# Further insights into regucalcin actions as a potential tumour suppressor in human prostate

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# Dedicatória

Dedico este trabalho ao meu avó João, que não estando presente fisicamente, está presente espiritualmente! Obrigado pelo orgulho constante que sempre manifestaste e por teres acreditado desde o primeiro segundo!

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# Resumo

A regucalcina (RGN) é uma proteína de ligação ao cálcio que controla diversos processos biológicos, nomeadamente importantes marcos no desenvolvimento do cancro, como a proliferação celular e a apoptose. Estudos prévios do nosso grupo de investigação demonstraram que a perda de expressão da RGN acompanha o aparecimento e progressão do cancro da próstata (PCa). Contudo, não se sabe se a diminuição dos níveis de expressão da RGN é uma causa ou uma consequência do PCa. A presente tese teve como objetivo investigar a relação dos níveis de expressão da RGN com os marcos do cancro e com o prognóstico clínico de pacientes com PCa. Uma análise bioinformática realizada com o software CancerTool[1] e recorrendo a um conjunto de bases de dados de pacientes demonstrou que a perda de expressão da RGN se correlaciona com o aparecimento e progressão do PCa para formas metastáticas da doença. No entanto, não se identificou uma correlação entre os níveis de expressão da RGN e a classificação histológica de Gleason ou a recorrência do PCa. Para além disso, verificou-se que pacientes com PCa com maiores níveis de expressão da RGN apresentavam maior taxa de sobrevivência livre de doença. A análise bioinformática da correlação da expressão gene-a-gene mostrou ainda que em tumores primários da próstata a expressão do gene da *RGN* se correlaciona diretamente com a expressão dos genes *cyclin-dependent kinase inhibitor 1A (CDKN1A)* e *IL6*. O gene *CDKN1A* codifica o inibidor do ciclo celular p21, o qual tem sido indicado como um alvo da RGN na próstata de rato e em amostras tumorais humanas. Nenhum outro estudo prévio identificou a ligação entre a RGN e a IL-6 em células ou tecidos cancerígenos. Numa segunda fase, investigámos se a perda de expressão da RGN pode alterar o destino celular das células de próstata humana. Usando uma abordagem de silenciamento génico através de siRNA, a expressão do gene da *RGN* foi diminuída em células não-neoplásicas humanas PNT1A, o que foi confirmado por PCR quantitativo em tempo real. O *knockdown* (*KD*) do gene da *RGN* aumentou a viabilidade e a capacidade proliferativa das células PNT1A, como indicaram os resultados dos ensaios de MTT e sulforodamina B, e da imunocitoquímica fluorescente do marcador de proliferação Ki-67. Verificou-se ainda que células PNT1A *KD* para a RGN apresentavam uma redução da atividade enzimática da caspase-3 o que demonstrou que a perda da RGN pode contribuir para a resistência à apoptose. Os resultados do *Western Blot* mostraram que as alterações no destino celular das células da próstata foram

[1] Cortazar, A.R., et al., *CANCERTOOL: a visualization and representation interface to exploit cancer datasets*. Cancer research, 2018. **78**(21): p. 6320-6328.

acompanhadas por alterações na expressão de oncoproteínas, como a AKT e a sua forma fosforilada, ou o c-myc; e também de reguladores chave da via extrínseca da apoptose, nomeadamente, FasR, FasL e caspase-8. O *KD* do gene da *RGN* não alterou o metabolismo glicolítico das células PNT1A, como indicaram os resultados do consumo de glucose e da produção de lactato. Globalmente, os resultados obtidos mostraram que a perda da expressão da RGN altera o comportamento das células de próstata humana e promove a agressividade e progressão do PCa, o que suporta o papel da RGN como uma proteína supressora de tumor. Para além disso, estes resultados suportam igualmente a ideia de que a manutenção de níveis elevados de expressão da RGN pode prevenir o processo carcinogénico e retardar a progressão da doença.

# Palavras-chave

Regucalcina;Cancro da Próstata;Proliferação;Sobrevivência;Apoptose;Metastização.

# Resumo Alargado

A proteína de ligação ao cálcio ($Ca^{2+}$) regucalcina (RGN) tem como principal função a regulação da homeostase intracelular do $Ca^{2+}$ através da modulação do transporte deste ião através da membrana plasmática e organelos celulares, nomeadamente, mitocôndria, retículo endoplasmático e núcleo. A RGN também tem uma importante ação antioxidante. Para além de aumentar a capacidade antioxidante e ativar enzimas antioxidantes, a RGN inibe a enzima sintase do óxido nítrico, diminuído assim os níveis do radical livre óxido nítrico. Há ainda um conjunto de outras funções essenciais que têm sido associadas à RGN, as quais envolvem a regulação da proliferação celular, da apoptose e do metabolismo glicolítico, e que são importantes marcos no desenvolvimento do cancro. Vários estudos *in vitro* e *in vivo* demonstraram o efeito anti proliferativo da RGN, assim como a sua ação na regulação do processo apoptótico, o que parece acontecer através da regulação da expressão e atividade de oncogenes, genes supressores de tumor e reguladores do ciclo celular. Relativamente à regulação do metabolismo glicolítico, estudos *in vitro* e *in vivo* mostraram que a RGN regula a expressão dos transportadores de glucose, e inibe a glicólise e a produção e exportação de lactato.

O nosso grupo de investigação estabeleceu pela primeira vez a relação entre a perda de expressão da RGN e o aparecimento e progressão do cancro da próstata (PCa). Uma análise de casos de PCa humanos demonstrou que 40% dos adenocarcinomas bem diferenciados apresentam elevados níveis de expressão da RGN, enquanto que apenas 12% dos tumores pouco diferenciados apresentavam este padrão de expressão. De grande relevância é o facto da perda de expressão da RGN associada ao aparecimento e desenvolvimento de tumores não ser exclusiva do PCa. Esta relação tem sido encontrada numa variedade de cancros humanos, o que permite sugerir a RGN como uma supressora de tumor. Contudo, permanece por averiguar se a diminuição dos níveis de expressão da RGN é uma causa ou uma consequência do PCa. A presente tese teve como objetivo investigar a relação dos níveis de expressão da RGN com os marcos do cancro e com o prognóstico clínico de pacientes com PCa.

Uma análise bioinformática realizada com o software CancerTool[2] e recorrendo a um conjunto de bases de dados de pacientes demonstrou que a perda de expressão da RGN

[2] Cortazar, A.R., et al., *CANCERTOOL: a visualization and representation interface to exploit cancer datasets*. Cancer research, 2018. **78**(21): p. 6320-6328.

se correlaciona com o aparecimento e progressão do PCa para formas metastáticas da doença, sendo este resultado consistente em três das cinco bases de dados analisadas. No entanto, não se identificou uma correlação entre os níveis de expressão da RGN e a classificação histológica de Gleason ou a recorrência do PCa. Para além disso, verificou-se em uma das três bases de dados analisadas que pacientes com PCa com maiores níveis de expressão da RGN apresentavam maior taxa de sobrevivência livre de doença. Apesar de ser necessário trabalho de investigação adicional, é possível, com estes dados, propor a potencial utilização da RGN como um biomarcador de prognóstico no PCa.

A análise bioinformática da correlação da expressão gene-a-gene mostrou ainda que em tumores primários da próstata a expressão do gene da *RGN* se correlaciona diretamente com a expressão dos genes *cyclin-dependent kinase inhibitor 1A (CDKN1A)* e *IL6*, sendo este resultado consistente nas duas análises de correlação utilizadas. O gene *CDKN1A* codifica o inibidor do ciclo celular p21, o qual tem sido indicado como um alvo da RGN na próstata de rato e em amostras tumorais humanas. Nenhum outro estudo prévio identificou a ligação entre a RGN e a IL-6 em células ou tecidos cancerígenos. Clarificar esta relação é de crucial importância dado que a IL-6 está identificada como uma proteína envolvida na sobrevivência das células de PCa, angiogénese e metastização. Para além disso, em pré-adipócitos embrionários, a sobre-expressão da RGN foi associada à diminuição dos níveis de IL-6. Para todos os outros genes considerados, e diferentes condições analisadas (tecido não-neoplásico, tumores, e tumores metastizados), não foi encontrada qualquer correlação com a expressão da RGN.

Numa segunda fase, investigámos se a perda de expressão da RGN pode alterar o destino celular das células de próstata humana. Usando uma abordagem de silenciamento génico através de siRNA, a expressão do gene da *RGN* foi diminuída em células não-neoplásicas humanas PNT1A, o que foi confirmado por PCR quantitativo em tempo real. O *knockdown* (*KD*) do gene da *RGN* aumentou a viabilidade e a capacidade proliferativa das células PNT1A, como indicaram os resultados dos ensaios de MTT e sulforodamina B, e da imunocitoquímica fluorescente do marcador de proliferação Ki-67. Embora existam estudos que mostram que a sobre-expressão da RGN diminui a capacidade proliferativa de células neoplásicas humanas, este é o primeiro trabalho que demonstra que a perda da expressão da RGN altera o comportamento das células não-neoplásicas humanas da próstata levando ao aumento da proliferação. Verificou-se ainda que células PNT1A *KD* para a RGN apresentavam uma redução da atividade enzimática da caspase-3 o que demonstrou que a perda da RGN pode contribuir para a resistência à apoptose. Os resultados do *Western Blot* mostraram que as alterações no destino celular das células da próstata foram acompanhadas por alterações na expressão de oncoproteínas, como a

AKT e a sua forma fosforilada, ou o c-myc fosforilado; e também de reguladores chave da via extrínseca da apoptose, nomeadamente, FasR, FasL e caspase-8. A enzima AKT e a sua forma ativa têm sido indicadas como alvos da RGN, mediando os seus efeitos na regulação da atividade proliferativa em várias linhas celulares tumorais humanas. Contudo, nenhum outro estudo reportou uma relação da RGN com o fator de transcrição c-myc fosforilado. Tanto a via PI3K/AKT como o fator de transcrição c-myc são importantes reguladores do desenvolvimento do PCa, o que suporta a importância da RGN nesta doença. A supressão da via extrínseca da apoptose pelo silenciamento da expressão génica da RGN é corroborada por estudos prévios na próstata de rato. Para além disso, tendo em conta a suscetibilidade das células de PCa para a apoptose mediada pelo recetor Fas, este efeito reforça a importância do RGN na prevenção do processo carcinogénico.

O *KD* do gene da *RGN* não alterou o metabolismo glicolítico das células PNT1A, como indicaram os resultados do consumo de glucose e da produção de lactato. Tendo em conta que foi descrito o efeito supressor da RGN no metabolismo glicolítico na próstata de rato, este resultado foi algo inesperado e aumenta a curiosidade sobre a ação da RGN ao nível do metabolismos das células de PCa. Globalmente, os resultados obtidos mostraram que a perda da expressão da RGN altera o comportamento das células de próstata humana e promove a agressividade e progressão do PCa, o que suporta o papel da RGN como uma proteína supressora de tumor. Para além disso, estes resultados suportam igualmente a ideia de que a manutenção de níveis elevados de expressão da RGN pode prevenir o processo carcinogénico e retardar a progressão da doença.

# Palavras-chave

Regucalcina;Cancro da Próstata;Proliferação;Sobrevivência;Apoptose;Metastização.

# Abstract

The calcium-binding protein regucalcin (RGN) regulates several biological processes, namely the hallmarks of cancer, such as cell proliferation and apoptosis. Previous work of our research group reported that the loss of RGN expression accompanies the onset and progression of prostate cancer (PCa). However, it remains largely unknown if the decrease of RGN expression is a cause or consequence of PCa. The present thesis aims to investigate the relationship of RGN expression levels with the hallmarks of cancer and PCa patients' outcomes. An *in silico* analysis using the CancerTool[3] software and patients' datasets demonstrated that the loss of RGN correlates with the onset and progression of PCa to metastatic forms of disease. However, no correlation was found between the expression levels of RGN and the histological score of Gleason or PCa recurrence. Moreover, it was found that PCa patients with higher RGN expression levels displayed higher disease-free survival. Bioinformatic analysis of gene-to-gene expression correlation showed that the *RGN* gene expression in primary prostate tumours correlates directly with the expression of *cyclin-dependent kinase inhibitor 1A* (*CDKN1A)* and *IL6* genes. *CDKN1A* gene encodes the cell cycle inhibitor p21, which has been indicated as a target of RGN in rat prostate and human cancers. No other previous study has identified a connection of RGN with IL-6 in cancer cells or tissues. Secondly, we investigated whether the loss of RGN expression may alter human prostate cell fate. Using a siRNA gene silencing approach, *RGN* gene expression in human non-neoplastic PNT1A cells was knockdown (KD), which was confirmed by quantitative real-time PCR. *RGN* gene KD increased the viability and proliferative ability of PNT1A cells as indicated by the MTT and sulforhodamine B assays' results, and the fluorescent immunocytochemistry of the Ki-67 proliferation marker. The reduced caspase-3-like activity found in PNT1A cells KD for RGN demonstrated that the loss of this protein might contribute to apoptosis resistance. Western Blot results showed that the changes in prostate cell fate were accompanied by the altered expression of oncoproteins, such as AKT and its phosphorylated form, as well as c-myc; and also key regulators of the extrinsic pathway of apoptosis, namely, FasR, FasL and caspase-8. *RGN* gene KD did not alter the glycolytic metabolism of PNT1A cells, as indicated by the results of glucose consumption and lactate production. Altogether, the present findings showed that the loss of RGN expression alters the behaviour of human prostate cells and promotes the aggressiveness

[3] Cortazar, A.R., et al., *CANCERTOOL: a visualization and representation interface to exploit cancer datasets*. Cancer research, 2018. **78**(21): p. 6320-6328.

and progression of PCa, which supports the RGN's role as a tumour suppressor protein. Moreover, the obtained results also support the idea that maintaining high RGN expression levels may prevent the prostate carcinogenic process and delay the progression of the disease.

# Keywords

Regucalcin;Prostate Cancer;Proliferation;Survival;Apoptosis;Metastization.

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# List of Publications

Below are listed the scientific outcomes related with the theoretical background or the methodological approach of this thesis.

## Published papers

Cardoso, HJ, Carvalho, TMA, **Fonseca, LRS**, Figueira, MI, Vaz, CV, Socorro, S. Revisiting prostate cancer metabolism: From metabolites to disease and therapy. *Med Res Rev*. 2020; 1– 40. https://doi.org/10.1002/med.21766 (IF: 9.3; Pharmacology & Pharmacy **Q1 D1**; Medicinal Chemistry **Top 5**).

## Published abstracts

Vaz C, Silva GR, Carvalho TMA, Serra C, Brás L**, Fonseca L**, Figueira MI, HJ Cardoso, AP Duarte, S Socorro (2019) Human neoplastic and non-neoplastic prostate cells are sensitive to metabolic (de) regulation induced by endocrine disrupting chemicals and dietary factors *European Urology Supplements* 18 (8), e315

## Book chapters

**Fonseca L.**, Correia S., Vaz C.V., and Socorro S. (2020) The relationship of calcium-binding protein regucalcin with cancer and mammalian reproduction. In: "Advances in Comparative Endocrinology. Vol. X". Association Ibérica de Endocrinologia Comparada (AIEC), in press. (Appendix)

## Oral communications

**Lara R.S. Fonseca**, Henrique J. Cardoso, Cátia V. Vaz, Sílvia Socorro. Does the loss of regucalcin protein contribute to the onset and progression of prostate cancer? XV ANNUAL CICS-UBI SYMPOSIUM 2020, 1 and 2 October 2020, Covilhã, Portugal

## Posters

**Fonseca L.R.S.**, Figueira M. I., Brás L.P., Serra C.M.D., Cavaco E. J., Cardoso H.J., Vaz C.V., **Socorro S.** Downregulated expression of calcium-binding protein regucalcin correlates with prostate cancer development and aggressiveness. *Annual International (bio)Medical Students (AIMS) Meeting*. 18-21 March 2021, Lisbon, Portugal. (for presentation)

Brás LP, Cardoso HJ, Serra CMD, **Fonseca LRS**, Vaz CV, Socorro S The obesogen tributyltin disrupts prostate cancer cells fate. *Annual International (bio)Medical Students (AIMS) Meeting*. 18-21 March 2021, Lisbon, Portugal. (for presentation)

Serra CMD, Brás LP, **Fonseca LRS**, Cardoso HJ, Vaz CV, Socorro S Deregulation of glycolytic and lipid metabolism induced by the obesogen tributyltin in human prostate cells. *Annual International (bio)Medical Students (AIMS) Meeting*. 18-21 March 2021, Lisbon, Portugal. (for presentation)

**Fonseca LRS**, Cardoso HJ, Vaz CV, Socorro S. Loss of regucalcin protein contributes for prostate cancer development and aggressiveness. Cancer Biology: From Basic to Translational Research, 25 September 2020, Lisbon, Portugal.

**Fonseca L**, Alves L, Serra C, Maia CJ, Vaz CV, Socorro S. Regucalcin overexpression prevents aging-associated alterations in rat prostate: oxidative stress, apoptosis and metabolism. III Congress in Health Sciences Research: Towards Innovation and Entrepreneurship Trends in Aging and Cancer, 14-16 November 2019, Covilhã, Portugal

Brás LP, Cardoso HJ, Serra C, **Fonseca L**, Vaz CV, Socorro S. Tributyltin stimulates the glycolytic and lipid metabolism of human neoplastic prostate cells: a missing link between obesity and prostate cancer? III Congress in Health Sciences Research: Towards Innovation and Entrepreneurship Trends in Aging and Cancer, 14-16th November 2019, Covilhã, Portugal

Vaz CV, Silva GR, Carvalho TMA, Serra C, Brás L, **Fonseca L**, Figueira MI, Cardoso HJ, Duarte AP, Socorro S. Human neoplastic and non-neoplastic prostate cells are sensitive to metabolic (de)regulation induced by endocrine disrupting chemicals and dietary factors. 26th Meeting of the EAU Section of Urological Research (ESUR), 10th-12th October 2019, Oporto, Portugal. Abstract published in European Urology Supplements 18 (8), e315

# List of Figures*

* - Figures containing schematic representations of signalling pathways and protein interaction networks made use of cells structure, protein receptors and respective ligands, complex protein structure, organelles and nuclei acids available at https://smart.servier.com/.

