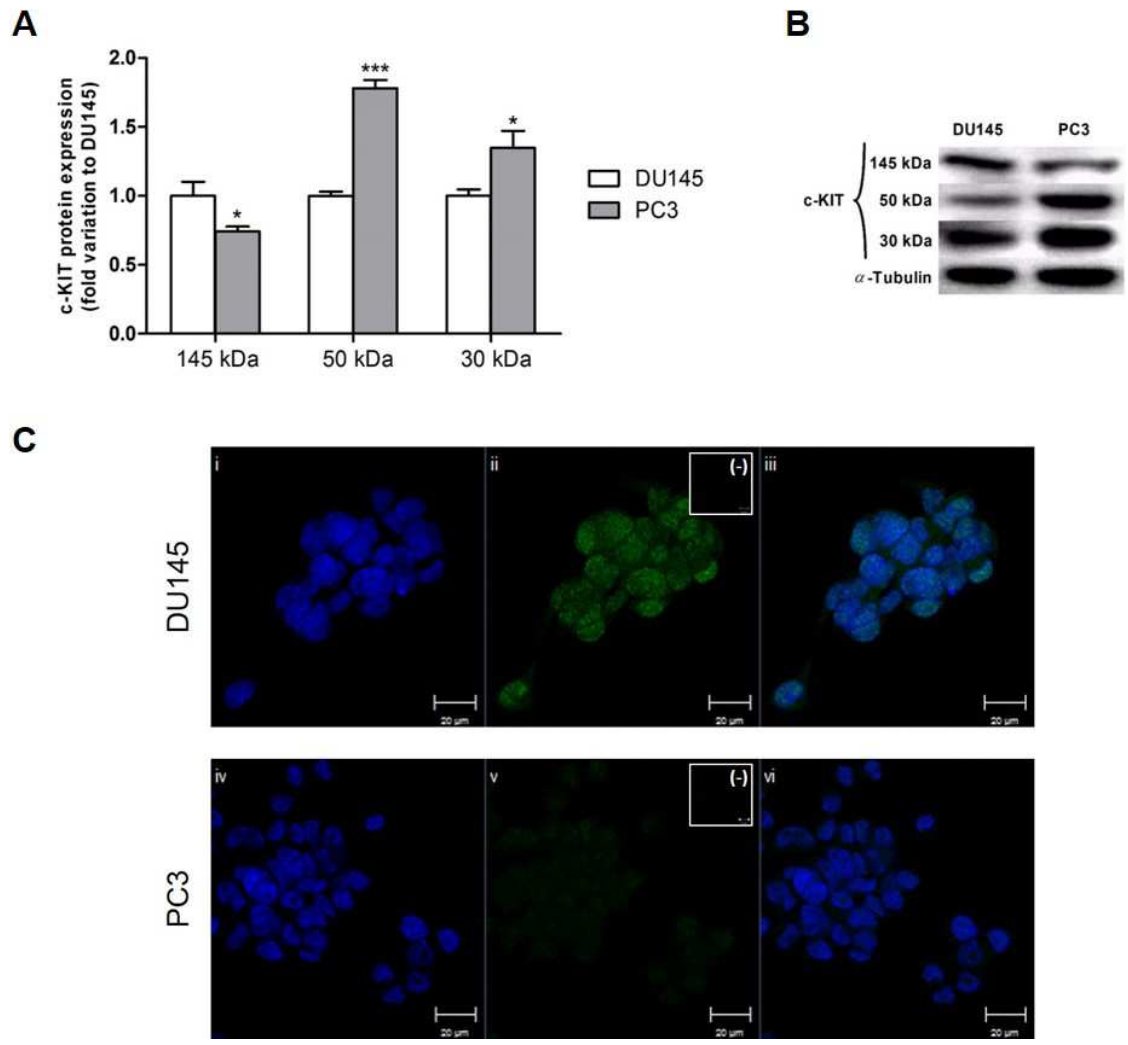

Figure IV.12. Expression of full-length (145 kDa), and 50 and 30 kDa isoforms of c-KIT in DU145 and PC3 cells. (A) Expression of c-KIT proteins determined by WB analysis. Results are expressed as fold-variation relatively to DU145 cells after normalization with α-tubulin. Error bars indicate mean ± S.E.M (n≥ 5). *p<0.05; ***p<0.001. Representative immunoblots are shown in panel B. (C) Representative confocal microscopy images showing c-KIT positive staining in DU145 and PC3 cells. Nuclei are stained with Hoechst 33342 (blue, i and iv), and fluorescence c-kit-positive staining is green (ii and v). Merged images are shown in iii and vi. Negative controls for c-kit obtained by omission of the primary antibody are provided as insert panels (-).

5. The expression of SCF/c-KIT system is decreased in the prostate of Tg-RGN rats

Previous studies have demonstrated that RGN is able to suppresses protein kinase and tyrosine phosphatase activities in hepatoma cells and liver (Inagaki and Yamaguchi, 2001;Fukaya and Yamaguchi, 2004), and that its overexpression in rat prostate is linked with inhibited cell proliferation and altered expression of cell-cycle regulators (Vaz et al., 2014), which raised the question whether RGN actions may alter the expression of SCF/c-KIT system. Thus, the expression of SCF and c-KIT in the prostate of Tg-RGN animals, comparatively with their wild type counterparts, was analyzed both at mRNA and protein level.

The mRNA expression of c-KIT was strongly reduced in the prostate of Tg-RGN rat comparatively with their wild-type counterparts (0.70 ± 0.20 variation, $p<0.01$, Fig. IV.13A). WB analysis allowed detecting the 50 and 30 kDa isoforms of c-KIT, which were both significantly decreased in the prostate of Tg-RGN rats (respectively, 0.18 ± 0.05 and 0.40 ± 0.06 fold variation relatively to wild type, Fig. IV.13B). The full-length c-KIT (145 kDa) was not detected. Relatively to SCF protein, also a significant diminution was observed in the prostate of Tg-RGN in comparison with wild-type rats, both at mRNA (0.30 ± 0.12 fold variation, Fig. IV.13D) and protein level (0.70 ± 0.24 fold variation, Fig. IV.13C).