# List of Tables

# List of Abbreviations

| | |
|---|---|
| β2M | Beta-2-microglobulin |
| ADT | Androgen-deprivation therapy |
| AP-1 | Activator protein 1 |
| AR | Androgen receptor |
| Bak | Bcl-2 homologous antagonist killer protein |
| Bax | Bcl-2 associated-X protein |
| Bcl-2 | B-cell lymphoma 2 protein |
| Bcl-XL | B-cell lymphoma- extra-large protein |
| Bcl-w | B-cell lymphoma-w protein |
| Bid | BH3 interacting domain death protein |
| BPH | Benign prostatic hyperplasia |
| BRCA1 | Breast cancer predisposition gene 1 |
| BRCA2 | Breast cancer predisposition gene 2 |
| $Ca^{2+}$ | Calcium |
| CaM | Calmodulin |
| CaSR | $Ca^{2+}$-sensing receptor |
| cdc2a | Cell division control protein 2 homolog A |
| CDK | Cyclin-dependent kinase |
| *CDKN1A* | *Cyclin dependent kinase inhibitor 1A* |
| chk2 | Checkpoint kinase 2 |
| CRPC | Castration-resistant prostate cancer |
| CZ | Central Zone |
| DHT | 5α-dihydrotestosterone |
| DR | Death Receptor |
| $E_2$ | 17β-estradiol |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |
| EMT | Epithelial-mesenchymal transition |
| ER | Oestrogen receptor |
| ERK | Extracellular signal-regulated kinase |
| FasL | Fas-ligand |
| FasR | Fas receptor |
| FBS | Fetal bovine serum |
| GLUT | Glucose Transporter |
| GNL | Gluconolactonase |
| GS | Gleason score |
| GSK-3β | Glycogen synthase kinase 3 β |
| GST | Glutathione-S-transferase |
| HER2 | Human epidermal growth factor receptor-type 2 |
| IL | Interleukin |
| IGF | Insulin-like growth factor |
| KD | Knockdown |
| LDH | Lactate dehydrogenase |
| LPS | Lipopolysaccharide |

| | |
|---|---|
| MAPK | Mitogen activated protein kinase |
| MCU | Mitochondrial $Ca^{2+}$ uniporter |
| MMP | Matrix metalloproteinase |
| MTT | 3-(4,5-dimethylthiazol-2- thiazolyl)-2,5-diphenyltetrazolium bromide |
| NF1-A1 | Nuclear factor1-A1 |
| *NFKB1* | *Nuclear factor kappa B subunit 1* |
| NF-kβ | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NLS | Nuclear localization signal |
| NOS | Nitric oxide synthase |
| Nuclear SERCA pump | Nuclear outer membrane sarco/endoplasmic reticulum $Ca^{2+}$-ATPase pump |
| OS | Oxidative stress |
| OXPHOS | Oxidative phosphorylation |
| P/S | Penicillin/streptomycin |
| PBS | Phosphate buffer saline |
| PBS-T | Phosphate buffer saline containing 0.1% (w/v) Tween-20 |
| PCa | Prostate cancer |
| PFK | Phosphofructokinase |
| PI3K | Phosphoinositide 3-kinase |
| PIN | Prostatic intraepithelial neoplasia |
| PKB | Protein kinase B |
| PKC | Protein kinase C |
| PMCA pump | Plasma membrane $Ca^{2+}$-ATPase pump |
| pNA | p-nitro-aniline |
| PSA | Prostate-specific antigen |
| PTEN | Phosphatase and tensin homolog |
| PTH | Parathyroid hormone |
| PZ | Peripheral zone |
| qPCR | Real-time polymerase chain reaction |
| RGN | Regucalcin |
| RGPR-p117 | *RGN* gene promoter region-related protein p117 |
| RT | Room temperature |
| RTK | Receptor tyrosine kinase |
| SAPK/JNK | Stress-activated protein kinases/Jun amino-terminal kinases |
| SCF | Stem cell factor |
| SERCA pump | Sarco/endoplasmic reticulum $Ca^{2+}$-ATPase pump |
| siRNA | Small interfering RNA |
| si-RGN | Small interfering RNA targeting regucalcin |
| si-scramble | Scramble siRNA |
| SMP30 | Senescence Marker Protein 30 |
| SRB | Sulforhodamine B |
| STAT | Signal transducer and activator of transcription |
| t-BHP | Tert-Butyl hydroperoxide |
| TCA | Tricarboxylic acid |
| TF | Transcription factor |
| TGF | Transforming growth factor |
| TNF | Tumour necrosis factor |

| | |
|---|---|
| TNFR | Tumour necrosis factor receptor |
| *TP53* | *Tumour protein P53* |
| TZ | Transition zone |
| WB | Western Blot |
| Wnt | Wingless-related integration site |

# I. Introduction

# 1. Brief overview of prostate anatomy and physiology

The prostate gland is a walnut-sized organ, weighing about 15-20 g [1]. This organ is the largest male accessory gland and is located in the subperitoneal compartment between the pelvic diaphragm and the peritoneal cavity [1-3]. Prostate is located below the urinary bladder, surrounding the prostatic urethra and the ejaculatory ducts (Figure I.1) [1].

The human prostate is an extremely slow-growing organ and its development begins with the growth of prostatic buds from the urogenital sinus at about 10 weeks of fetal development [4]. This male organ is small in childhood, weighing approximately 2 g. At puberty, with the increase of serum testosterone levels, prostate undergoes a phase of exponential growth, and its weight increases about 20 g. The prostate weight stabilizes and remains constant until the men has the age of 30, then prostatic weight begins to rise slowly. This rise may reflect the onset of benign prostatic hyperplasia (BPH), which mainly affects men above 50 years old [4-6].

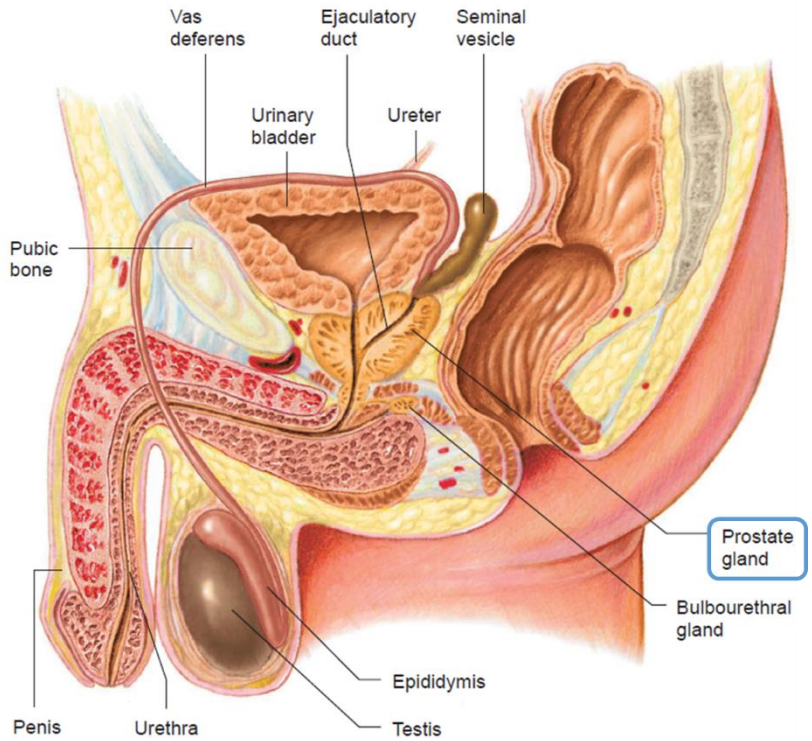


**Figure I.1.** Localization of the prostate gland in the male reproductive system and its relationship with urinary bladder. The prostate gland surrounds the bladder neck, the first portion of the urethra and the ejaculatory ducts [7].

According to McNeal's concept of zonal anatomy, prostate can be divided in three histological different zones (Figure I.2): the peripheral zone (PZ), the transition zone (TZ) and the central zone (CZ). The three zones display different embryologic origins, anatomic landmarks, biological functions, and susceptibility to pathologic disorders, like cancer [2]. The main characteristics of the three prostate zones are summarized in Table I.1.

The PZ represents 70% of the glandular tissue of the adult prostate and is the origin of the majority of prostate cancer (PCa) cases. This zone is derived from the urogenital sinus and

surrounds most of the CZ (Figure I.2), extending caudally to partly surround the distal portion of the urethra [2, 8]. The TZ is located near the prostatic urethra and displays the same embryologic origin of PZ. However, the percentage of PCa arising from this zone is much lower, which may be due to the differences in the stromal components of these two areas (Table I.1). Moreover, it is postulated that BPH, predominantly arises in the TZ [2]. Furthermore, the CZ is a cone-shaped region, with the wider portion at the base of the prostate and the apex at the verumontanum surrounding the ejaculatory ducts. This zone, displays a very low incidence of PCa and is derived from Wolffian ducts (Table I.1) [1, 2, 8]. Histologically, the human prostate is formed by stromal elements and glandular epithelium (Figure I.2), tightly fuse inside a pseudocapsule, which consists in a thin fibroelastic tissue layer [1, 2, 5]. The pseudocapsule gives to the prostate an unlobulated appearance. Moreover, the fibroelastic capsule forms a septa that subdivide the prostate into five lobes: an anterior, a posterior, a medial and two laterals [5], allowing this alternative anatomical classification.

The prostatic stroma is fibromuscular, being composed by abundant smooth muscle cells mixed with fibroblast, blood vessels and nerves. The prostate glandular epithelium is composed by acini and ducts lined by four types of cells (Figure I.2), luminal, basal, stem and neuroendocrine [1, 9]. The luminal cells are columnar epithelial cells specialized in the secretion of a variety of products, like prostate-specific antigen (PSA) and citrate, into the lumen that contribute to the formation of the seminal fluid. This type of cells is the most abundant in the prostate glandular epithelial and are the main cells in the PZ. The basal cells are non-secretory cells located adjacent to the basement membrane essential in the maintenance of cell-growth [1, 3, 10]. The neuroendocrine cells are the less abundant cells and secrete neuropeptides and other hormones that promotes the growth of prostate. This cells often display a dendritic-like process that contacts with the glandular lumen [3].

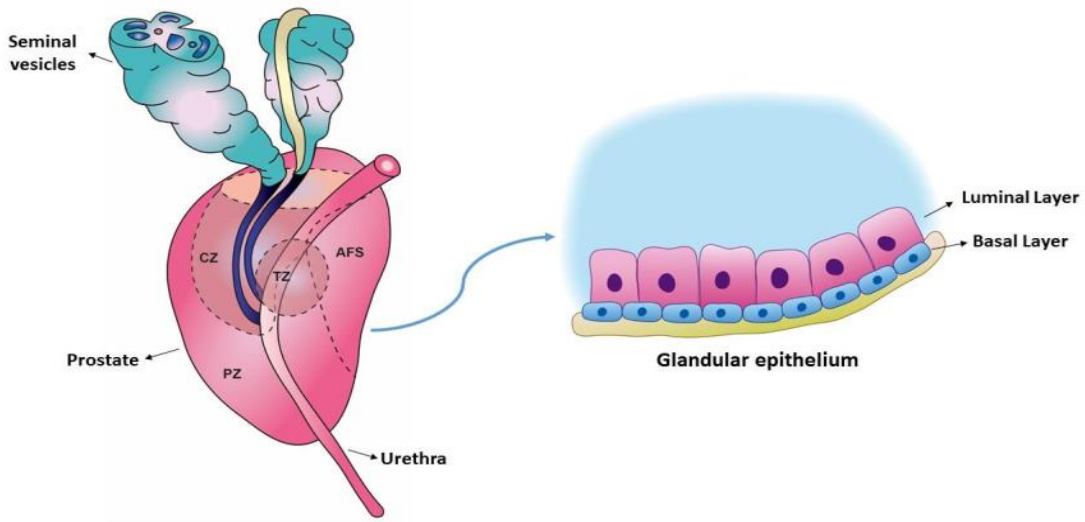


**Figure I.2.** Representation of the zonal anatomy of prostate gland and organization of the glandular epithelium. According to the zonal anatomy concept, the prostate can be divided in three zones, the transition zone (TZ), the central zone (CZ) and the peripheral zone (PZ), which has the more incidence of prostate cancer (PCa). At histological level, two distinct histological epithelial layers can be observed in the prostate glandular tissue, the luminal layer and the basal layer. The luminal layer is constituted by columnar cells responsible for the production of prostatic secretions. The basal layer is constituted by basal cells that are involved in the maintenance of cell growth. Additionally, in the basal layer exist a stem cell like reservoir, these cells differentiate into columnar secretory cells. Located among the epithelial cells also exist neuroendocrine cells (not showed), that are involved in the regulation of secretory activity and cell growth of prostate [11].

**Table I.1.** Main characteristics of three human prostate zones [2].

| | **Central zone** | **Transition zone** | **Peripheral zone** |
|---|---|---|---|
| Volume of normal prostate (%) | ≈ 25 | ≈ 5 | ≈ 70 |
| Proposed embryonic origin | Wolffian duct | Urogenital sinus | Urogenital sinus |
| Epithelium | Complex, large polygonal glands | Simple, small rounded glands | Simple, small rounded glands |
| Stroma | Compact | Compact | Loose |
| Origin of prostatic adenocarcinoma (%) | ≈ 5 | ≈ 25 | ≈ 70 |
| Benign prostatic hyperplasia (%) | - | ≈ 100 | - |

The main function of prostate is the production of the prostatic fluid that represents over 30% of the semen volume [12]. The prostatic fluid consists on a complex mixture of components that facilitates and enhances sperm motility and is fundamental to sperm viability, protecting sperm cells during their cross through the female tract [13, 14]. Moreover, the prostatic secretions contain several factors that control ejaculation, regulate the processes of semen clotting and liquefaction, sperm activation and capacitation and nutrition of spermatozoa [13]. Moreover, the prostatic secretion also induces several changes in the cervix, in the endometrium and in the maternal immune system that contribute to successful implantation and embryo growth [5, 13, 14]. Additionally, prostate also has an important role in the control of urine output from the bladder, through its mass and musculature, affecting prostatic urethra (Figure I.1). Furthermore, this gland also helps

in the rapid metabolism of testosterone, by its conversion to 5α-dihydrotestosterone (DHT), influencing the hypothalamic and pituitary functions [5].

# 2. Notes on prostate cancer

## 2.1. Aetiology and development of prostate cancer

PCa is the second most common cancer worldwide, and one of the major causes of cancer-related mortality [15]. World Health Organization data indicate that in Portugal, and excluding non-melanoma skin cancer, PCa was the most frequent cancer in men until December 2020 [16]. In 2018, PCa was the 3rd cause of cancer-related mortality [17]. However, most recent data reported that until December 2020, this human neoplasia was the 4th cause of cancer death [16]. These statistic data support that the mortality rate of PCa is decreasing in Portugal and that this disease is not one of the deadliest cancers [17, 18].

Epidemiologic evidence links factors, such as aging, family history, race, ethnicity, diet and lifestyle with the development of PCa [19, 20]. Aging is one of the major causes for the development of PCa [21]. This human neoplasia is more common above 50 years of age [20] and displays a peak of incidence in older men, at approximately 70 years [21].

Despite all the advances made in analysing the genetic basis of PCa, the ability to define syndromes of hereditary PCa and identify hereditary PCa genes has been limited [22]. However, PCa pedigrees studies suggest dominant, recessive and X-linked models of PCa development [23, 24]. Moreover, it is widely demonstrated that first-degree relatives of men with PCa have a risk of developing the disease twice than that of the general population [22, 23]. Also, a recognized family association exists between the development of breast cancer and PCa [25]. It was demonstrated that first or second male relatives of women with breast cancer display a higher risk of PCa development [26]. Moreover, inherited mutations in the breast cancer predisposition gene 2 (BRCA2) and breast cancer predisposition gene 1 (BRCA1) have been associated with an increased risk of PCa development [25, 27, 28]. However, BRCA2 mutations further increase the risk of PCa development compared to BRCA1 mutations [25, 28], and were associated with poor clinical outcomes [29, 30]. More studies are needed to definitely confirm the use of BRCA2 as an independent prognosis biomarker.

Concerning diet, and despite some inconsistency in epidemiological evidence, an association has been found between the consumption of red, smoked and seasoned meat and a higher risk of PCa and associated mortality [20, 31-34]. An unbalanced diet, with high-calorie intake, and rich in saturated animal fat, has been shown to increase testosterone and insulin-like growth factors (IGFs) levels, and thus, basal metabolism, cell proliferation and tumour growth [20, 35, 36]. Accordingly, obesity, one of the main chronic diseases of XXI century [37], has been associated with the development of PCa and aggressiveness of disease. The accumulation of adipose tissue induces the deregulation of

the IGF-1 axis, alterations in the secretion of sex hormones and an increase of oxidative stress (OS) promoting tumour progression [38]. Furthermore, lipid metabolism generates free radicals and leukotrienes and prostaglandins, which are pro-inflammatory factors that contribute to the development of PCa [20, 39]. Also, the consumption of milk and dairy products, because of the saturated animal fat and calcium ($Ca^{2+}$) content, has been associated with the proliferation of PCa cells [20, 23, 40, 41].

Lifestyle and habits, such as smoking and the level of physical exercise also play an important role in the development of PCa [42, 43]. Specifically, it was demonstrated that a sedentary lifestyle increases the risk of PCa development [20, 44]. On the contrary, moderate to vigorous exercise reduces PCa-specific mortality and significantly increases the quality of life of PCa patients [45-47]. Besides, other factors, such as vasectomy, infection and sexually transmitted diseases, may augment the probability of the developing PCa [20].

The incidence of PCa largely depends on race, ethnicity and geographic context. West African men descent from the Caribbean or South America and African American men display higher incidence and mortality of PCa compared to white man [24, 48-50]. Contrastingly, South Central Asian men have the lowest incidence of PCa [24, 49, 50]. The reasons for this geographic contrast are not totally understood, but it is accepted that these findings can be explained by the fact that the development of PCa depends on the interaction between environmental factors and genes [20]. Also, habits and the type of diet have been shown to contribute to the discrepancies among geographic regions. The low incidence of PCa in South Central Asian men is explained by the low consumption of fat and meat and the high content of plants and soy, specifically phytoestrogens, in the Asian diet [51, 52]. Therefore, it is possible to affirm that the distinct incidence of PCa attributed to race, ethnicity and geography, may also be a consequence of cultural habits and lifestyle.

Considering the onset of PCa, there is a good agreement that this cancer develops from prostate epithelial cells [53]. However, conflicting evidence exists regarding if the tumour arises from basal or luminal epithelial cells [54-56]. The current models of prostate carcinogenesis strongly defend the existence of an “activated” pre-malignant state [58]. This model assumes that tissue damage, likely by inflammation due to infection or prostatitis (reviewed by [57]), induces the development of a pre-neoplastic state called prostatic intraepithelial neoplasia (PIN) (Figure I.3) [58]. The PIN lesions can develop to high-grade PIN progressing to invasive prostate adenocarcinoma [58].

Other important feature of PCa is its androgen dependency in early stages of disease, the so-called androgen-sensitive PCa. This allows the use of androgen-deprivation therapy (ADT) to counteract tumour growth and treat PCa [58]. However, with the continuous tumour development PCa cells became androgen-insensitive, and the disease progresses to the castration-resistant PCa (CRPC) or hormone refractory PCa [59, 60].

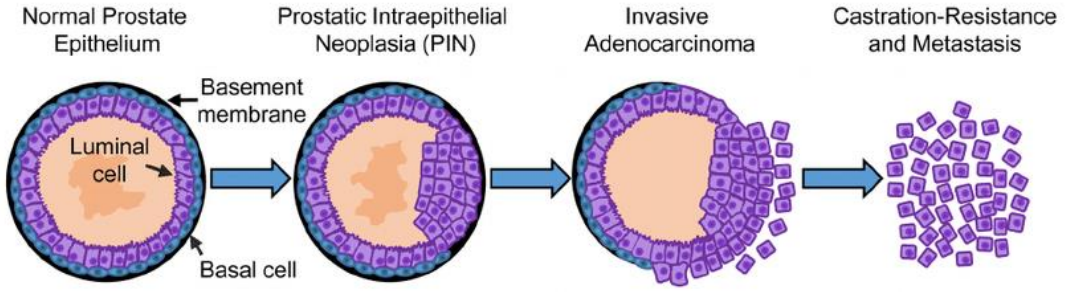


**Figure I.3.** Model of the development of prostate cancer (PCa). Damage in the prostate epithelium induces the development of pre-neoplastic lesions called prostatic intraepithelial neoplasia (PIN). This stage progress to high-grade PIN, prostate adenocarcinoma and invasive adenocarcinoma. PCa becomes gradually more aggressive with the subsequent development of castration-resistant prostate cancer and metastization [60].

The mechanisms that drive the progression androgen-sensitive to CRPC are not totally understood. However, it is thought that androgen receptor (AR) signalling plays a central role in this process. In fact, the possible mechanisms underlying the establishment of CRPC can be divided in AR-independent and -dependent. AR independent mechanisms include alterations in steroid metabolism, coactivator expression/activity and activation of alternative intracellular signalling pathways [58, 61]. The AR-dependent mechanisms include AR mutations, over-expression/amplification of AR and occurrence of AR splice variants [58, 61, 62]. Currently, five types of potential mechanisms explain the emergence of CRPC as proposed by [61]:

**Type 1. Hypersensitive pathway:** PCa overpass the effects of ADT by the increased sensitivity to very low levels of androgens, thus continuing to be dependent on the AR signalling [58, 61, 62], or by the intraprostatic androgens biosynthesis (Figure I.4A**)** [63, 64]. The mechanisms linked with the increased sensitivity to androgens include the amplification of the AR, which is one of the most common gene alterations found in CRPC [58, 62], allowing the sufficient ligand-receptor interactions to develop a response even in the presence of low levels of androgens [65, 66]. The increased sensitivity of AR also results from the high expression levels of AR, increased protein stability and enhanced nuclear localization of receptor [58, 61]. Recurrent tumour cells, display intracrine androgen biosynthesis, converting adrenal androgen precursors and cholesterol into testosterone and DHT in PCa cells [63, 64]. PCa cells not only contain all the components necessary and the key enzymes to androgens biosynthesis, but also, these enzymes, usually, are overexpressed in recurrent or metastatic tumours [67, 68].

**Type 2. Promiscuous pathway:** ADT obligates PCa cells to adapt to the low concentration of androgens, which has been related with the acquisition of AR mutations that lead to an aberrant activation of the androgen signalling [58, 69]. The genetic changes that usually happen in the *AR* gene are missense mutations, that decrease the specificity of the ligand-binding domain for testosterone and DHT, allowing the inappropriate activation of AR by non-androgen steroids (oestrogens and progesterone) and also anti-androgens [58, 70]. Besides these somatic mutations, spliced variants of AR have been described. Many alternative spliced variants of AR lack the C-terminal ligand-binding domain but retain the transactivating N-terminal domain, leading to the constitutive activation of AR (Figure I.4B**)** [62, 70, 71].

**Type 3. Outlaw pathway:** The AR can also be activated in a ligand-independent way. Several studies showed that various growth factors, cytokines, kinases and other proteins interact and activate the cytosolic AR in a ligand-independent manner (Figure I.4C**)** [61, 62, 70]. IGF-1 is the most potent factor that activates AR signalling in the absence of androgens [72]. In addition, it has also been shown that IGF-1 potentiates AR signalling in an indirect way because it induces the expression of transcriptional intermediary factor-2, a co-activator of AR [73]. Furthermore, studies demonstrated that the human epidermal growth factor receptor-type 2 (HER2), a receptor tyrosine kinase (RTK) overexpressed in CRPC, can directly activate AR signalling in PCa cells [74, 75]. On the contrary to IGF-1, interleukin (IL)-6 and IL-8, the action of HER2 in the presence of antiandrogens is not interrupted, what indicates that the action of this receptor in AR signalling is independent of the ligand binding domain of AR [70]. Other RTK, like IGF and epidermal growth factor (EGF) receptors activate essential downstream survival pathways like protein kinase B (PKB)/AKT, mitogen activated protein kinase (MAPK) and signal transducer and activator of transcription (STAT), which are dysregulated in PCa and induce the progression to CRPC [70, 76].

**Type 4. Bypass pathway:** The AR-independent mechanisms also play an important role in the development of CRPC [61, 70]. Other signalling pathways allow the progression of PCa (Figure I.4D**)**. For example, the IGF-1 signalling pathway induces the expression of genes that are able to promote the progression of PCa [70, 77]. Furthermore, it has been found that B-cell lymphoma 2 (Bcl-2) protein is overexpressed in many cases of CRPC [78, 79]. Moreover, was also demonstrated that when the expression of Bcl-2 is blocked in human PCa LNCaP cells xenograft mice model, the emergence of CRPC is delayed [80]. In addition, other studies showed that the glucocorticoid receptor can replace AR signalling when it is blocked, and induce the progression of PCa [62, 81].

**Type 5. The Lurker cell pathway:** Isaacs J. T. *et al* demonstrated that the progression to CRPC may be due to a subpopulation of androgen-independent tumour cells that already exist before the administration of ADT (Figure I.4E**)** [82]. According to this model, the

androgen-independent epithelial stem cell within the basal cells' population, transform and became the origin of PCa. In the presence of androgens, most of the epithelial stem cells progeny differentiate into androgen-dependent epithelial cancer cells. This cancer cells that form most of the tumour are destroyed by ADT. However, androgen-independent malignant epithelial stem cells continue to proliferate and originate CRPC [82]. Craft *et al.* showed that the latter stage of androgen independence results from clonal expansion of androgen-independent cells [83]. This finding suggested that PCa contains a heterogeneous mixture of cells that vary in their dependence on androgens, supporting this hypothesis.

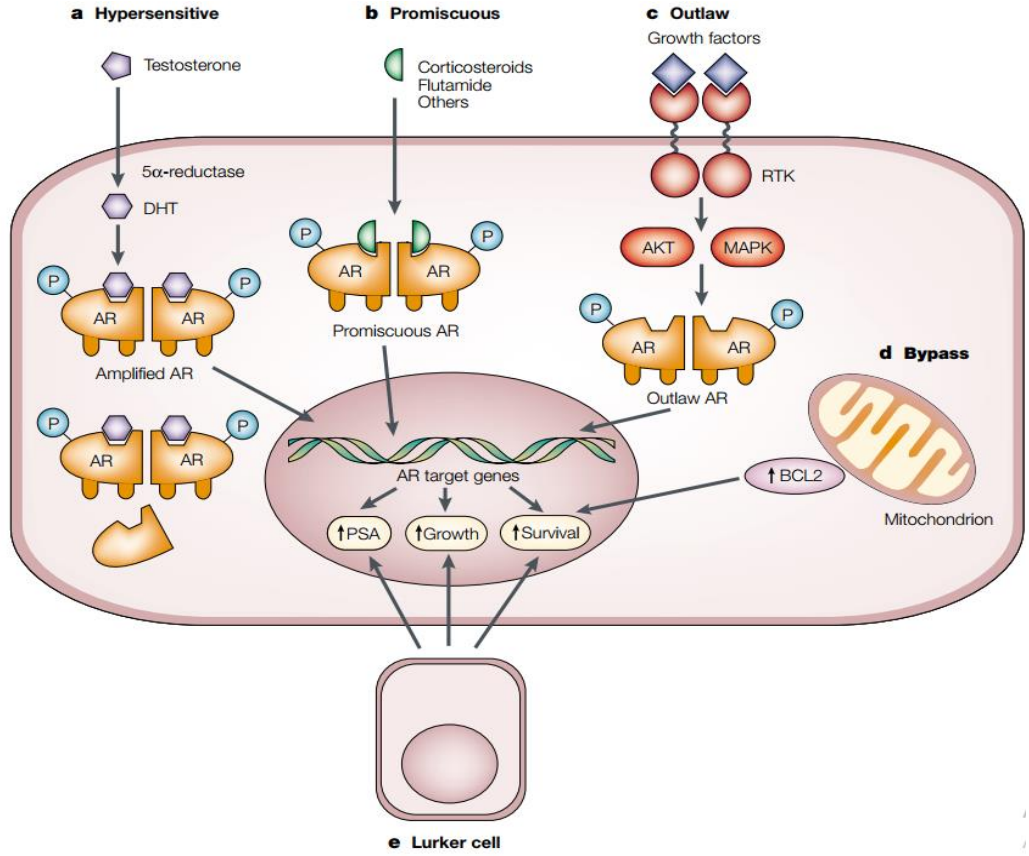


**Figure I.4.**Five possible pathways underpinning the development of castration-resistant prostate cancer [61]. **a.** In the hypersensitive pathway, the enhanced sensitivity to androgens, for example, by the amplification of the androgen receptor (AR), compensates the low levels of androgens maintaining cancer cell growth. In addition, the intraprostatic production of 5α-dihydrotestosterone (DHT) is increased in consequence of 5α-reductase overexpression. **b.** In the promiscuous pathway the specificity of the AR to androgens is decreased, and it can be activated by other type of ligands, such as oestrogens, progesterone and anti-androgens. **c.** In the outlaw pathway, receptors tyrosine kinase (RTK) are overactivated and, through the AKT or the mitogen-activated protein kinase (MAPK) pathway, activate the AR by phosphorylation. **d.** In the bypass pathway, the activation of parallel survival pathways, such as the pathways that involve the B-cell lymphoma 2 (Bcl-2) protein, remove the need for AR or its ligand. **e.** In the lurker pathway, a minor sub-population of androgen-independent cells present in prostate tumours are selected by the androgen deprivation therapy (ADT). Legend: P, Phosphorylation.

## 2.2. Prostate cancer cells growth and proliferation

PCa is a multifactorial disease, being its progression, aggressiveness and development induced and controlled by several factors. As mentioned above, androgens play a central role in the control of PCa cells' proliferation and growth. Androgens are the main regulators of the proliferation/apoptosis ratio, stimulating proliferation and inhibiting apoptosis of

prostate cells, and, thus, inducing the development of PCa [58, 61, 84, 85]. Testosterone is secreted by testes and produced by peripheral conversion of adrenal steroid hormones [86, 87]. Although most of the testosterone circulates in the blood bound to albumin and sex hormone binding globulin, a small fraction circulates freely in the serum and enters to prostate cells. After entering the cells, testosterone is mostly converted into DHT, a more active androgen [88-91]. DHT binds to the AR, induces its activation and translocation to the nucleus. The complex DHT/AR acts as a transcription factor (TF) inducing the expression of proliferation inducer genes, such as *cyclin-dependent kinase (CDK) 2* and *CDK4* [92], and apoptosis inhibitors, namely inhibitors of caspase activation (Figure I.5) [61, 93, 94].

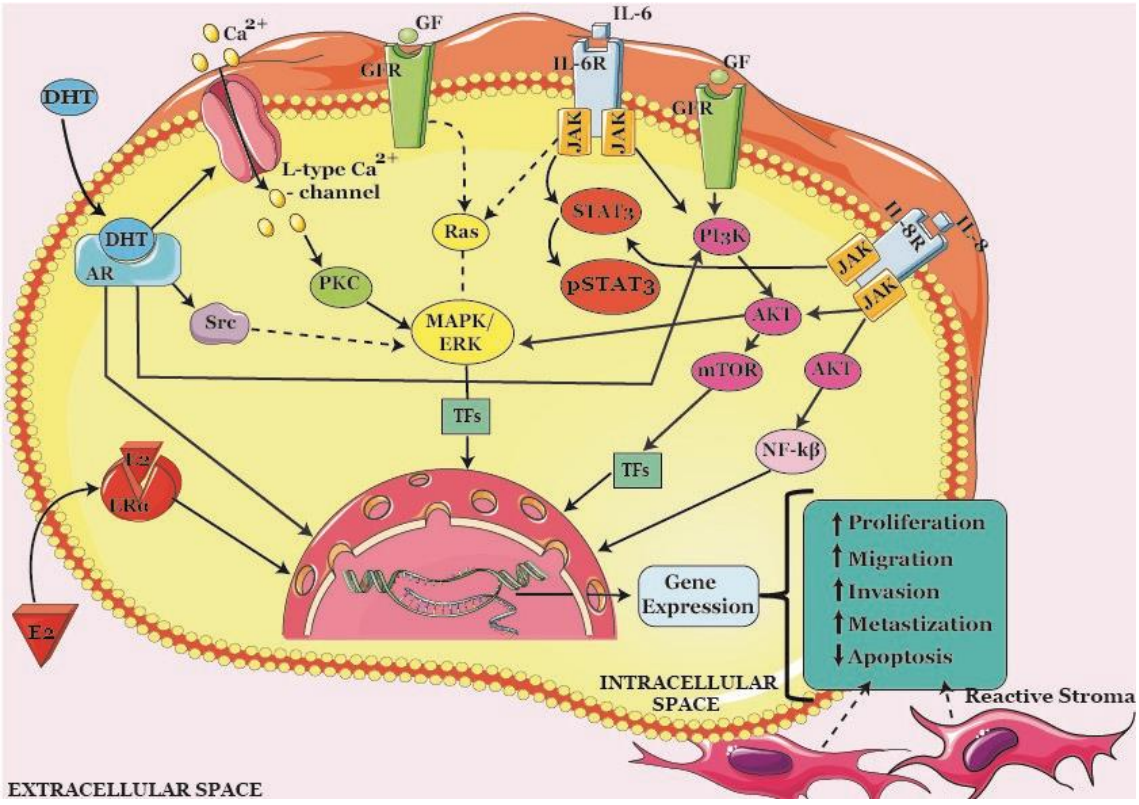


**Figure I.5.** Schematic representation of the major survival and growth signalling pathways activated in prostate cancer (PCa). Androgen receptor (AR) signalling induces the progression of PCa through the activation of genomic and non-genomic pathways. After binding 5α-dihydrotestosterone (DHT), the AR undergoes a conformational change and translocates to the nucleus, where induces the expression of genes that favour cell survival and growth. The non-genomic signalling involves the increase of intracellular calcium ($Ca^{2+}$) and the consequent activation of protein kinase C (PKC). Furthermore, the AR-non genomic signalling activates the Src protein, activating the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway. Moreover, the AR through phosphoinositide 3-kinase (PI3K) phosphorylation activates the PI3K/AKT pathway. Also, growth factors (GF) activate the MAPK and PI3K pathways, which activate transcription factors (TFs) that translocate to the nucleus inducing the expression of pro-proliferation and anti-apoptotic genes. Interleukin-6 (IL-6) increases the expression of genes that promote the progression of PCa by activating the signal transducer and activator of transcription 3 (STAT3), MAPK and AKT pathways. Furthermore, interleukin-8 (IL-8) activates the STAT3, AKT and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kβ) pathways. The oestrogen receptor α (ERα), after binding 17β-estradiol ($E_2$) undergoes a conformational change and translocates to the nucleus, acting as a TF with a role in the progression of PCa. The activation of all these signalling pathways inhibits apoptosis and induce the proliferation, invasion, and migration of PCa cells, being also implicated in tumour metastization. The reactive stroma through factors released to the tumour microenvironment also inhibits apoptosis and promotes PCa cells' proliferation, invasion, migration and the metastatic process. Legend: GRFR, Growth Factor Receptor; IL-6R, Interleukin-6 Receptor; IL-8R, Interleukin-8 Receptor; JAK, Janus Kinase; mTOR, rapamycin. Head-arrows black lines mean protein activation. Head-arrows dashed black lines mean multiple activation steps not represented.

Besides the classical AR genomic signalling, also non-genomic actions of the AR have been reported. A feature of non-genomic signalling is its fast and reversible responses, which in the case of AR encompasses the capability of regulating and modify PCa cells' proliferation [95, 96]. Nevertheless, rapid effect mediated by changes in intracellular $Ca^{2+}$ concentration and phosphorylation events can cross-talk with the regulation of gene transcription (Figure I.5). Studies performed in LNCaP cells demonstrated that androgens stimulate the activity of L-type $Ca^{2+}$ channels increasing the intracellular $Ca^{2+}$ levels [97] with the activation of protein kinase C (PKC), which in turn activates the MAPK/ extracellular signal-regulated kinase (ERK) pathway, inducing PCa cells proliferation (Figure I.5) [84, 98].

DHT treatment has been shown to increase the levels of phosphorylated ERK 1/2 [96], which translocates to the nucleus, where directly interacts and phosphorylates TFs that upon activated control the expression of proliferation-related genes (e.g. c-fos) [99-102]. This DHT-induced ERK activation is also mediated by the phosphoinositide 3-kinase (PI3K) /AKT/ phosphatase and tensin homolog (PTEN), Src, and Ras-Raf pathways (Figure I.5) [95]. Importantly, this response was not observed in the AR-negative PC3 human PCa cells, corroborating the androgen dependence of this pathway [96].

The PI3K/AKT/PTEN signalling pathway is frequently activated in PCa cases as a consequence of PTEN loss [58, 103-105]. Moreover, the AR directly activates the PI3K/AKT pathway through the interaction with the PI3K p85α regulatory subunit [106]. Once activated, PI3K activates the serine-threonine enzyme, PKB/AKT, regulating several downstream targets, namely, the mechanistic target of rapamycin, forkhead box protein O1 and the MAPK/ERK cascade. These targets activate TFs, such as c-myc, that induce the expression of proteins associated with cell survival and proliferation, cell cycle progression, migration and angiogenesis, and, thus, contributing to the progression of PCa [95, 107-109]. Indeed, c-myc has been shown to be overexpressed at the early stages of disease [109]. In non-tumour conditions c-myc is a short lived protein, which is degraded by the ubiquitin-proteasome pathway after phosphorylation by protein kinases, such as, glycogen synthase kinase 3 β (GSK-3β) [110, 111]. Available studies also reported that inflammatory cytokines, EGF receptor and HER2 families can enhance the development and progression of PCa through the PI3K/AKT pathway (Figure I.5) [107, 112].

The Src/Raf/ERks pathway is also activated by the AR signalling. Magliaccio et al. demonstrated that AR activation triggers the entry of LNCaP cells into the cell cycle S-phase through the activation of the Src/Raf/ERK pathway [113]. After ligand-induced activation, AR activates Src by relieving of its autoinhibition [95]. In turn, activated Src activates Shc, an upstream regulator of the MAPK pathway, leading to the activation of this signalling pathway [95, 114]. Furthermore, studies reported that the inhibition of the Src/MAPK pathway decreased the levels of DHT-induced ERK-1/2 phosphorylation [95]. Src, through the MAPK pathway, activated also the expression of receptors, such as IGF-1 receptor,

which activates the PI3K/AKT pathway, contributing to PCa cells proliferation, migration and angiogenesis (Figure I.5) [115].