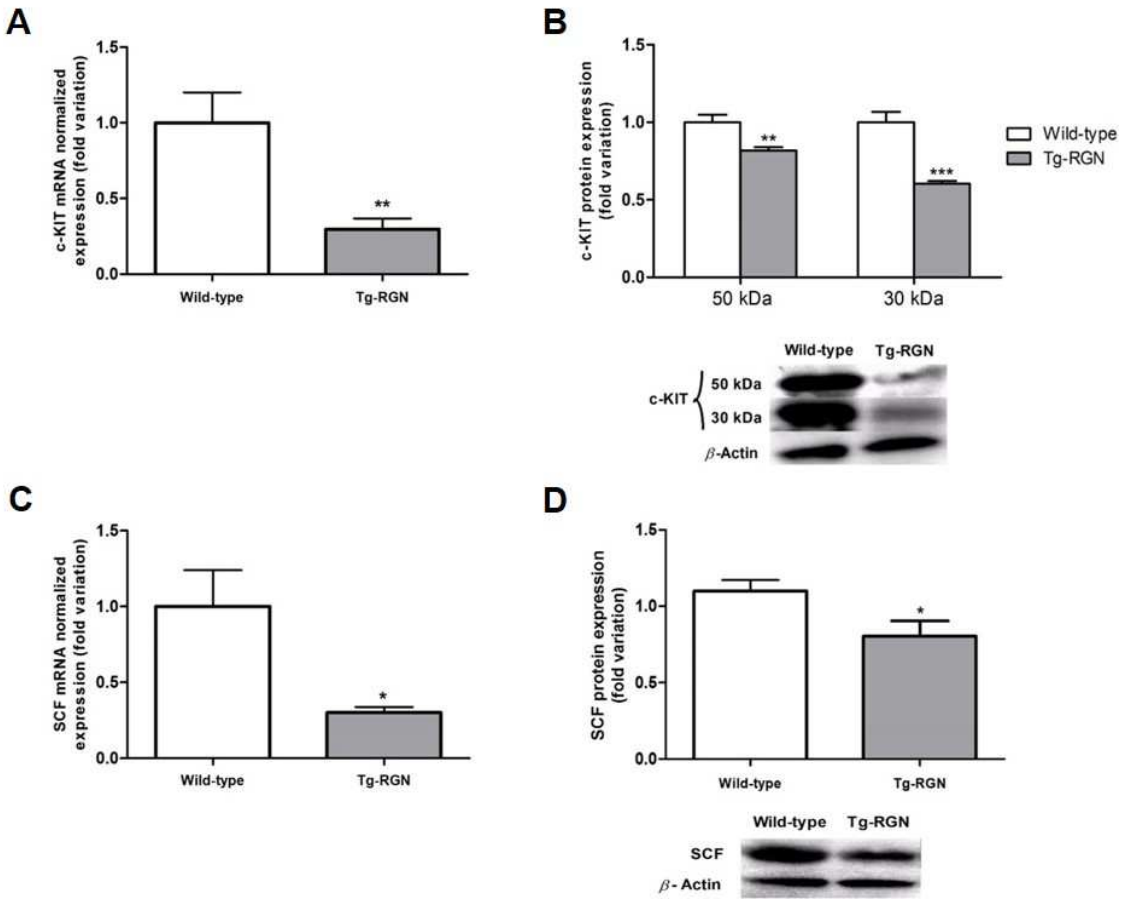

Figure IV.13. Expression of c-KIT and its ligand SCF in the prostate of Tg-RGN rats comparatively with their wild-type counterparts. mRNA expression of c-KIT (A) and SCF (C) were analyzed by qPCR. Results are expressed as fold-variation relatively to wild-type group after normalization with ß-Actin and GAPDH. Protein expression of c-KIT (B) and SCF (D) were analyzed by WB. Representative immunoblots for c-KIT and SCF are shown under panels B and D, respectively. Results are expressed as fold-variation relatively to wild-type rats after normalization with ß-actin. Error bars indicate mean ± S.E.M (n≥5). *$p<0.05$; ** $p<0.01$; ***$p<0.001$.

6. RGN is expressed in cell line models of HRPC and co-localizes with α-tubulin

Prior studies of the research group have shown that RGN is expressed by LNCap cells (Maia et al., 2009), being located at cytoplasm and also at cell nucleus. It was also described that a

down-regulated expression of RGN accompanies the progression of PCa (Maia et al., 2009). However, there are no studies analyzing the expression of RGN in cell line models of HRPC.

Immunofluorescence analysis confirmed the presence of RGN in two cell line models of HRPC, DU145 and PC3 cells, both at cytoplasm and nucleus (Fig. IV.14). Moreover, we investigated the co-localization of RGN with the α-tubulin, a protein essential for microtubule formation and organization of cell division. For the first time, it was shown that RGN co-localizes with α-tubulin, indicating its association with components of the cell division machinery (Fig. IV.14).