Besides the unquestionable role of androgens as promoters of PCa, other steroid hormones and proteins factors can influence the survival and proliferation of prostate cells. A substantial amount of evidence showed that oestrogens also can induce prostate cells' proliferation and progression of PCa [93, 116-118]. In men, testosterone secretion and plasma levels decline with aging [119, 120], whereas oestrogens levels increase modestly, what results in the increased oestrogen/testosterone ratio [93, 121]. Furthermore, African-American men, with higher incidence of PCa, display higher plasma-free oestrogens levels [122]. Moreover, administration of 17β-estradiol ($E_2$) with low doses of testosterone increased the incidence of PCa to nearly 100% in noble rats [93, 116, 117]. Based on these findings, it has been proposed that oestrogens display an important role in the establishment of PCa.

Oestrogen receptors (ER), the mediators of oestrogens actions, are present in the prostate gland, being ERα mostly expressed in the mesenchymal cells and the ERβ in the epithelial cells [93, 117]. Moreover, a dual role for ERα and ERβ has been proposed. Several studies have demonstrated that ERβ exhibits a protective role against the development of PCa. ERβ expression is partly lost during carcinogenesis, and is the mediator of anti-proliferative and pro-apoptotic oestrogenic actions, being suggested as a tumour suppressor [116, 123]. Concerning ERα, studies showed that during the prostatic malignant transformation, the expression of this receptor extends from basal to luminal cells. Moreover, it was demonstrated that ERα induces the expression of several genes that trigger the progression of PCa (Figure I.5) [116-118]. Also, $E_2$ administration was shown to induce, through the ERα pathway, chronic inflammation in mouse prostate, an etiological factor involved in the development and progression of PCa [116, 117]. Based on these findings, and contrastingly with ERβ, ERα was proposed to act as an oncogene inducing the progression of PCa [116-118].

As mentioned above, inflammation contributes to development, progression, aggressiveness and invasiveness of PCa [124]. Inflammation regulates the tumour microenvironment through the altered balance of cytokines, chemokines, TFs and reactive oxygen species [57, 124-127]. Both IL-8 and IL-6 have been pointed out to influence the proliferation of PCa cells [126]. IL-6 is the cytokine extensively studied in PCa and considered one of the major regulators of progression of disease [124]. The first indication that IL-6 is involved in PCa biology was that patients with CRPC presented elevated serum levels of IL-6 when compared with healthy men or patients with localized PCa [128, 129]. Also, PCa patients with localized PCa that underwent radical prostatectomy and developed bone metastasis displayed higher levels of IL-6 [130]. Moreover, it was demonstrated that the expression of IL-6 in PCa epithelial cells increases with the progression of PCa [124].

Furthermore, Giri *et al.* reported that the increased expression of IL-6 receptor in an *in vivo* model correlates with a higher proliferation of PCa cells [131]. Concerning the mechanistic involved, studies in LNCaP cells demonstrated that IL-6 induces growth through the induction of STAT3 and MAPK signalling pathways [131-133]. It was also demonstrated that IL-6 induced survival and angiogenesis with the activation of the PI3k/AKT pathway [133, 134]. Moreover, this effect is also a consequence of the increased expression of vascular endothelial growth factor and epithelial-mesenchymal transition (EMT) molecules induced by the activation STAT3 pathway [134, 135]. In addition, through the STAT3 pathway, IL-6 also induced the expression of IGF-1, inhibiting apoptosis (Figure I.5) [136]. IL-8 also promoted cell proliferation, migration, and invasion and inhibited apoptosis of PCa cells (Figure I.5) [137-139], which occurred through the activation of the STAT3 and AKT pathways [140]. It was also demonstrated that IL-8 activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kβ) pathway dependently on the AKT pathway (Figure I.5) [140].

Similarly to other cancers, growth factors and peptides are also important proliferation inducers in PCa. PCa stromal cells and malignant epithelial cells express EGF and transforming growth factor (TGF)-α. These two growth factors transmit their message to other PCa cells, through EGF receptors, inducing the autonomous growth of human PCa. Also, EGF stimulates the invasiveness of PCa through the promotion of the chemomigration of tumours cells [141, 142].

The PCa stroma also plays a relevant role inducing growth and proliferation of PCa cells. PCa stroma is composed by smooth muscle cells and in less abundance fibroblasts, vascular, nerve and immune cells and extracellular matrix (ECM) [141, 143]. In prostate carcinoma, the phenotypic histological change in the stroma induces alterations in the tumour microenvironment, it is the so-called the reactive stroma. This phenotypic histological change leads to a loss of well-differentiated smooth muscle cells, increase of fibroblast population and increase of secretion and deposition of ECM components, such as matrix metalloproteinase (MMP). All these changes can lead to epithelial cell depolarization and formation of conduits for tumour cell migration [144-146]. Another study with ECM isolated from prostate reactive stroma and LNCaP cells demonstrated that ECM promotes proliferation, survival, motility and induce the expression of MMPs, the proteins involved in the degradation of ECM (Figure I.5) [147].

## 2.3. Activation of apoptotic cell death in prostate cancer cells

Apoptosis is the highly regulated process of cell death that plays a critical role in normal development and tissue homeostasis through the elimination of unnecessary and unwanted cells [148]. A correct apoptotic signalling is also essential to the maintenance of a healthy

cell population, being activated, for example, in response to DNA damage, and, thus, protecting the genome's integrity [148, 149].

Apoptosis can be triggered by the intrinsic and extrinsic signalling pathways (Figure I.6), which converge at activation of caspase-3, the endpoint of apoptosis [150]. However, the intrinsic and extrinsic pathways are activated by different factors. The intrinsic pathway usually is activated by the loss of growth factor signals or in response to intracellular damage, for example DNA damage or OS, and depends on the release of cytochrome-c from the mitochondria [148, 149, 151]. The extrinsic pathway is initiated when transmembrane death receptors (DR) , such as Fas receptor (FasR) or tumour necrosis factor (TNF) receptor (TNFR) bind to their specific ligands [152]. Independently of the activated pathway, apoptosis is characterized by cell shrinkage, dynamic membrane blebbing, loss of adhesion to ECM, chromatin condensation and DNA fragmentation [148, 149]. In addition, a set of biochemical alterations also occur, namely, externalization of phosphatidylserine and activation of caspases [148].

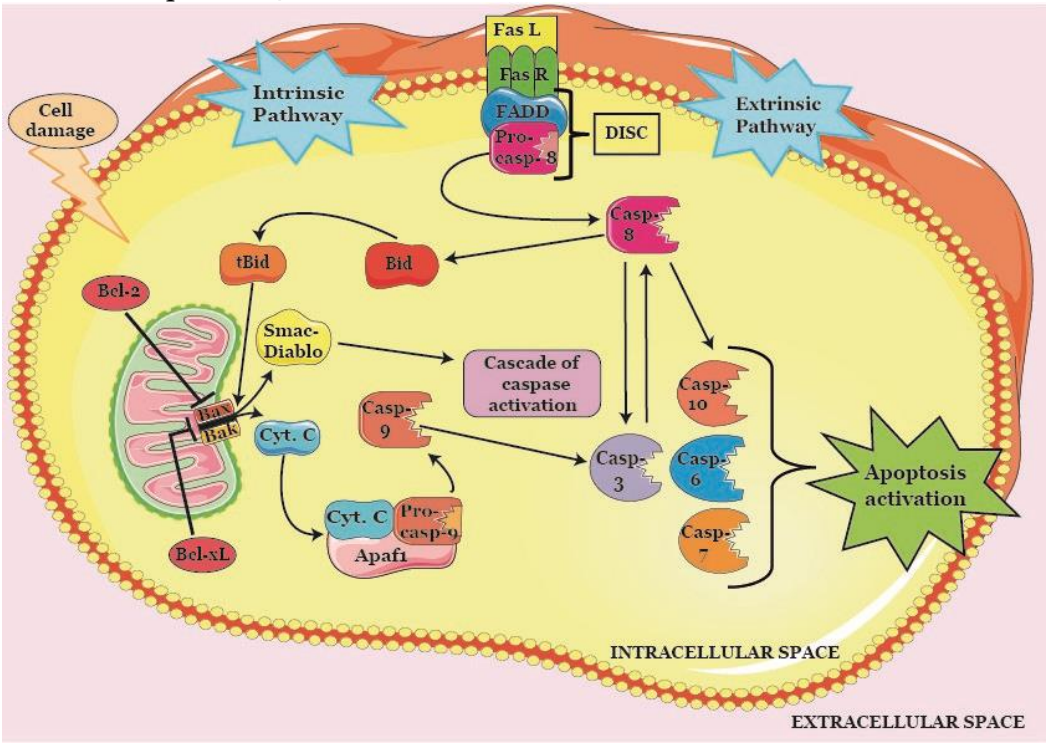


**Figure I.6.** Schematic representation of the extrinsic and the intrinsic pathways of apoptosis. The extrinsic pathway is activated by the interaction of membrane death receptors (DR) with their extracellular ligands, such as the Fas receptor (Fas R) and Fas ligand (Fas L). This interaction induces a signalling cascade that culminates in the formation of the death-inducing signalling complex (DISC) and activation of caspase-8 (Casp-8) through its autocleavage. In turn, Casp-8 activates the effector caspase-3 (Casp-3), which, not only initiates the cascade of caspase activation, but also feeds back itself. Casp-8 can also activate the intrinsic pathway by the cleavage of BH3 interacting domain death (Bid) protein to truncated Bid (tBid). Cell damage and triggering of mitochondrial response are the main activators of the intrinsic pathway. The altered balance between pro- and anti- apoptotic members of the B-cell lymphoma-extra-large protein (Bcl-2) family, lead to the formation of pores in the mitochondrial membrane and release of cytochrome C (Cyt. C) and second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI protein (Smac/Diablo). Cyt. C, with the apoptotic protease-activating factor 1 (APAF1) protein and pro-caspase-9 (Pro-Casp-9) form the apoptossome, which induces the autocleavage of the pro-Casp 9 to caspase-9 (Casp-9). Consequently, Casp-9 activates Casp-3 that initiates the cascade of caspase activation. The Smac/Diablo protein supports the cascade of caspase activation. Legend: Bak, Bcl-2 homologous antagonist killer protein; Bax, Bcl-2 associated-X protein; Bcl-XL, B-cell lymphoma- extra-large protein; Casp-6, Caspase-6; Casp-7, Caspase-7; Casp-10, Caspase-10; FADD, Fas-Associated protein with Death Domain; Pro-caspase-8 (Pro-Casp-8). Head-arrows black lines mean protein activation. Bar-ended black lines indicate protein inhibition.

As mentioned above, the intrinsic pathway may be activated, for example, in response to DNA damage or other stress factors [153]. Significant DNA damage induces the accumulation of the tumour suppressor protein p53, which triggers the apoptotic process mainly by regulating the transcription of pro- and anti-apoptotic proteins [154]. The major modulators of the intrinsic pathway are proteins that belong to the Bcl-2 family [152]. This family includes pro-apoptotic proteins, such as Bcl-2 associated-X (Bax) and Bcl-2 homologous antagonist killer (Bak), and anti-apoptotic proteins, like Bcl-2 and B-cell lymphoma-extra-large (Bcl-XL). The activation of the intrinsic pathway induces a conformational change in the pro-apoptotic proteins, namely Bax and Bak [148, 155] that allows their oligomerization and insertion into the mitochondrial membrane, inducing the formation of apoptotic pores [148, 155]. Subsequently, cytochrome C is released from the mitochondrial intermembrane space to the cytosol forming together with the apoptotic protease activating factor 1 and procaspase-9 the apoptossome. Caspase-9 is activated through autocleavage and initiates the cascade of caspase activation through the activation of caspase-3, -6 and -7 [148, 156-159]. Also, the pro-apoptotic protein second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI is released from mitochondria and supports the cascade of caspase activation (Figure I.6) [156]. The effector caspases, like caspase-3, caspase-6 and caspase-10, play an essential role in the cytoskeletal reorganization and in the formation of cytoplasmic bleeds and apoptotic bodies [148, 160]. Noteworthy, the intrinsic pathway can also be activated by the extrinsic pathway (Figure I.6). The cross-talk between the two apoptotic pathways is due to the cleavage of BH3 interacting domain death (Bid) protein to the truncated Bid by caspase-8 [151, 156]. The truncated Bid protein interacts with Bax and Bak in mitochondria, destabilizes the outer membrane and induces the release of cytochrome C [156].

The activation of the extrinsic pathway depends on the interaction between extracellular ligands and the extracellular domain of DR (Figure I.6). The most well characterized ligand-DR complexes are the Fas-ligand (FasL)/ FasR and TNF-α/TNFR1 [160]. The interaction between the ligand and the respective DR induces the trimerization of the receptor, that once activated recruits through the intracellular death domains adaptor proteins, which, in turn, recruit the pro-caspase-8 and cellular FLICE inhibitory proteins [148, 156, 160, 161]. This macromolecular complex is called the death-inducing signalling complex and once is formed pro-caspase-8 is activated [148]. The cascade of caspase activation triggered by caspase-8 induces the activation of several effector caspases, such as caspase-3, -7 and -10. Besides, the effects mentioned earlier, caspase-3 also increases the activity of caspase-8 through a positive feedback loop (Figure I.6) [157].

Resistance to apoptosis is a well-established hallmark of cancer, contributing to tumour progression, aggressiveness and also resistance to treatment [149, 162]. In PCa, the characteristic androgen-independency associated with the progression of disease has been

associated with the alterations of apoptotic response. It was described the increased expression of anti-apoptotic proteins, namely, Bcl-2, B-cell lymphoma-w (Bcl-w), Bcl-xL and myeloid cell leukaemia 1 [163-170]. Also, it was reported that Bcl-2 and Bcl-w overexpression contributes to the therapeutic resistance, disease recurrence and shortened survival observed in CRPC patients [170-173]. Moreover, the protein Bcl-xL is commonly overexpressed in metastatic CRPC [163]. On the contrary, the inhibition of the expression of these pro-survival proteins sensitized tumour cells to chemotherapy [163-170], with this pathway being suggested as a possible target for treatment.

Also, the expression of pro-apoptotic proteins has been found to be deregulated in PCa. It is the case of *p53* that is one of the most commonly mutated genes in cancer. p53 expression is decreased in 40% of PCa cases [174]. The expression of DR receptors has been shown to be diminished in PCa and related with the inhibition of the extrinsic pathway, and by consequence to the inhibition of apoptosis [175, 176]. Also, it has been reported that PCa cells overcome apoptosis by becoming insensitive to the activation of Fas-mediated extrinsic pathway [177]. On the contrary, when Fas-mediated apoptosis is activated, the rates of programmed cell death increase [177, 178]. This suggested that drugs that activate this pathway may be used in the treatment of PCa.

## 2.4. Diagnosis and treatment

Even though PCa is one of the most common cancers worldwide, both diagnosis and treatment continue inefficient [15, 59, 61].

PCa screening encompasses the physical rectal examination followed by a PSA analysis. PSA measurement has been widely used as a PCa biomarker; however it has important limitations because elevated PSA levels also can be found in other prostate pathologies, such as BPH or prostatitis [70, 179, 180]. Therefore, the most powerful diagnosis tool is prostate biopsy using transrectal ultrasound-guided scan [180, 181]. Nevertheless, this invasive diagnosis procedure, after obtaining 10 to 12 tissue samples in a grid-like pattern, still misses 21 to 28% of PCa cases and undergraduates 14 to 17% of the cases [180].

Upon histological analysis, systematic prostate biopsies are graded according to the Gleason patterns [180]. The Gleason patterns range from 1 to 5, where 1 looks like normal prostate tissue with well differentiated cellular architecture and 5 represents an aggressive and un-differentiated cellular architecture [70, 182, 183]. One of the best and more used prognostic indicators in PCa is the Gleason score (GS), which characterizes the glandular architecture of prostate based on a histological score that represents the level of “de-differentiation” of PCa. This score comprises the sum of the two grades that represent the most common Gleason patterns found in a prostate specimen [70]. In this way, the highest GS can be is 10 and means a highly aggressive PCa. On the other hand, grades 1 and 2 are not often used for biopsies, which means that the lowest GS of a cancer found on a prostate biopsy is 6.

However, management of PCa risk do not depends exclusively on GS and include other factors, such as PSA levels [70, 183].

It was suggested that a higher degree of AR positivity correlates with a greater degree of differentiation or lower GS; however this is not a universal observation [112]. Taking into account the limitations of the diagnosis procedures currently used, new diagnosis strategies, including novel molecular biomarkers and gene panels in different clinical scenarios have been studied. However, the use of these molecular biomarkers still needs further investigation and validation to be incorporated into the clinical practice [15].

Concerning PCa treatment, the first approach is prostatectomy or radiation to destroy the cancerous cells confined within the prostate capsule [184]. However, frequently this treatment fails and the cancer recurs [61].

Similarly to the non-neoplastic prostate, PCa cells need androgens to growth and survive, what makes the ADT an effective first-line therapy. This therapy through surgical or pharmacological castration, the latter with, for example, luteinizing hormone releasing hormone or gonadotropin-releasing hormone agonists, reduces serum testosterone levels by 90-95% [62]. However, the intraprostatic DHT levels only decline about 50% [62], and the antiandrogens drugs, antiandrogens blocking the AR action or inhibitors of 5α-reductase (Figure I.4) are also used [62, 185, 186]. Although most men show positive outcomes for 2 to 3 years with ADT, PCa continues progressing and resistance to therapy is developed with the disease entering the stage of CRPC (Figure I.3 and I.4) [187-189]. PCa is considered castrate resistant when, despite the reduced levels of serum testosterone, occur an increase of the PSA serum levels (biochemical progression), the development of symptoms in the presence of the pre-existing cancer (clinical progression) or the detection of new metastatic lesions on imaging (radiographic progression) [62, 190, 191]. This aggressive and lethal form of PCa progresses and metastasizes, not existing currently an effective therapy, only palliative treatments [59, 61]. Life-extending treatments encompass the use of new hormone therapies, chemotherapy, radioisotopes and immunotherapy [184, 192]. From all the possible treatments and combinations tested in clinical trials, the most commonly used is the docetaxel chemotherapy combined with the steroid drug prednisone [193]. However, if PCa patients are resistant to docetaxel, other agents could be used, such as cabazitaxel [192, 193].

# 3. Characterization of regucalcin protein

## 3.1. Regucalcin gene, protein structure and function

Regucalcin (RGN) or Senescence Marker Protein 30 (SMP30) is a $Ca^{2+}$-binding protein discovered in 1978 by Yamaguchi M [194]. The *RGN* gene its located in the p11.3–q11.2 and q11.1–12 segments of the human [195] and rat [196] X chromosomes, respectively. The organization of both human (Figure I.7A) and rat *RGN* gene consists of 7 exons, 6 introns and several consensus regulatory elements that exist upstream of the 5'-flanking region [197, 198]. The full-length RGN mRNA (1354 bp) comprises a 897 bp open-reading-frame that gives rise to a protein with 229 amino acid residues (Figure I.7B) and a molecular weight of approximately 33 kDa [195]. Besides the full-length transcript two alternative RGN mRNA spliced variants, RGN Δ4 and RGN Δ4,5 were identified (Figure I.7B) [198-200]. The RGN Δ4 transcript arises from the deletion of exon 4 and encodes a protein with 227 amino acid residues [198] and an estimated molecular weight of 25 kDa [201]. In a similar way, RGN Δ4, 5 transcript has deletion of exons 4 and 5 and gives rise to a protein with 183 amino acid residues [198] and approximately 20 kDa [201]. It is not completely understood if this transcripts are translated, because three distinct proteins variants with 24, 28 and 27 kDa were identified arising from the proteolytic processing of full-length RGN protein [201, 202]. Also, the functional role of the RGN protein spliced variants and the proteins that are originated from proteolytic processing remains largely unknown.