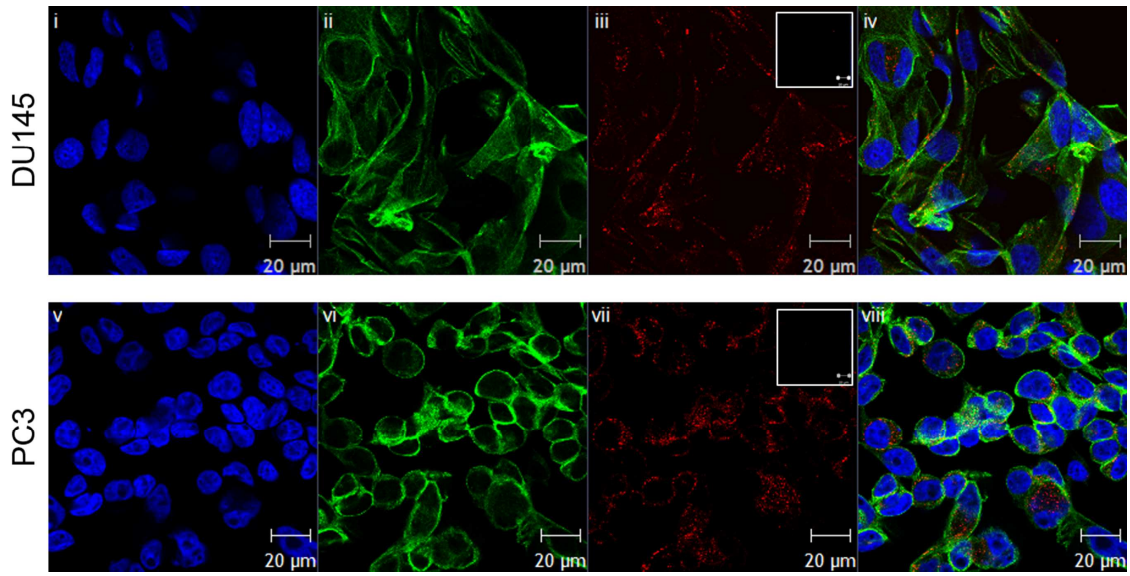

Figure IV.14. Expression of RGN in cell line models of HRPC, namely, DU145 and PC3 cells. Representative confocal microscopy images showing RGN positive staining in co-localization with α-tubulin in DU145 and PC3 cells. Nuclei are stained with Hoechst 33342 (blue, i and v). Fluorescence positive staining for α-tubulin (ii and vi) and RGN (iii and vii) is, respectively, green and red. Merged images are shown in panels iv and viii. Negative controls for RGN obtained by omission of the primary antibody are provided as insert panels (-).

V. Discussion

The present dissertation investigated the cytotoxic effects of imatinib in DU145 and PC3 cells, two cell line models of HRPC, which do not express the AR and have been widely used to mimic metastatic stages of PCa (Tilley et al., 1990;Yu et al., 2009)

Imatinib treatment (20 μM) decreased the number of viable cells in the DU145 cell line at 48 and 72 h, which is in agreement with other reports using the same dose of imatinib (Kubler et al., 2005). These results are also supported by the half-life time of the active metabolite of imatinib that is 40 hours (Nikolova et al., 2004;Peng et al., 2005).

However, an opposite effect was observed in the PC3 cell line. Although diminishing the number of viable cells 6 h after administration, at 24 h and thereafter imatinib significantly increased the viability of PC3 cells.

The contradictory response of DU145 and PC3 cells to imatinib was accompanied by a distinct expression pattern and activity of target regulators/effectors of apoptosis. The caspases enzymes belong to the homologous cysteine-dependent cell death proteases and are the central executioners of apoptosis (Fiandalo and Kyprianou, 2012;Hensley et al., 2013) being activated by internal or external stimuli (intrinsic or mitochondrial pathway) or by several receptors at cell membrane (extrinsic pathway) (Fig. V.15). Independently of the activated pathway, execution of apoptotic cell-death depends on the activation of caspase-3, which has been recognized as an end point of apoptosis (Fig V.15) (Ghobrial et al., 2005). Therefore, we measured the enzymatic activity of caspase-3 as an indicator of cell-death in response to imatinib. Imatinib induced cell-death by apoptosis in DU145 cells, evident by a 50% increase in the activity of caspase-3 enzymatic activity. Contrarily, the increased viability observed in PC3 cells was underpinned by a 40% reduction in the activity of caspase-3.

The activation of caspase-3 in extrinsic and intrinsic pathways of apoptosis is triggered by initiator caspase-8 and caspase-9 (Fig. V.15), respectively. In DU145 cells, imatinib treatment induced augmented expression of caspase-8 and caspase-9 proteins, which indicates that both pathways of apoptosis were activated. In PC3 cells no differences were observed on the expression levels of caspase-8 and caspase-9 indicating again the non-cytotoxic stimulation of imatinib in this cell line.

The protein ratio of proapoptotic (Bax)/antiapototic (Bcl-2) members of Bcl-2 family of apoptosis regulators also has been used as an indicator of cell commitment to apoptosis, and also as a useful prognostic marker in cancer (Mackey et al., 1998;Scopa et al., 2001). Bcl-2 stabilizes the mitochondrial membrane and prevents the release of cytocrohome c into the cytosol, whereas the Bax have the opposite function, which conducts to the activation of caspases, and subsequently apoptosis (Fig V.15) (Lucken-Ardjomande and Martinou, 2005;Chipuk and Green, 2008;Garcia-Saez et al., 2010). Imatinib treatment decreased the protein levels of Bcl-2 in DU145 cells, but no differences were seen in PC3 cells. Relatively to Bax no differences were obtained in PC3 cells and in DU145 cell line no protein expression were observed. This is in accordance with other studies that indicate a differential expression of Bax protein between DU145 (barely detectable) and PC3 (relatively high expression) cells,

and a mutated Bax gene was identified in DU145 cells, while PC3 cells display a functional Bax protein (Honda et al., 2002;Kwok et al., 2005). The results obtained also assume particular relevance considering that Bcl-2 protein, has been described in samples of androgen-independent prostate carcinoma, whereas 70% of androgen-dependent carcinoma samples were negative and 30% represent weak staining (McDonnell et al., 1992). Also, a metastatic variation of a prostatic carcinoma cell line was characterized by increased expression levels of Bcl-2 and low levels of Bax protein, when compared to the non-metastatic variant of the same cell (McConkey et al., 1996;Westin et al., 1997).