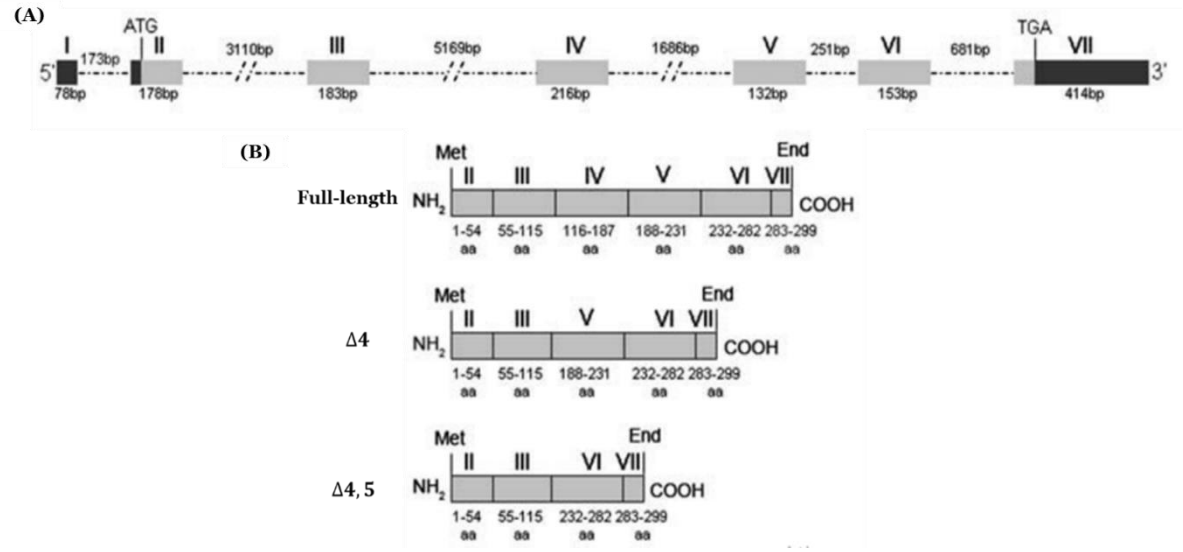


**Figure I.7.** Representation of the *regucalcin* (*RGN*) gene organization and hypothetical proteins [200]. **(A)** Human *RGN* gene organization. **(B)** The full-length RGN protein and the proteins encoded by the products of alternative splicing. Legend: Arabic numerals indicate the base pairs (bp) and number of amino acids (aa) encoded by each exon in panel **(A)** and **(B)**, respectively. Roman numerals represent each exon. ATG, translation initiation codon; End, end of the protein; Met, Methionine; TGA, translation stop codon. Full-length, full-length RGN protein; Δ4, protein of RGN Δ4 transcript; Δ4, 5, protein of RGN Δ4,5 transcript.

The transcription of the *RGN* gene is controlled by several TFs, namely the activator protein 1 (AP-1), nuclear factor1-A1 (NF1-A1), *RGN* gene promoter region-related protein p117 (RGPR-p117), β-catenin and other factors like NF-kβ [203-206]. Additionally, studies demonstrated that the nuclear translocation of AP-1, NF1-A1, RGPR-p117 is mediated

through phosphorylation by PKC, calmodulin (CaM)-dependent protein kinase, MAPK kinase and PI3K, which are activated by several hormonal and non-hormonal factors and physiological conditions [201, 207].

The expression of *RGN* gene is also regulated by a set of non-hormonal (e.g. OS) and hormonal factors (Figure I.8). Among all the non-hormonal factors, one of the most studied in the regulation of RGN expression is OS. Studies performed with OS inducers, such as lipopolysaccharide (LPS) and tert-Butyl hydroperoxide (t-BHP), demonstrated that the increase of OS decreases the expression of RGN [208-211]. Additionally, it was also demonstrated that caloric restriction, an anti-oxidant factor, diminishes the levels of OS and induces an increase of RGN expression [209, 211].

Besides OS, $Ca^{2+}$ is the most important non-hormonal factor in the regulation of RGN expression. Several reports demonstrated that $Ca^{2+}$ stimulation induces the expression of RGN [212-218]. Moreover, it has been proposed that this regulation, both *in vivo* and *in vitro*, may be partly mediated by the $Ca^{2+}$/CaM signalling pathway (Figure I.8) [212, 217, 219, 220].

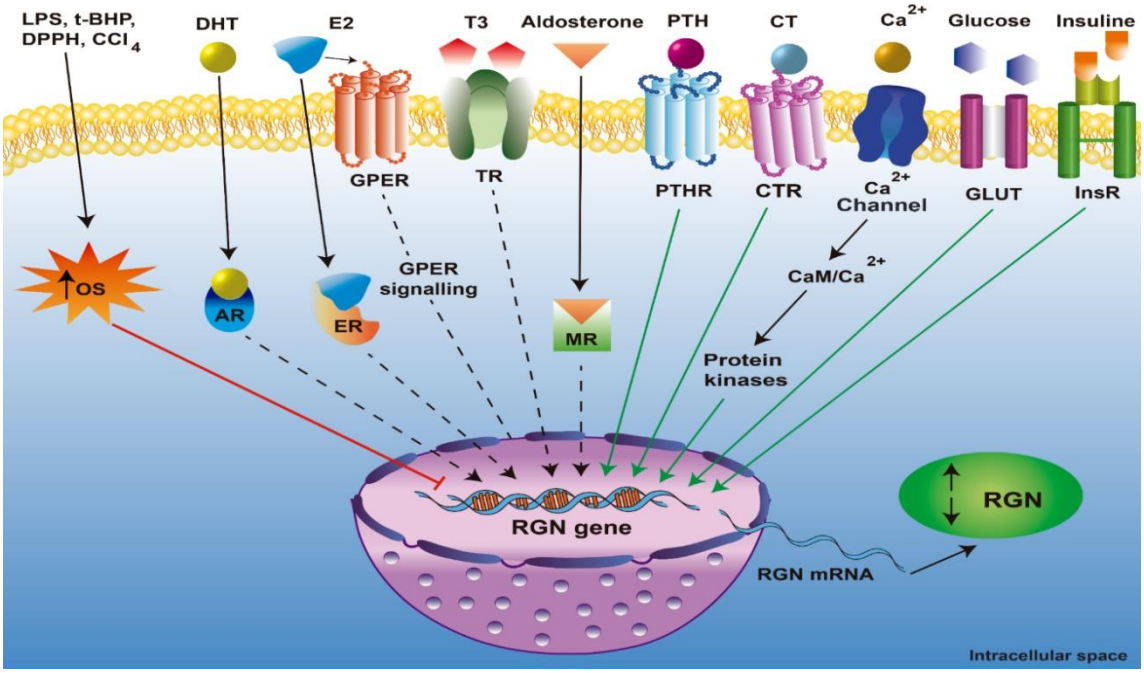


**Figure I.8.** Schematic representation of the diverse hormonal and non-hormonal factors that regulate regucalcin (RGN) expression in tissues and cell lines [221].
Legend: AR, Androgen Receptor; ; $Ca^{2+}$, Calcium; CaM, Calmodulin; CCl4, carbon tetrachloride; CT, Calcitonin; CTR, Calcitonin Receptor; DHT, 5α-dihydrotestosterone; DPPH, 1,1-diphenyl1-2-picryl-hydrazyl; $E_2$, 17β-estradiol; ER, Estrogen Receptor; GLUT, Glucose Transporter; GPER, G Protein-coupled Estrogen Receptor; InsR, Insulin Receptor; LPS, Lipopolysaccharide; MR, Mineralocorticoid Receptor; OS, Oxidative Stress; PTH, Parathyroid Hormone; PTHR Parathyroid Hormone Receptor; T3, 3,3', 5-triiodo-L-thyronine; t-BHP, tert-Butyl hydroperoxide; TR, thyroid hormone receptor. Head-arrow lines and bar-ended lines indicate activation and inhibition, respectively. Dashed arrows indicate that up- or down-regulation may occur dependently on the dose, time of stimulation and specific cell type.

A panoply of hormone factors has been shown to regulate RGN expression (Figure I.8). In this context, steroid hormones are among the most well study and important regulators of RGN expression levels. The sex steroid hormones, oestrogens and androgens seem to

regulate the expression of RGN through receptor-mediated effects [198]. Concerning the estrogenic regulation, reports demonstrated that $E_2$ increased the RGN mRNA expression in rat liver [222] and in rat seminiferous tubules cultured *ex vivo* [223]. Contrarily, in rat kidney [224], mammary gland and prostate [225], and in bovine testis and accessory sex glands [226], RGN expression decreased in response to $E_2$. These findings indicate that the estrogenic effects in the regulation of RGN are tissue-dependent and species-specific. Furthermore, Maia *et al.* demonstrated that the regulation of RGN levels by $E_2$ in MCF-7 human breast cancer cells was time-dependent [198].

The action of androgens in the regulation of RGN expression also was shown to be tissue specific. Interestingly, orchidectomized rats displayed reduced expression of RGN in the kidney [227], and increased levels in the prostate, an effect that was abrogated after androgens replacement [228]. In reproductive organs, namely in the testis of veal calves and beef cattle, and in rat seminiferous tubules cultured *ex vivo*, it was demonstrated that DHT administration decreased the RGN levels [226, 229]. Furthermore, Maia *et al.* demonstrated that DHT-treatment suppressed the expression of RGN in LNCaP cells [198].

Adrenal hormones also were indicated in the control of RGN expression. Studies demonstrated that both adrenalectomy and saline ingestion diminished the expression levels of RGN [230-232]. Furthermore, it was demonstrated that aldosterone regulate RGN expression in a tissue-dependent manner, enhancing expression levels in rat renal proximal tubular NRK52E cells and reducing it in the rat kidney cortex [224, 232]. Vitamin D is other important steroid hormone that was shown to regulate the expression of RGN in a tissue-dependent manner [232, 233].

Parathyroid and thyroid hormones were shown to modulate RGN expression in the kidney and liver and in several cell types. Calcitonin and parathyroid hormone (PTH) stimulated the expression of RGN in the liver of thyroparathyroidectomised rats and in osteoblastic MC3T3-E1 cells and NRK52E cells [232-234]. Furthermore, Nakagawa T. *et al* demonstrated that PTH induced the transcription of *RGN* gene in NRK52E cells through the stimulation of the nuclear translocation of RGPR-p117 TF [232]. Regarding thyroid hormones, 3,3', 5-triiodo-L-thyronine regulated the expression of RGN in a time-dependent manner in the liver of female rats and in breast cancer cells [235]. A study showed that thyroxine do not affected RGN expression in the rat liver [222]. Additionally, glucose and insulin seem to regulate RGN expression. Yamaguchi *et al.* demonstrated that both glucose and insulin up-regulated RGN expression in rat liver, suggesting its role in the control of liver metabolism [213].

Concerning the structure of RGN protein it has been characterize as containing 24 β-strands forming 6 β-sheets, which form a closed circular arrangement around a central solvent filled tunnel that has one metal binding site [236]. Motif analysis showed that RGN do not contains the typical EF-hand $Ca^{2+}$-binding sequence typical of the majority of $Ca^{2+}$-binding

proteins [236]. However several techniques, as X-ray diffraction and circular dichroism proved that RGN is able to bind to this ion [236, 237]. However, despite displaying a higher affinity to $Ca^{2+}$ [238], the human RGN protein also was shown to bind others divalent cations, namely, $Zn^{2+}$, $Mg^{2+}$, $Mn^{2+}$ and $Co^{2+}$ [236, 238].

The major recognized role of RGN is the regulation of $Ca^{2+}$ homeostasis through the direct and/or indirect modulation of plasma membrane $Ca^{2+}$-ATPase (PMCA), sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA), nuclear outer membrane SERCA (nuclear SERCA) pumps and mitochondrial $Ca^{2+}$ uniporter (MCU) [239, 240]. Moreover, RGN also influences the expression of L-type $Ca^{2+}$-channels and $Ca^{2+}$-sensing receptor (CaSR) (Figure I.9) [241, 242].

The stimulatory effect of RGN on the activity of PMCA pump has been described in several studies and cell types [243-247]. RGN seems to bind directly to the plasma membrane [244, 248] and/or interacting with the SH groups of the ATPase [248, 249]. Moreover, it was also demonstrated that CaM and $Ca^{2+}$ modulate the action of RGN in the regulation of PMCA pump activity [244, 250]. Studies demonstrated that RGN also increases SERCA pump activity through the modulation of SH groups [250-252], phosphorylation [250] and/or membrane association [252]. Similarly, the nuclear SERCA pump also is regulated by RGN. A study showed that RGN decreases its activity [253].

Concerning the mitochondrial $Ca^{2+}$-ATPase and MCU (Figure I.9), RGN modulates the SH groups of the pump, and transporter activity in a CaM-dependent way, which result in the increase of $Ca^{2+}$ uptake into the mitochondrial fraction [254-258]. The action of RGN on the expression of L-type $Ca^{2+}$ channels was studied in kidney cells, being demonstrated that RGN suppress this channel expression [241]. Moreover, in non-neoplastic human prostate PNT1A cells, human PCa LNCaP cells, transgenic rats overexpressing RGN (Tg-RGN) and NRK52E cells it was demonstrated that RGN decreases the expression of CaSR, leading to the inhibition of $Ca^{2+}$ signalling [241, 242].

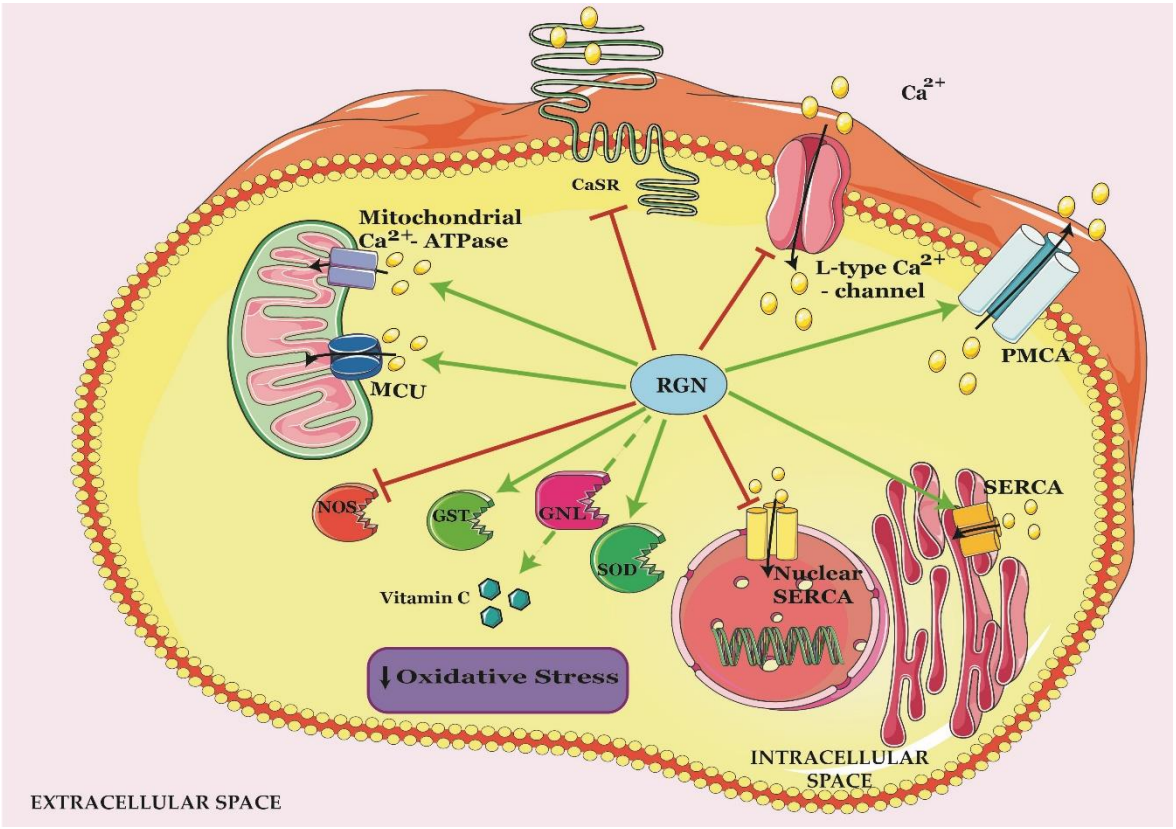


**Figure I.9.** Schematic representation of regucalcin (RGN) actions in calcium ($Ca^{2+}$) homeostasis and in the protection against oxidative stress (OS). RGN maintain intracellular $Ca^{2+}$ homeostasis by inducing the expression and/or activity of mitochondrial $Ca^{2+}$-ATPase, mitochondrial $Ca^{2+}$-uniporter (MCU) and plasma membrane $Ca^{2+}$-ATPase (PMCA) and sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) pumps and inhibiting the expression of $Ca^{2+}$-sensing receptor (CaSR), L-type $Ca^{2+}$-channel and nuclear SERCA pump. RGN protein also up-regulates the expression and activity of antioxidant enzymes, namely glutathione-S-transferase (GST) and superoxide dismutase (SOD), whereas inhibiting the activity of nitric oxide synthase (NOS). Additionally, RGN also has been identified as a gluconolactonase (GNL), an enzyme involved in the biosynthesis of Vitamin C, a well-known antioxidant agent. The actions of RGN in the anti-oxidant enzymes, in NOS and in Vitamin C biosynthesis, allows the decrease of OS. Legend: Green arrows mean a RGN stimulatory effect. Bar-ended red arrows indicate an inhibitory effect of RGN.

Besides the regulation of intracellular $Ca^{2+}$ levels, RGN has been shown to play other relevant roles in cell physiology, which may depend on its wide subcellular location. The RGN protein has been shown to be present at cell nucleus and perinuclear space [198, 202, 225, 229, 241, 259-264], cytoplasm [202, 225, 229, 259-262], and in the mitochondrial fraction [202]. The mechanisms related with the nuclear translocation of RGN are unknown at the moment, but a bioinformatic analysis identified an importin α-dependent putative nuclear localization signal (NLS) in the RGN protein sequence [265]. Importantly, approximately 72% of the residues of the putative NLS are 100% conserved in all species analysed [265], which is indicative of its potential functionality.

Other relevant role of RGN protein is related with the protection against OS (Figure I.9) [266, 267]. RGN's actions include the increase of antioxidant capacity, stimulation of the activity of antioxidant enzymes, namely glutathione-S-transferase (GST) and superoxide dismutase , and inhibition of nitric oxide synthase (NOS) [262, 268-276]. Moreover, the anti-oxidant effect of RGN is also due to its activity as a gluconolactonase (GNL) [277], at least in rodents. GNL catalyses the penultimate step of Vitamin C biosynthesis, a feature

that was lost during evolution, being absent in humans but maintained in rodents [277]. Indeed, studies in the SMP30-knockout rats demonstrated that the GNL activity of RGN displays an important anti-oxidant role [266, 267, 278-281]. Besides the GNL activity, it was also indicated that RGN presents organophosphate hydrolase activity, what suggests that this protein may protect against the maleficent effects of organophosphates [236, 238, 282].