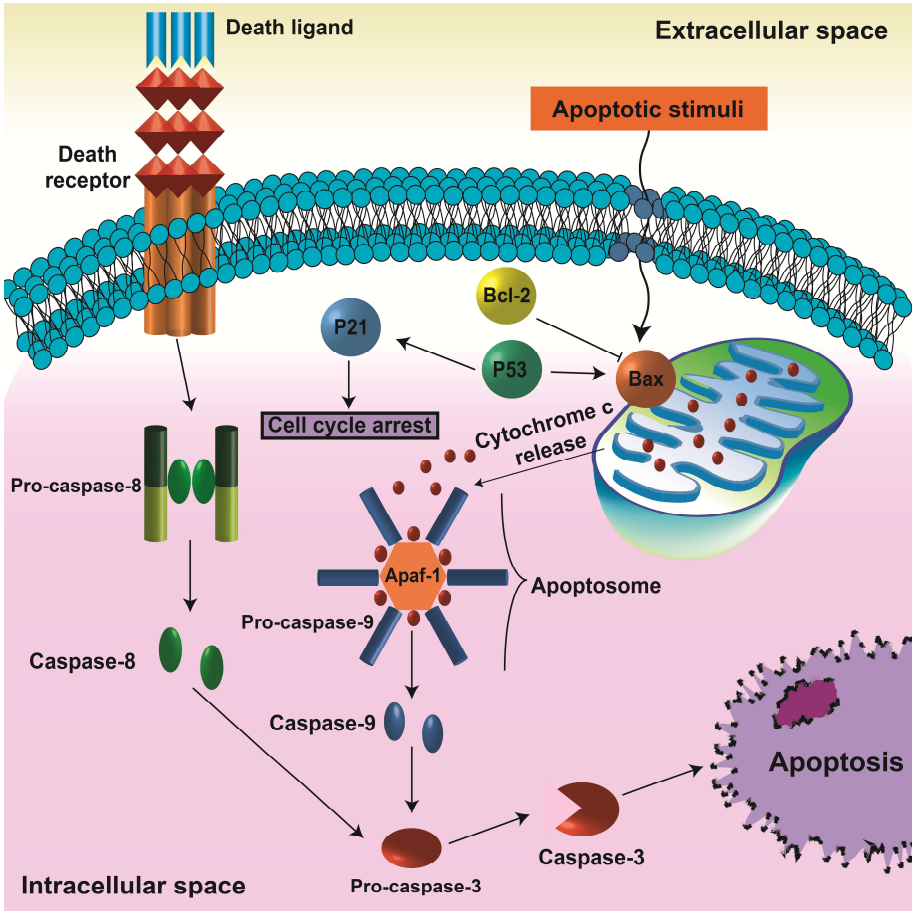

Figure V.15. Two alternative pathways to induce apoptosis. The apoptosis can be activated by internal or external stimuli (intrinsic or mitochondrial pathway) or by several receptors at cell membrane (extrinsic pathway) and the executioners are caspase-9 and caspase-8 respectively. In both pathways, induction of apoptosis leads to activation of an initiator caspase (pro-caspases) and caspase-9 is activated through apoptosome. The pathways converges to induce the activation of caspase-3, which has been recognized as an end point of apoptosis. Bcl-2 is the protein that stabilizes the mitochondrial membrane and prevents the release of cytocrohome c into the cytosol, whereas the Bax have the opposite function activating apoptosis. p53 functions as a transcription factor regulating downstream genes important in cell cycle arrest and apoptosis, including, p21, Bax and Bcl-2. And p21 is a cyclin-dependent kinase inhibitor capable to arrest the cell cycle at the G1 phase. Adapted from (Correia et al., 2014c)

This study described for the first time the effects of imatinib in the apoptotic machinery, a fundamental process in PCa treatment resistance, in non-androgen PCa lines. The results obtained demonstrated that there was an increase in the executioners of apoptosis in DU145 cells, namely caspases, but these results were not observed in the PC3 cell line. The differential response to imatinib can be connected with the distinct characteristics of DU145 and PC3 cells. For example, it has been demonstrated the differential sensitivity of DU145 and PC3 cells to several kinase and phosphatase inhibitors (Rokhlin and Cohen, 1995). Guo et al. (2010) described a different sensibility to apoptosis with protein kinase C inhibitor in PC3 and DU145 cells. Also, in AR-transfected PC3 and DU145 cells the differential effects were observed. In PC3-AR9 cells with natural AR promoter, AR functions as suppressor of *in vivo* growth, *in vitro* and *in vivo* metastasis (Niu et al., 2008), whereas in DU145-AR cells, AR acts as suppressor of proliferation. Moreover, this effect were restored by treatment with testosterone (Scaccianoce et al., 2003). Thus, it is likely that the different phenotypes that both of cell lines present may contribute to the resistance to imatinib observed in PC3 cells.