Finally, other multiple essential roles for RGN have been identified in the regulation of several intracellular signalling pathways that control cell proliferation, apoptosis and also energy metabolism [221, 283], as will be detailed in section 3.3.

## 3.2. Tissue expression pattern of regucalcin and the effect of aging

The RGN protein was first identified in the rat liver, where it is highly expressed [194, 284]. Nevertheless, rapidly RGN was found in a wide variety of species, being expressed in fungi, bacteria, invertebrates and vertebrates. In the last few years, the use of bioinformatics tools demonstrated that 18% of the amino acid residues of RGN are conserved in all species analysed [200]. Moreover, it was reported that the human RGN displays a high homology with the other species RGN. Similarity values are 98, 88-96, 79-85, 41-30, 26 and 32-22% when comparing human RGN with primates, other mammalian species, non-mammalian vertebrates, invertebrate species, and fungi and bacteria RGN, respectively [200, 283]. These findings showed that RGN is a protein highly conserved through evolution, which reinforces its biological importance and important function.

Also, the expression of RGN in different tissues, physiological conditions and diseases has been characterized [200, 283]. RGN was found in a broad range of non-reproductive and reproductive organs in different species (reviewed by [283]). Besides the rat liver, immunohistochemistry and Northern blot analysis demonstrated that RGN is also strongly expressed in the rat kidney, more precisely in proximal tubular epithelia [285]. Other rat tissues, such as skeletal muscle, duodenum, lung, heart, spleen, brain and hippocampus, bone, and submandibular gland also express RGN [286-290]. Furthermore, our research group demonstrated that RGN is expressed in rat mammary gland and prostate [225], epididymis and all testicular cell types, somatic and germ line [229].

In humans, RGN is highly expressed in the liver and kidney, being also detected in other tissues (reviewed by [283]), such as heart [283], testicular cell types, somatic and germ line [229], mammary gland, prostate [198]. Similarly, the RGN mRNA alternative spliced variants have been described in healthy human tissues, namely, liver, kidney, brain, lung, breast, prostate and testis [198, 201, 229]. Additionally, a differential expression of RGN alternative transcripts has been described. For example, MCF-7 cells only express the RGN Δ4 transcript whereas RGNΔ4,5 is detected in LNCaP cells [198]. Moreover, the 24 and 28 kDa alternative proteins arising from the proteolytic processing of full-length RGN were identified in the rat liver and kidney, and in human liver cell lines [202] and tissue

specimens [201]. Concerning the other proteolytic product, the 27 kDa protein it has only been found in the human kidney [201].

In parallel with the indication of the relevant physiological role of RGN, its deregulated expression has been described in pathological conditions, namely with the onset and progression of the carcinogenic process. RGN was identified as a down-regulated protein in a set of animal and human cancers, namely, liver, kidney, brain, lung, breast, prostate and cervix [198, 201, 291-295]. Furthermore, a relationship was found between the diminished expression of RGN and the histological grade of infiltrating ductal carcinoma of breast and cellular differentiation of prostate adenocarcinoma [198]. Also, a microarray gene expression profile study in the rat liver proved that the expression of RGN starts to reduce during the establishment of pre-neoplastic lesions before acquisition of tumoral phenotype [296]. Altogether, these findings lead to the assumption that the loss of RGN protein is related with tumour onset, progression and aggressiveness.

Another interesting feature of RGN protein is its down-regulated expression with aging. It was firstly reported in the rat liver and kidney. RGN expression seems to be biologically required only after birth as 18 days embryos did not express RGN, increasing from day 1 after birth till adulthood [297], but decreasing markedly in older rats [297]. This expression pattern of downregulated expression of RGN with the aging process also was found in the rat testis, prostate and brain [229, 271, 288].

Aging triggers the appearance of aging-related alterations that have been linked with tissue damage and the appearance of diseases, namely PCa. It is the case of increased OS, deregulation of cell proliferation mechanisms, apoptosis and metabolism [298]. Interestingly, in the rat prostate, it was reported that the decrease of RGN levels with aging is underpinned by alterations in the expression of proliferation and apoptosis regulators and increased OS, which was suppressed in Tg-RGN animals [271]. These findings highly support the tissue-protective role of RGN as a putative anticancer protein.

## 3.3. Regucalcin and the hallmarks of cancer

### 3.3.1. Cell proliferation and Apoptosis

Deregulated proliferation and resistance to apoptosis are well-known and established hallmarks of cancer, contributing to tumour onset, progression and aggressiveness [162, 299, 300]. Several studies have been indicating the anti-proliferative and pro(anti)-apoptotic actions of RGN (Figure I.10). In the case of proliferation, it includes the regulation of DNA replication and RNA synthesis. As mentioned above, RGN is located in the cytoplasm and translocated to the nucleus [198, 260, 263, 301], in a process mediated by PKC signalling pathway [264]. Therefore, considering RGN localization, it is not surprising

that this protein controls DNA replication, which was shown to have an impact in proliferation [221, 283].

RGN activity in the cell nucleus is mediated through binding to both nuclear proteins and DNA [221, 283, 302]. For example, studies in the rat liver reported that RGN binds directly to DNA and the $Ca^{2+}$- dependent endonuclease inhibiting its activity [302-304]. Moreover, it has been shown that RGN inhibits nuclear protein import and export through the inhibition of Small GTPase ras-related nuclear protein [305].

It has also been shown that RGN modulates the transcriptional activity and RNA synthesis. Indeed, the regulatory role of RGN in cell proliferation seems to be mediated by controlling the expression of tumour suppressor genes, oncogenes and cell-cycle regulators (Figure I.10) [221, 283, 306]. Studies in rat liver H4-II-e, rat kidney NRK52E, human lung A549 , human pancreatic MIA PaCa-2, human colon RKO, human liver HepG2, human breast MDA-MB-231, human kidney A498 and human cervix HeLa cancer cells showed that the overexpression of RGN suppresses cell proliferation [295, 307-314]. Additionally, in all cell types mentioned, except He-La cells, RGN induced G1 and G2/M phase cell cycle arrest [307-314]. In the specific case of HeLa cells, RGN induced G2/M phase cell cycle arrest only [295]. Moreover, it seems that the RGN influence on the expression of cell fate regulators is cell-specific [295, 306-314]. In H4-II-E cells, it was demonstrated that RGN increased p21 levels not altering significantly the expression of cell division control protein 2 homolog A (cdc2a) and checkpoint kinase 2 (chk2) [315]. However, in NRK52E cells RGN suppressed the expression of c-jun and chk2, not affecting the expression of c-myc, c-fos, cdc2a and p21 [316]. Additionally, was also reported that RGN regulated the expression levels of important proteins, such as Ras, AKT, MAPK, p38-MAPK, stress-activated protein kinases/Jun amino-terminal kinases , c-fos, c-myc, NF-kβ, p65, STAT3, β-catenin, GSK-3β and p53 and pRb [228, 271, 295, 306-310, 312-318]. Moreover, the RGN protein inhibited proliferation by modulating the activity of protein kinases, phosphatases and RNA polymerase II. In addition, RGN also suppressed signalling pathways that are known to be altered in PCa, namely MAPK/ERK, PI3K and NF-kβ [295, 307-317, 319, 320].

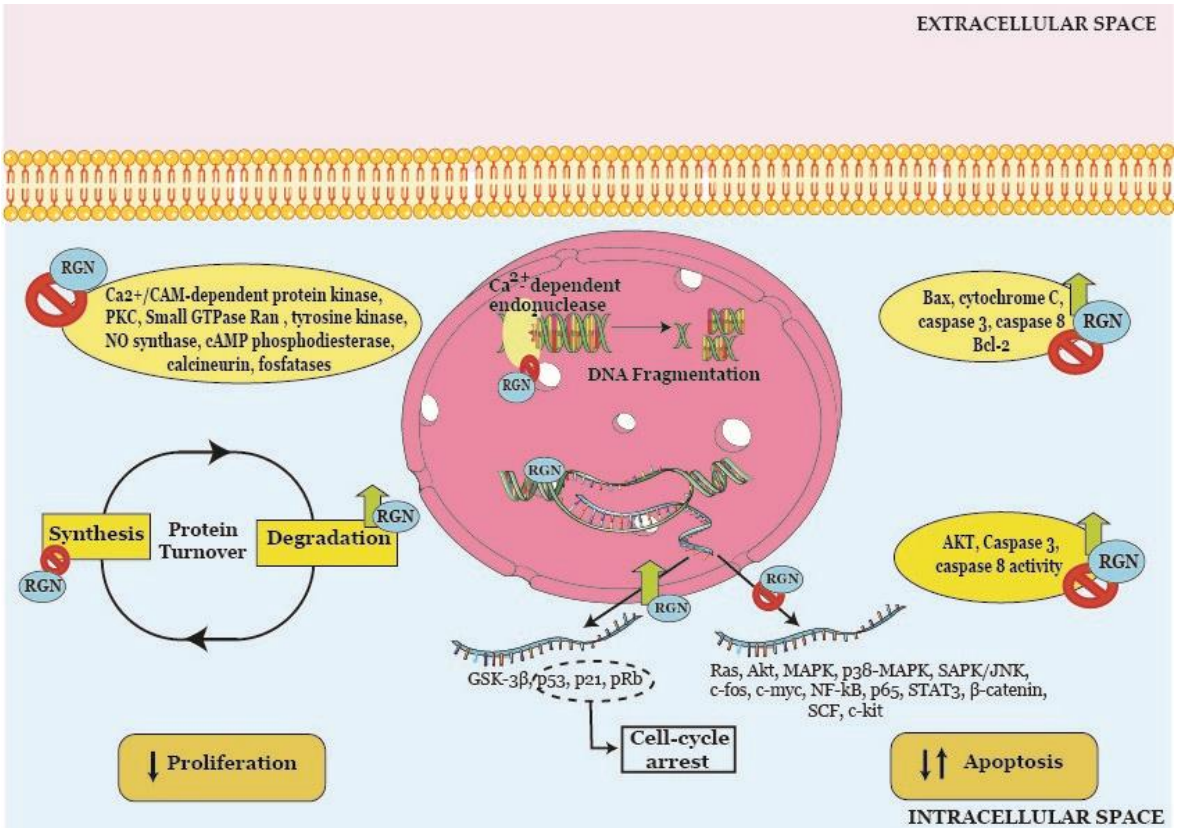


**Figure I.10.** Schematic representation of the regucalcin (RGN) actions in intracellular signalling, cell proliferation and apoptosis. RGN inhibits calcium ($Ca^{2+}$)/Calmodulin (CAM)-dependent protein kinase, protein kinase C (PKC), Small GTPase ras-related nuclear protein (Ran), tyrosine kinase, nitric oxide synthase (NOS), cyclic-adenosine monophosphate (cAMP) phosphodiesterase, calcineurin and phosphatases. Cytoplasmic RGN controls protein turnover by inhibiting protein synthesis and activating protein degradation. In the nucleus, RGN inhibits DNA fragmentation through the inhibition of $Ca^{2+}$-dependent endonuclease. Moreover, RGN enhances the expression of tumour suppressor genes (glycogen synthase kinase 3 β (GSK-3β) , p53, p21 and pRb) and diminishes the expression of oncogenes (Ras, AKT, mitogen activated protein kinase (MAPK), p38-MAPK, stress-activated protein kinases/Jun amino-terminal kinases (SAPK/JNK), c-fos, c-myc, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), p65, signal transducer and activator of transcription (STAT)3, β-catenin, stem cell factor (SCF), and c-kit), inducing cell-cycle arrest. Concerning apoptosis, RGN may increase (or decrease) AKT expression and activity, and the expression of caspase-3 and -8, Bcl-2 associated-X (Bax), cytochrome C and B-cell lymphoma 2 (Bcl-2), dependently on the age group. In general, RGN suppresses apoptosis; however, in aged animals the overexpression of RGN induced apoptosis, which may be a protective mechanism against the development of age-associated diseases. Legend: Green arrows mean a stimulatory effect of RGN. Proinhibition signals indicate a RGN inhibitory effect. Both green arrows and a prohibition signal indicate a reported dual role for RGN with induction or inhibitory effects.

Studies performed in several cell types also demonstrated that nuclear RGN, as well as the cytoplasmic, inhibits several modulators of intracellular $Ca^{2+}$ signalling and protein phosphorylation/dephosphorylation processes, like $Ca^{2+}$/CAM-dependent protein kinase and PKC, which mediate signal transduction from cytoplasm to nucleus [221, 283, 306, 321]. In addition, RGN also regulates tyrosine kinase, NOS, cyclic-adenosine monophosphate phosphodiesterase, calcineurin and other phosphatases that enhance proliferation [221, 283, 306, 321]. Other actions of RGN in the cytoplasm were related to the protein turnover (Figure I.10) [322, 323]. It was demonstrated that RGN inhibited protein synthesis by inhibiting aminoacyl-tRNA synthetase activity [322] and inducing protein degradation by the activation of neutral cysteinyl-proteases [196].

Concerning cell survival and death, studies performed in different cell types, namely testicular cells, cardiomyocytes, and mouse embryonic P19, rat kidney NRK52E, rat liver

H4-II-E, human lung A549 , human pancreatic MIA PaCa-2 , human colon RKO, human liver HepG2, human breast MDA-MB-231, human kidney A498 and human cervix HeLa cancer cells, demonstrated that RGN inhibits apoptosis triggered by OS- or apoptosis-inducers, such as thapsigargin, actinomycin D, hydrogen peroxide , t-BHP, Bay K 8644, insulin, TGF-β, TNF-α, IGF or LPS [295, 307-314, 317, 324-330]. Based on these findings the protective role of RGN against cell damage and chemical-induced cell death was proposed.

Similarly to proliferation, the protective role of RGN against apoptosis is related to the suppression and stimulation of several proteins and signalling pathways [221, 283, 324]. Studies in H4-II-E cells showed that RGN suppressed $Ca^{2+}$/CaM-dependent NOS activity due to the binding to CaM and/or due to the direct $Ca^{2+}$-binding [274]. Moreover, RGN inhibited apoptosis mediated by factors that increase intracellular $Ca^{2+}$ content and, by consequence, DNA fragmentation [325, 326]. These RGN's effects may be mediated by its capacity to bind $Ca^{2+}$, regulate the $Ca^{2+}$ pumps present at the cell membrane and by the direct inhibition of nuclear endonuclease [324]. Additionally, the suppressive effect of RGN on apoptosis is also related with the inhibition of the expression and activity of various protein kinases, phosphatases, Bax, cytochrome C and caspase-3 [324]. Curiously, the inhibition of caspase-3 seems to be a major way by which RGN inhibits apoptosis, as in several cell lines the RGN effect was not enhanced by caspase-3 inhibitors [295, 307-314]. Nevertheless, studies performed in NRK52E and HepG2 cells showed that RGN was linked with inhibition of apoptosis, increased expression of anti-apoptotic proteins, such as Bcl-2 and AKT, and increased AKT activity [228, 324, 329, 331]. Studies in knockin and knockout *in vivo* animal models strongly demonstrated that RGN protects against apoptosis. Ishigami *et al.* showed that SMP-30 knockout mouse liver is very susceptible to apoptosis mediated by TNF-α and Fas (Figure I.10) [332]. Accordingly, a study performed in the prostate of aged Tg-RGN rats demonstrated that RGN counteracts the resistance to apoptosis characteristic of aging. Vaz *et al.* [271] showed that RGN overexpression was related to higher rates of apoptosis, as indicated by the increased caspase-3 activity, and also increased expression levels of p53, Bax, FasL, FasR and caspase-8. This study also found that RGN suppressed cell proliferation concomitantly with the reduced expression of the tyrosine kinase receptor c-kit and its ligand stem cell factor (SCF) (Figure I.10) [271]. Based on these findings, it is likely that RGN plays a relevant role in maintaining cell and tissue homeostasis by balancing cell proliferative activity and apoptosis. Moreover, as cancer arises from an imbalance between these two cellular processes a potential anti-tumour action of RGN can be anticipated.

### 3.3.2. Migration and invasion

Cell migration and invasion are critical hallmarks of cancer, related to the metastization process, and represent major barriers to good clinical outcomes in cancer therapy [333]. The association of RGN with the development of metastasis still is limited. However, recent information has been detailing the relationship of RGN with the control of cell invasion, migration and the metastatic capacity of cancer cells (Figure I.11). *In vitro* approaches using lung A549, liver HepG2 and pancreas MIA PaCa-2 cancer cells showed that RGN overexpression suppressed cell migration [307, 308, 310]. Moreover, Li *et al.* [295] demonstrated that RGN suppressed migration, invasion, and the metastization capacity of human cervical adenocarcinoma He-La cells, by inhibiting EMT through the suppression of the wingless-related integration site (Wnt)/ β-catenin signalling pathway. The TF β-catenin is the major player in the Wnt/β-catenin pathway with a pivotal role promoting migration and invasion of cancer cells [334]. Wnt/β-catenin activation also has been implicated in the EMT [335]. RGN overexpression increased the levels of GSK-3β and decreased the levels of phopho-GSK-3β and β-catenin (Figure I.11). As a consequence, the expression levels of β-catenin downstream targets, namely, MMP-3, MMP-7 and MMP-9, was also inhibited [295]. In the same way, it was found that the expression levels of the epithelial marker E-cadherin were increased in response to the suppression of β-catenin, whereas vimentin and N-cadherin (mesenchymal markers) expression remained unchanged [295]. On the other hand, RGN knockdown (KD) was shown to induce the opposite effect, increasing vimentin and N-cadherin levels (Figure I.11) [313].

Additionally, it was demonstrated that RGN influences the expression or activity of several modulators of signalling pathways that promote migration and invasion of cancer cells [336-341]. RGN was shown to decrease the levels of Ras, AKT, phospho-AKT, MAPK, phospho-MAPK, PI3K, NF-kβ, STAT3 and phospho-STAT3 [307-310, 312, 314, 317, 331]. Although further research is needed to understand the liaison of RGN with the migration and invasion capacities of cancer cells, these findings support its relevant role in suppressing these cancer hallmarks. Ras/MAPK and PI3K pathways are known to induce the expression of MMPs, EMT inductors and activate the NF-kβ pathway [336-338]. Furthermore, the activation of the NF-kβ pathway, induces the expression of cell adhesion molecules (ICAM, E-selectin), MMPs and angiogenic factors, whereas inhibiting the expression of MMPs inhibitors [341]. Also, activated STAT 3 translocates to the nucleus and induces the expression of MMP and angiogenic factors [339, 340].

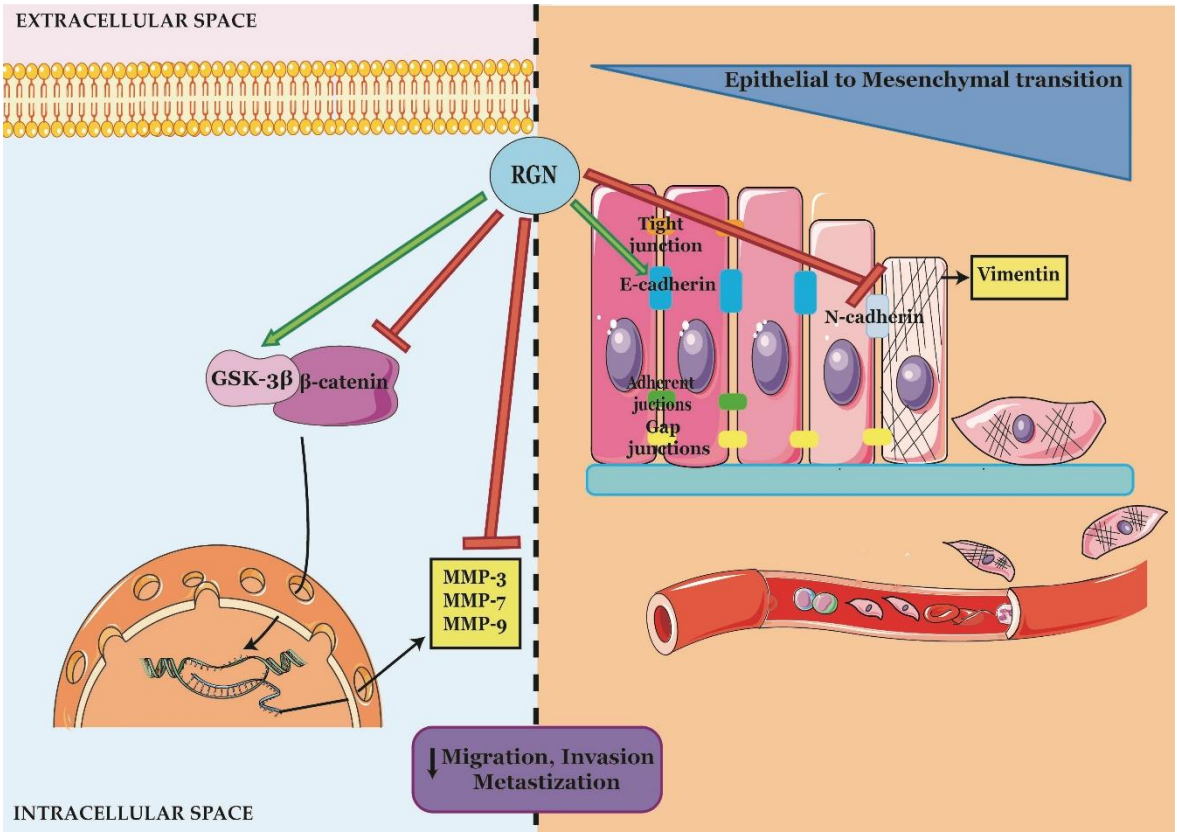


**Figure I.11.** Schematic representation of the effects of regucalcin (RGN) in the control of cell migration, invasion and metastization. RGN inhibits wingless-related integration site (Wnt)/β-catenin signalling pathway by decreasing the expression of β-catenin whereas increasing the expression of glycogen synthase kinase 3 β (GSK-3β). Inhibition of (Wnt)/β-catenin signalling pathway leads to the increase of E-cadherin and to the down-regulation of other downstream targets, namely, matrix metalloproteinase (MMP)-3, MMP-7 and MMP-9. Moreover, RGN inhibits epithelial to mesenchymal transition by inhibiting vimentin and N-cadherin expression. Legend: Green arrows mean a RGN stimulatory effect. Bar-ended red arrows indicate an inhibitory effect of RGN. Head-arrow black lines indicate the normal cellular process occurring upon activation of the Wnt/β-catenin signalling pathway.

### 3.3.3. Reprogramming of glycolytic metabolism

Metabolic reprogramming, namely the establishment of an hyperglycolytic phenotype, is a common feature of cancer cells, which is determinant to sustain excessive and uncontrolled proliferation [162]. Recognized as a cancer hallmark in 2011 [342], this cancer cells' feature is known almost for a century. Otto Warburg was the first researcher describing cancer cells' metabolism and their dependency on glucose as an energy source [343]. In aerobic conditions, normal cells convert glucose to pyruvate in the cytosol through glycolysis. Thereafter pyruvate is converted to acetyl-CoA entering the mitochondria to follow the tricarboxylic acid (TCA) cycle. Under anaerobic conditions, glycolysis is favoured and most of the pyruvate produced is converted to lactate to rapidly regenerate $NADP^+$ to be used in glycolysis. Nevertheless, a small fraction of pyruvate is sent to the mitochondria [162, 300, 344-346]. Otto Warburg pioneer work showed that, even in the presence of oxygen, cancer cells reprogram metabolism, using the end-product of glycolysis pyruvate to actively produce lactate [343]. This state is termed "aerobic glycolysis" and known as the Warburg effect [162, 300, 343-346]. The efficiency of ATP production through glycolysis is much

lower, but this metabolic switch with the increase of glucose uptake through the upregulation of glucose transporters (GLUTs) allows cancer cells to rapidly obtain energy [162, 300, 344, 345].

Considering PCa, this type of neoplasia has been shown to have unique metabolic features [347]. Contrarily to what happens in other cancers, PCa, in the initial phases of disease, relies on oxidative phosphorylation (OXPHOS) as an important source of energy [347]. Only with the progression to advanced stages, PCa acquires the hyperglycolytic phenotype using ATP from the aerobic glycolysis as energy [347, 348]. Even so, glucose consumption and lactate production are increased in androgen-sensitive human PCa cells being stimulated by androgens actions [348, 349]. Nevertheless, the CRPC PC3 cells displayed a higher glycolytic phenotype [348] .

*In vitro* and *in vivo* studies have linked RGN with the glycolytic state of cells and tissues (Figure I.12). In the rat liver, RGN reverted the effect of $Ca^{2+}$ and/or CaM inducing glycolysis, gluconeogenesis, TCA cycle and OXPHOS [350-353]. In the rat prostate, RGN *in vivo* overexpression was shown to inhibit glycolysis, and lactate production and export (Figure I.12) [354].

Glucose utilization starts with its entry into the cells by the activity of GLUTs. RGN actions concerning GLUTs were studied in H4-II-E cells and rat prostate, though distinct responses were obtained. Yamaguchi *et al.* showed that RGN overexpression in H4-II-E cells enhanced the mRNA expression of GLUT2 [355]. In the rat prostate, Vaz *et al.* reported that RGN overexpression decreased both mRNA and protein GLUT3 expression levels [354]. These findings indicate that the RGN action modulating glucose uptake may depend on the type of GLUT present in a specific tissue. This tissue-specific effect was corroborated by studies performed in bone marrow cells and rat prostate. In bone marrow cells was shown that RGN stimulated glucose uptake [356]. Contrarily, in the rat prostate, it was demonstrated that RGN inhibited glucose entry into the cells [354].

After entering the cell, glucose may be metabolized through glycolysis or stored as glycogen, with both mechanisms being regulated by RGN. RGN protein suppressed the activity of glycogen phosphorylase in the rat liver, which was concomitant with glycogen degradation (Figure I.12) [357]. Concerning glycolysis, studies in the rat prostate and liver showed that RGN suppressed the glycolytic activity by inhibiting phosphofructokinase (PFK) expression [354] and pyruvate kinase activity [350]. PFK and pyruvate kinase catalyse two rate-limiting steps of glycolysis, being the reaction catalysed by PFK (Figure I.12) indicative of "commitment" to glycolysis [346].

As mentioned above, pyruvate can be converted to lactate. Existent data showed that this metabolic step also is controlled by RGN. In bone marrow cells, it was reported that RGN decreased lactate production [356]. Conversely, in the rat prostate RGN protein decreased lactate production by inhibiting the activity and expression of lactate dehydrogenase (LDH)

[354]. Also, in the rat prostate, it was reported that RGN negatively regulates the expression levels of the monocarboxylate transporter 4, consequently decreasing lactate export [354].

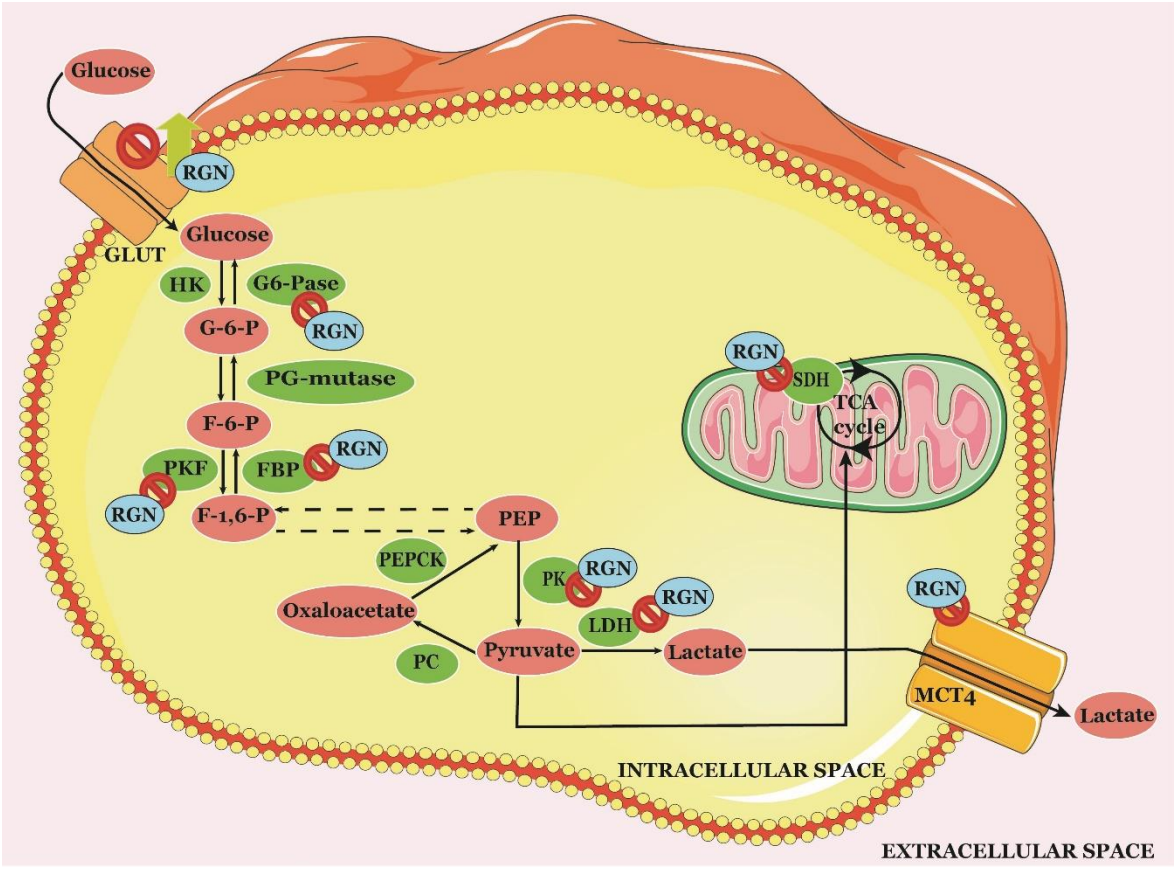


**Figure I.12.** Schematic representation of the regucalcin (RGN) actions over glycolytic metabolism. RGN seems to control glucose uptake, which was shown to be tissue dependent by increasing the expression of glucose transporter (GLUT) 2 (H4-II-E cells) and inhibiting the expression of GLUT3 (rat prostate). After entering the cell, glucose undergoes glycolysis, a metabolic process inhibited by RGN, as it decreased the expression of phosphofructokinase (PFK) and pyruvate kinase (PK). Moreover, RGN also inhibited tricarboxylic acid (TCA) cycle and oxidative phosphorylation through the inhibition of mitochondrial succinate dehydrogenase (SDH). The production and export of lactate also was inhibited by RGN concomitantly with the decreased expression and activity of lactate dehydrogenase (LDH) and reduced expression of monocarboxylate transporter (MCT) 4. Additionally, RGN also decreased the expression of glucose-6-phosphatase (G-6-Pase) and fructose-1,6-diphosphatase (FBP), inhibiting glucose synthesis. Legend: F-6-P, Fructose-6-phosphate; F-1,6-P, Fructose-1,6-biphosphate; G-6-P, Glucose-6-Phosphate; HK, Hexokinase; PC, Pyruvate carboxylase; PEPCK, PEP carboxykinase; PEP, Phosphoenolpyruvate; PG-mutase, Phosphoglucomutase. Green arrow means a RGN stimulatory effect. Prohibition signals indicate inhibition by RGN. Head-arrow dashed black lines indicated that there are intermediate steps of the metabolic pathway not shown.

RGN protein also suppressed gluconeogenesis in the rat liver, which similarly to glycolysis relied on the inhibition of rate-limiting steps of this pathway [358], namely, fructose-1,6-diphosphatase [351] and glucose-6-phosphatase (Figure I.12) [352].

Besides glycolysis, RGN suppressed TCA cycle and OXPHOS in the rat liver due to the inhibition of mitochondrial succinate dehydrogenase, an enzyme common to both metabolic pathways (Figure I.12) [353].

Gathering the data on the RGN's actions in regulating glycolytic metabolism and its deregulated expression in cancer, a role for RGN in the metabolic reprogramming of tumour cells can be proposed. More precisely, it is possible to assume that the loss of RGN found in several cancer cases could contribute to the metabolic switch of neoplastic cells and cancer

progression. On the other, it is also predictable that maintaining RGN expression levels may prevent the Warburg effect and, by consequence, tumour progression and aggressiveness. Both hypotheses need to be determined and open new avenues of research in cancer biology.

# 4.Regucalcin and prostate cancer

The background information presented in section 3 highlighted the relationship between RGN and PCa, indicating that this protein may have a suppressive role in prostate carcinogenesis. Herein, the data concerning the expression levels of RGN and its different actions in neoplastic and non-neoplastic prostate are summarised.

Our research group first reported the RGN expression in the rat prostate [225], and neoplastic and non-neoplastic human prostate tissues and cell lines [198, 242]. Moreover, immunohistochemistry analysis revealed that the RGN expression levels decrease with the cellular differentiation of prostate adenocarcinoma [198]. High RGN expression levels were detected in 40% of well differentiated prostate adenocarcinoma whereas only 12% of poorly differentiated adenocarcinoma presented this expression pattern [198]. It is not established that the loss of RGN contributes to prostate carcinogenesis, but RGN overexpression was shown to suppress the development of carcinogen-induced mammary gland tumours [293]. Also, the mechanisms that trigger the downregulation of RGN with cancer progression remain to clarify. However, several factors have been shown to influence the RGN expression pattern in prostate cells (Figure I.13).

Sex steroid hormones and $Ca^{2+}$ play an important role in the development and function of prostate [6, 41], which have prompted the research to determine whether these factors regulate RGN expression in the prostate. It was reported that $E_2$ decreased RGN expression levels in the rat prostate [225]. However, remains unknown if this effect is mediated by the genomic or non-genomic pathways (Figure I.13). Orchidectomized rats displayed enhanced RGN expression, an effect that was abrogated by the administration of DHT [228]. This DHT inhibitory effect over RGN expression was also found in LNCaP cells (Figure I.13), and implicated the classical AR genomic signalling [198]. Concerning $Ca^{2+}$, it was shown that physiological concentrations of this ion increased RGN expression in human non-neoplastic PNT1A and neoplastic LNCaP prostate cells (Figure I.13) [242]. However, this effect was faster in PNT1A cells, and higher $Ca^{2+}$ concentrations only increased the RGN expression levels in the non-neoplastic cell line. Interestingly, evidence from this study demonstrated that RGN might trigger a negative feedback-loop, contributing to the maintenance of its basal expression levels [242].

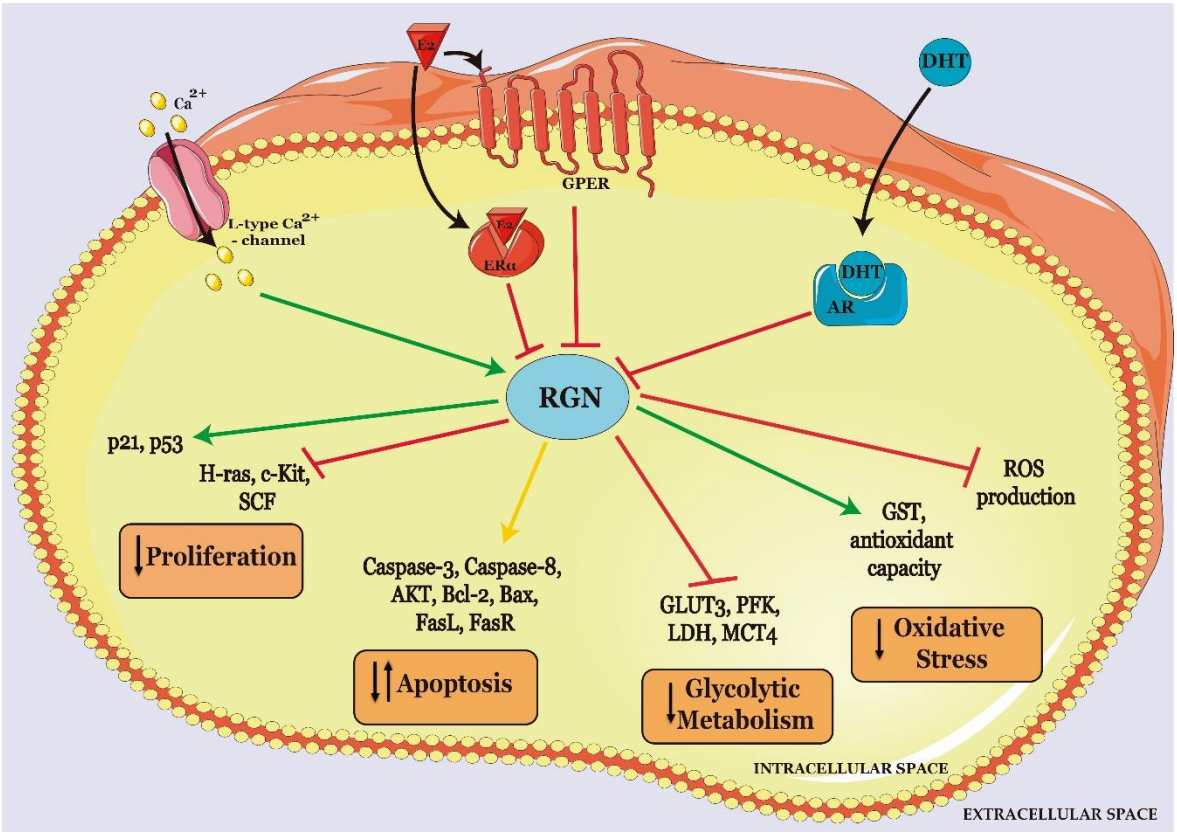


**Figure I.13.** Schematic representation of regucalcin (RGN) actions in prostate. RGN inhibited prostate cells' proliferation by inducing the expression of tumour suppressor proteins (p21 and p53) and inhibiting the expression of oncogenes (H-ras, c-Kit and stem cell factor (SCF)). RGN also influenced survival and apoptotic pathways in the prostate of Tg-RGN rats by modulating the expression and activity of AKT and caspase-3, and the expression of caspase-8, B-cell lymphoma 2 (Bcl-2) and Bcl-2 associated-X (Bax). In the prostate of older Tg-RGN rats, RGN induced apoptosis linked with Fas ligand (FasL) and Fas receptor (FasR) expression. Glycolytic activity, a known feature of cancer cells, was inhibited by RGN by decreasing glucose transporter 3 (GLUT3), phosphofructokinase (PFK) and monocarboxylate transporter (MCT) 4 expression, and lactate dehydrogenase (LDH) expression and activity. Additionally, RGN displayed a protective effect against oxidative stress in the rat prostate. It decreased the production of reactive oxygen species (ROS) and increased the anti-oxidant defence and glutathione-S-transferase (GST) activity. Several factors have been identified in maintaining RGN expression levels in prostate. Oestrogens inhibited RGN expression in the rat prostate, an effect that may be mediated by genomic and/or non-genomic pathways. Also, androgens decreased RGN expression dependently on the genomic AR signalling. Calcium ($Ca^{2+}$) was shown to increase RGN expression in PNT1A and LNCaP cells. Legend: DHT, 5α-dihydrotestosterone; $E_2$, 17β-estradiol; ERα, oestrogen receptor α; GPER, G protein-coupled oestrogen receptor. Green arrows mean a RGN stimulatory effect. Bar-ended red arrows indicate an inhibitory effect of RGN. Yellow arrows mean a RGN stimulatory or inhibitory effect.