p53 functions as a transcription factor regulating downstream genes important in cell cycle arrest and apoptosis, including, p21, Bax and Bcl-2 (Fig. V.15) (Knillova and Kolar, 2003;Ghobrial et al., 2005;McKenzie and Kyprianou, 2006). In the present dissertation no differences were observed on protein expression of p53 upon imatinib treatment in DU145 cells, and no expression was detected in PC3. These results are supported by other studies also indicating that DU145 cells present a mutated p53 and that PC3 cells are negative for this protein (Rubin et al., 1991;Moretti et al., 2014).

The p21 protein is a cyclin-dependent kinase inhibitor capable to arrest the cell cycle at the G1 phase (Fig. V.15) (Gotoh et al., 2003). The increased expression of p21 mRNA in response to imatinib is an interesting result indicating that imatinib can block cell cycle, which seems to be occurring in DU145 cells, since their number decreased with imatinib treatment. The relationship between p21 and p53 is complex, and it is established that the ability of p21 to induce cell cycle arrest is depend p53 mediated gene expression (Hernandez et al., 2003). However, PC3 do not express p53, and also in these cells an up-regulated expression of p21 was observed. This indicates that other factors besides p53 are controlling the diminished proliferation of DU145 cells. On the other hand, there are reports indicating that p21 can inhibits the apoptosis, which it is not clear how occurs, but it was described that cytoplasmic p21 binds and inhibits the activity of proteins directly involved in the induction of apoptosis, namely, procaspase 3, caspase-8, caspase-10 (Zhang et al., 1999;Dotto, 2000;Zhou et al., 2012). In addition, it was also reported that p21 confers resistance to imatinib induced-apoptosis in human chronic myeloid leukemia cells (Ferrandiz et al., 2010), and it could not be excluded that a similar mechanism is occurring in PC3 cells.

Angiogenesis is other important process for cancer progression and metastatization (Roberts et al., 2013). The VEGF expression has been considered a reliable method for analyzing the angiogenic process. We verified that imatinib administration produced an opposite effect in PCa cell lines tested. VEGF mRNA expression decreased in DU145 cells and

increased in PC3. VEGF has been identified as a therapeutic target and various drugs have been developed aiming at reach this factor (Aragon-Ching and Dahut, 2009). In patients with pulmonary arterial hypertension and patients with GIST imatinib inhibited the expression of VEGF (McAuliffe et al., 2007;Hatano et al., 2010), but in PC3 xenografts imatinib treatment do not changed the VEGF levels (Kimura et al., 2007). So, this is the first report describing that imatinib alters the VEGF expression in PCa cells. However, imatinib produced contradictory effects on the VEGF expression in DU145 and PC3 cells, which causes concern about using imatinib treatment and stimulating the progression of metastasis.

The biochemical activity of imatinib includes inhibition of tyrosine kinase receptors, such as, the PDGFR and the c-KIT. Since only 16% of metastatic PCa present express the active subunit of PDGFR inducing proliferation, migration and angiogenic (Hofer et al., 2004), which is the subunit associated with cell proliferation, migration and angiogenic effects (Claesson-Welsh, 1994), this reinforces the importance of c-KIT as an imatinib target. Nevertheless, the expression of c-KIT in PCa cells is poorly studied and available studies have originated some discrepancies. Herein, it was shown that PC3 cells express significantly lower levels of the full-length membrane isoform of c-KIT relatively to DU145, while presenting increased levels of tr-KIT (30 kDa) and 50 kDa isoform. Previous studies also have identified the c-KIT mRNA in DU145, PC3 and LNCaP cells (Savarese et al., 1998;Wiesner et al., 2008). However, protein expression of c-KIT was only described for the DU145 cell line (Wiesner et al., 2008;Brooks et al., 2012). In this study, we showed by distinct approaches, including WB and fluorescent immunocytochemistry, that c-KIT is expressed in DU145 and PC3 cells, and that PC3 mainly express the tr-KIT and the 50 kDa isoforms. Although the function of c-KIT isoforms remains unclear, the expression of tr-KIT has been associated with the more advanced stages of prostatic tumors (Paronetto et al., 2004;Zhang et al., 2013), which supports our findings since PC3 mimic a more advanced stage of disease.

It was previously described that SCF only stimulates the cell growth in the DU145 cell line (Savarese et al., 1998), which, according with our results, may be explained by the highest expression of the functional full-length c-KIT in these cells. Moreover, this fact may strongly explain the differential response of DU145 and PC3 cells to imatinib.

Into the clinical setting, imatinib do not displayed great cytotoxic effects and no PSA responses were found upon imatinib treatment (Mathew et al., 2004a;Mathew et al., 2004b;Corcoran and Costello, 2005). Imatinib treatment in PCa models only has been effective in combination with other chemotherapeutics agents (Kimura et al., 2007;Choudhury et al., 2009;Pinto et al., 2011;Huang et al., 2012). In PC3 xenografts, Kimura et al. (2007) demonstrated that imatinib improves the efficacy of radioimmunotherapy. Also, the combination of imatinib with ionizing radiation, gemcitabine, and mitomycin C in PC3 prostate xenografts enhanced tumor cell chemosensitivity and radiosensitivity (Choudhury et al., 2009).

The contradictory and paradoxical results obtained with imatinib in DU145 and PC3 cells considering cell survival, and expression and activity of the apoptotic machinery, may

somehow explain why Imatinib treatment do not had produced effective results in treatment of PCa (Kimura et al., 2007;Choudhury et al., 2009;Huang et al., 2012).

RGN or SMP30 is a Ca^{2+} binding protein that regulates several cell functions like Ca^{2+} homeostasis, cell proliferation, apoptosis, DNA fragmentation, and DNA and RNA synthesis (Marques et al., 2014a) (Fig. I.6). Moreover, in hepatoma H4-II-E cells it was shown that RGN inhibits cell proliferation while diminishing the protein kinase activity (Inagaki and Yamaguchi, 2001). This finding supports the hypothesis that the RGN may alter the expression of RTK, c-KIT, or its ligand, SCF. In the present study it was shown that prostates of Tg-RGN animals display decreased expression of SCF/c-KIT system. The SCF/c-KIT system is actively involved in cell proliferation in several tissues, including prostate (Imura et al., 2012), and recently Vaz and co-authors (Vaz et al., 2014) demonstrated that Tg-RGN rats have a decreased proliferation index comparatively with wild types counterparts. Altogether, it is plausible to speculate that the anti-proliferative actions of RGN in rat prostate may depend on the downregulation of SCF and c-KIT. It was also studied the subcellular localization of RGN in HRPC cell lines and its association with α-tubulin was investigated. RGN was localized in the nucleus and cytoplasm of PC3 and DU145 cells, which is identical with the compartmentalization found in LNCap cells (Maia et al., 2009). Although the precise nuclear functions of RGN need to be clarified, it has been shown that it may regulate expression of genes involved in cell cycle arrest, namely, by increasing the levels of cell cycle inhibitor, p21 (Yamaguchi and Daimon, 2005;Vaz et al., 2014), which sustains its anti-proliferative role.

Moreover, it was shown for the first time that RGN co-localized with α-tubulin, which indicates its association with components of the cell division machinery. Normal and cancer cells are dependent on the organization of the cytoskeleton and microtubules are essential for cellular processes such as growth, motility and inter- and intra-cellular signaling (Musch, 2004). Microtubules are composed by polypeptides of α- and ß-tubulin heterodimers, and α-tubulin displays a differential expression in PCa cancer cells contributing to the development of cancer (Souček et al., 2006). The novelty of the present findings are of the uttermost importance to further disclose the roles of RGN in the control of cell proliferation opening new avenues of research to explore its cytoplasmic and nuclear activities.

VI. Conclusions and Future Perspectives

The last stages of aggressive PCa are associated with loss of androgen responsiveness, a condition described as HRPC. This causes the failure of ADT and markedly restricts the therapeutic options available for this usually lethal form of disease. Imatinib, an inhibitor of RTKs has not demonstrated effective results in treatment of PCa.

In this study, imatinib was effective decreasing cell viability and increasing the activity of apoptotic inducers in DU145 cells, an effect opposed to that seen in PC3 cells. Moreover, in PC3 cells imatinib stimulated cell viability and enhanced the expression of VEGF, which indicates that imatinib paradoxically may be promoting tumor growth and the angiogenic process. The present study contributed to understand the discrepancies in the effectiveness of imatinib in the treatment of PCa, which may be related with the differential expression of c-KIT isoforms. Future studies are needed to characterize the complete picture of the molecular targets of imatinib in both cell line models of HRPC. The combination of imatinib with other chemotherapeutic agents can be a future strategy. Moreover, it will be interesting to understand the importance of truncated isoforms of c-KIT in response of imatinib.

Previous findings of our research group have shown that RGN is a protein involved in the control of proliferation in prostate. Herein, it was demonstrated that RGN overexpression decreases the expression of SCF/c-KIT system further confirming the role of this protein in controlling the prostate tissue homeostasis, which may involve an altered expression of SCF and c-KIT. In a near future it will be important to determine whether imatinib actions counteracting cell proliferation may involve changes in the expression of RGN. The co-expression of RGN with α-tubulin in HRPC cells highlights for the importance of this protein in the control of cell cycle. New research perspectives are open to further explore the association of RGN with other components of the cell division machinery.

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VIII. List of publications

1. Publication in International Peer-reviewed 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

Correia S, **Cardoso HJ**, Cavaco JE; Socorro S (2014). Estrogens as apoptosis regulators in mammalian testis: angels or devils? Submitted to *Expert Reviews in Molecular Medicine* (under revision). IF: 5.912

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2. Oral communications

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.

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.

3. Posters

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.

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.