Other *in vitro* and *in vivo* studies were performed to understand the importance of RGN in prostate tissue homeostasis and carcinogenesis (Figure I.13). The effect of RGN in prostate cell proliferation and apoptosis was studied in young and aged Tg-RGN [228, 271]. The *in vivo* studies reported that the overexpression of RGN suppressed cell proliferation by regulating the expression of tumour suppressor genes and oncogenes [228, 271]. Specifically, it was demonstrated that young Tg-RGN rats displayed decreased expression levels of SCF and H-ras [228], whereas in aged Tg-RGN rats diminished expression of the proliferation factors SCF and c-kit was found (Figure I.13), indicating reduced proliferative rates [271]. Curiously, a distinct effect was observed concerning apoptosis (Figure I.13). In young Tg-RGN rats an anti-apoptotic effect was reported [228]; however, in older animals higher apoptotic rates were described in Tg-RGN compared with wild-type [271]. RGN's effects in modulating apoptosis in the prostate of young and older Tg-RGN rats resulted

from the altered expression and activity of key apoptosis regulators. Although RGN overexpression did not affect p53 expression in younger animals, suppression of apoptosis was underpinned by the diminished expression and activity of caspase-3 and reduced expression of caspase-8. Simultaneously, Bcl-2 (anti-apoptotic)/Bax (pro-apoptotic) ratio, AKT expression and activity, and p21 expression were increased (Figure I.13) [228, 271]. Concerning aged rats, RGN overexpression induced prostate cells apoptosis by increasing caspase-3 expression and activity, and p53, caspase-8, Bax, FasL and FasR expression (Figure I.13). These findings suggested that RGN can trigger apoptosis in the rat prostate by activating the extrinsic pathway [271]. Also, a protective role against the age characteristic resistance to apoptosis was disclosed [271].

It has been reported that $Ca^{2+}$ and CaSR are important drivers of PCa tumorigenesis, being strongly associated with metastization [359, 360]. In non-neoplastic PNT1A prostate cells, *RGN* gene silencing decreased the expression of CaSR [242], an effect that was not observed in LNCaP cells [242]. These findings proposed that the RGN inhibitory role in the expression CaSR may be lost in cancer cells and that this may be a pathway by which RGN could protect the development of prostate gland tumours.

The increase of OS has been identified as one of the major causes of aging-related diseases and cancer [361, 362]. RGN protein protected the prostate of young and old Tg-RGN rats against OS (Figure I.13) [271], but different underlying mechanisms were identified. In the prostate of young rats, RGN overexpression decreased OS by inhibiting the production of ROS (Figure I.13) [271]. On the other hand, in the prostate of older Tg-RGN animals, the age-related increase of OS was counteracted by the enhanced GST activity and total antioxidant capacity (Figure I.13) [271].

The last decade has witnessed the emergence of metabolic reprogramming as a hallmark of cancer [342], which together with the metabolic actions proposed for RGN (section 3.3.3) stimulated investigating the glycolytic metabolism in the prostate of Tg-RGN rats [354]. Vaz *et al.* [354] demonstrated that RGN overexpression decreased glucose content in the rat prostate due to the inhibition of GLUT3 expression (Figure I.13). The same report demonstrated that RGN also reduced lactate production and export by decreasing the activity and expression of LDH and the expression of monocarboxylate transporter 4 (Figure I.13) [354]. Moreover, it was reported that RGN suppressed the glycolytic flux by reducing the expression of PFK (Figure I.13) [354], a critical enzyme in glycolysis catalysing a rate-limiting step (Figure I.12). These findings are of utmost importance as the hyperglycolytic phenotype of PCa has been associated with increased proliferative ability and progression of disease [348].

In sum, broad RGN actions targeting several cancer hallmarks were reported in human PCa cells and rat prostate, namely counteracting the hyper-proliferative status, resistance to apoptosis, augmented OS and metabolic reprogramming [162, 299, 300]. Based on the

accumulated knowledge, it is liable to speculate that RGN can act as a tumour suppressor protein protecting against the development of PCa.

# II. Aim of the Thesis

The multifunctional protein RGN has been linked with the control of several biological processes, namely, cell survival and growth. Also, the down-deregulated expression of RGN has been identified as a typical feature of several human cancers. Our research group demonstrated that RGN is under-expressed in human PCa cases, with the loss of RGN expression accompanying the cellular differentiation of prostate adenocarcinoma. However, it is not established if it is the down-regulation of RGN that contributes to carcinogenesis, or if it is a consequence of cancer development itself. Nevertheless, the *in vivo* overexpression of RGN was shown to suppress OS and cell proliferation in rat prostate. Moreover, previous work of our team also showed that RGN overexpression markedly inhibited the development of carcinogen-induced mammary gland tumours. All these findings highly support the role of RGN as a putative tumour suppressor protein. However, it is entirely unknown if the altered RGN expression levels can affect the survival and proliferative behaviour of human prostate cells.
As a multifunctional protein, RGN actions in the regulation of cell metabolism also have been reported.

The present dissertation aims to disclose the relationship of RGN expression levels with the hallmarks of cancer and patients' outcomes. For this purpose, the following experimental approaches were implemented:

- *In silico* analysis of RGN mRNA expression in human prostate correlated with patient clinicopathological data, PCa progression, GS, disease-free survival and recurrence. RGN expression correlated with other key molecular targets also was analysed.
- Silencing of *RGN* gene in non-neoplastic human prostate cells using siRNA, and analysis of cell proliferation, apoptosis, and glycolytic metabolism in response to RGN-KD.

Establishing the relationship between RGN silencing and the altered proliferation, apoptotic activity and glycolytic profile of human non-neoplastic prostate cells will help in understanding the protective roles of RGN against carcinogenesis, and this protein's role as a tumour suppressor.

# III. Materials and Methods

## 1. Reagents

All chemicals, culture medium and antibodies unless otherwise stated were purchased from Sigma-Aldrich (St. Louis, MO, USA).

## 2. *In silico* analysis of regucalcin expression in human prostate cancer cases

The CancerTool resource (http://web.bioinformatics.cicbiogune.es/CANCERTOOL/) [363] was used to analyse the RGN expression in several patients datasets, namely, Glinsky *et al.* [364], Grasso *et al.* [365], Varambally *et al.* [366], Taylor *et al.* [104], Tomlins *et al.* [367] and Lapointe *et al.* [368] and TCGA [369] . RGN mRNA expression in human prostate cases was correlated with patient clinicopathological data, PCa progression, GS, disease-free survival and recurrence. The number of cases in each condition for the different datasets is provided in Appendix (Table VIII.1).

A gene-to-gene correlation of RGN expression with that of oncogenes, and target regulators of cell cycle, and apoptotic and survival pathways also was performed.

## 3. Regucalcin gene silencing in non-neoplastic PNT1A cells

The human non-neoplastic prostate epithelial cell line PNT1A was acquired from the European Collection of Cell Cultures (ECACC, Salisbury, UK). This epithelial cell line has been widely used as a model to study the proliferative behaviour of human prostate cells in different experimental conditions [370, 371].

PNT1A cells were maintained in RPMI 1640 - phenol red medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S), at 37 ºC in a humid atmosphere equilibrated with 5% $CO_2$. PNT1A cells were seeded in 6-well plates at a density of 1,5 x $10^5$ cells per well in 2 mL of RPMI 1640-phenol red medium supplemented with 10% FBS and 1% P/S. After 24 h, cell culture medium was replaced by antibiotics-free medium. At 50% confluence, PNT1A cells were transfected with 10 nM of a small interfering RNA (siRNA) targeting RGN (si-RGN, s17374, Ambion, Carlsbad, CA, USA), or scramble siRNA (si-scramble, sc-37007, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 24 h. For this purpose, the appropriated quantity of si-scramble and si-RGN (per well) was diluted in 150 µL of Opti-MEM® (mix A), and simultaneously, 9 uL of Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) were diluted in 150 µL of Opti-MEM® (mix B). After incubation for 5 minutes at room temperature (RT), mix A and B were combined, and the formation of siRNA: Lipofectamine RNAiMAX complexes occurred for additional 20 minutes at RT. Then, the siRNA: Lipofectamine RNAiMAX complexes were added to each well and cells were incubated at 37 ºC in a humid atmosphere equilibrated with 5% $CO_2$ for 24 h.

The si-RNA transfection protocol described was performed for different concentrations of si-RGN and time of transfection. The best efficiency of RGN knockdown was obtained with 10 nM of si-RGN for 24 h. The RGN silencing was confirmed by real time-polymerase chain reaction (qPCR, section 6).

si-RGN and si-scramble-transfected PNT1A cells (5 x $10^3$ cells/well in 96-well plates or ~ 3 x $10^5$ cells in 25 $cm^2$ tissue culture flasks) were maintained in culture for different experimental time points. At the end of experiments, cells in 25 $cm^2$ tissue culture flasks were trypsinized and harvested for RNA and protein extraction, and subsequent real-time polymerase chain reaction (qPCR, section 8) and Western Blot (WB, section 10) analysis, and determination of caspase-3 like activity (section 11). Cell culture medium was collected for quantification of glucose and lactate (section 12).

## 4. Cell viability assay

Viability of si-RGN and si-scramble-transfected PNT1A cells cultured in 96-well plates for 48, 72 and 96 h was evaluated by the MTT (3-(4,5-dimethylthiazol-2- thiazolyl)-2,5-diphenyltetrazolium bromide) assay. The yellow tetrazole MTT is reduced to formazan in metabolically active cells, which is used as an indicator of cell viability [372].

In brief, cell culture medium was removed and the MTT solution at a final concentration of 0.5 mg/mL was added. After incubation in the dark at 37 ºC for 4 h, the MTT solution was carefully removed and 100 uL of dimethyl sulfoxide was added to solubilize the formed purple formazan crystals. The absorbance of the resultant purple coloured solution was measured at 570 nm using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, CA, USA). The value of absorbance obtained is directly proportional to the number of viable cells in each experimental group.

## 5. Sulforhodamine B assay (SRB)

The cellular mass of si-RGN and si-scramble-transfected PNT1A cells cultured in 96-well plates for 48, 72 and 96 h was measured by the Sulforhodamine B (SRB) assay. SRB is a bright-pink aminoxanthene that binds basic amino-acid residues, which allows the determination of cell mass that is used as an indicator of cell proliferation [373].

Briefly, cells were fixed with 50 uL of 60% trichloroacetic acid, overnight at 4 ºC. After removing the fixing solution and washing with milli-Q water, fixed cellular proteins were stained with SRB solution (0.05% (w/v) in 1% acetic acid) for 1 h at 37 °C. The unbound dye was removed with 1% acetic acid, and cell bound dye solubilized with 10 mM Tris base solution, pH 10. The absorbance of the bright-pink coloured solution was measured at 540 nm using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). The absorbance measured is directly proportional to the cell mass in each experimental group.

## 6.Ki-67 fluorescent immunocytochemistry

PNT1A cells were fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 1% Triton X-100 for 5 minutes at RT. Then, nonspecific staining was blocked by incubation with phosphate buffer saline (PBS) containing 0.1% (w/v) Tween-20 (PBS-T) and 20% FBS for 1 h at RT. After washing, cells were incubated with a rabbit anti-ki-67 (1:50, ab16667, Abcam, Cambridge, United Kingdom) primary antibody for 1 h at RT. Next, cells were washed and incubated with the Alexa fluor 546 goat anti-rabbit IgG (1:1000, Invitrogen) secondary antibody for 1 h at RT. After washing cell nuclei were stained with Hoechst 33342 (5 μg/mL, Invitrogen) for 10 minutes at RT. Then, lamellae were washed with PBS-T and mounted using Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Specificity of the staining was assessed by the omission of primary antibody. Images were acquired using a Zeiss LSM710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany). Proliferation index was determined by the percentage of Ki-67-positive cells out of the total number of Hoechst stained nuclei in 15 randomly selected ×40 magnification fields for each section.

## 7. RNA Extraction

si-RGN and si-scramble-transfected PNT1A cells were homogenized in the adequate volume of Trizol (GRiSP, Oporto, Portugal) reagent and incubated for 5 minutes at RT. Next, 200 uL of chloroform per 1 mL of Trizol was added and samples were vigorously mixed and incubated at RT for 10 minutes. A centrifugation step at 12000 G, 4 ºC for 15 minutes separated the mixture into 3 phases. The upper aqueous phase containing the total RNA was collected to fresh tubes. RNA was precipitated with 500 μL of isopropanol per 1 mL of Trizol and an incubation at RT for 10 minutes. Next, a centrifugation at 12000 G for 10 minutes at 4 ºC was performed and the supernatant carefully rejected. The RNA pellet was washed with 1 mL of 75% cold-EtOH per 1 mL of Trizol and centrifuged at 7500 G for 5 minutes at 4 ºC. The RNA pellet was resuspended in 20 μL of diethylpyrocarbonate treated-water.

The quantity and integrity of total RNA was estimated by spectrophotometric analysis at 260 and 280 nm (NanoPhotometer, Implen, Munich, Germany), followed by an agarose gel electrophoresis.

## 8.Real-time polymerase chain reaction

qPCR was carried out to confirm the *RGN* gene KD in the si-RGN PNT1A cells. cDNA was synthetized using 1 ug of total RNA in a final volume of 20 uL using the NZY First Strand cDNA Synthesis kit (NZYtech, Lisbon, Portugal), following the manufacturer's instructions. Details about the amplification primers and cycling conditions are indicated in Table III.1. Gene expression was normalized using beta-2-microglobulin (β2M) as the reference gene.

qPCR reactions were carried out in the CFX Connect ™ Real-Time PCR Detection System (Bio-Rad) and the efficiency of amplification was determined for all primer sets using serial dilutions of cDNA (1, 1:10 and 1:100). PCR conditions and reagents concentrations were previously optimized and the specificity of the amplicons was determined by melting curves analysis. All qPCR reactions were performed in duplicate and a negative control without reverse-transcribed RNA was included in each qPCR. For each reaction, 1 uL of cDNA was used in a final volume of 20 µL containing 10 uL of iTaq Universal SYBR Green Supermix (2X) (Bio-Rad), sterile and nuclease-free $H_2O$ and sense and antisense primers (Table III.1). Reactions conditions were: initial denaturation at 95 ºC for 5 minutes, followed by 40 cycles of denaturation at 95 ºC for 10 seconds, annealing at 60 ºC for 30 seconds and extension at 72 ºC for 10 seconds. The melting curve was constructed by heating reactions from 60 ºC to 95 ºC with 5 seconds hold at each temperature. Normalized expression values were calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-(\Delta\Delta Ct)}$ [374].

**Table III.1.** Details of primers and real-time polymerase chain reaction cycling conditions.

| Gene | Primer Sequence (5' – 3') | Amplicon size (bp) | AT (ºC) | Number of amplification cycles |
|---|---|---|---|---|
| *Regucalcin* | Sense: GCAAGTACAGCGAGTGACC<br>Antisense: TTCCCATCATTGAAGCGATTG | 177 | 60 | 40 |
| *B-2-microglobulin* | Sense: ATGAGTATGCCTGCCGTGTG<br>Antisense: CAAACCTCCATGATGCTGCTTAC | 93 | 60 | 40 |

AT: annealing temperature

## 9.Protein extraction

si-RGN and si-scramble-transfected PNT1A cells were homogenized in the appropriated volume of radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris, 1 mM EDTA) supplemented with 1% protease inhibitor cocktail and 10% phosphatase inhibitor cocktail. Samples were kept on ice for 20 minutes, with vortex every 5 minutes, and centrifuged at 14000 rpm for 20 minutes at 4 ºC. Total protein extracts were recovered to fresh tubes. Protein concentration was determinated using the Pierce™ BCA Protein Assay Kit (Thermo Fisher, Waltham, MA, EUA), according to the manufacturer´s instructions.

## 10.Western blot

Protein extracts (25 ug) were resolved on a 12.5% acryl-bisacrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electro-transferred to polyvinylidene difluoride membranes (Bio-Rad). Subsequently, membranes were incubated overnight at 4 ˚C with rabbit anti-AKT (1:500, #9272; Cell Signaling Technology, Danvers, MA, EUA), rabbit anti-Phospho-AKT (1:500, #9271; Cell Signaling Technology), rabbit anti-MAPK

(1:500, #9102; Cell Signaling Technology), rabbit anti-phospho-MAPK (1:500, #9101; Cell Signaling Technology), rabbit anti-phospho-c-myc (1:500, #13748; Cell Signaling Technology), rabbit anti-p53 (1:1000, FL-393:sc-6243; Santa Cruz Biotechnology), rabbit anti-FasL (1:1000, C-178:sc-6237; Santa Cruz Biotechnology), rabbit anti-FasR (1:1000, A-20: sc-1023; Santa Cruz Biotechnology), rabbit anti-Caspase-9 (1:500, H-35:sc-8355; Santa Cruz Biotechnology) and rabbit anti-Caspase-8 (1:250, #4790; Cell Signaling Technology) primary antibodies. Protein expression levels were normalized using a mouse anti-β-actin (1:10000, A1978) primary antibody. Goat anti-rabbit IgG HRP-linked (1:10000, #7074, Cell Signaling Technology) or anti-mouse-IgGκ BP-HRP linked (1:20000, sc-516102; Santa Cruz Biotechnology) were used as secondary antibodies (1 h incubation at RT). The membranes were incubated with ECL substrate (Bio-Rad) for 5 minutes and immunoreactive proteins were visualized with the ChemiDoc™ MP Imaging System (Bio-Rad). Band densities were obtained according to standard methods using the Image Lab 5.1 software (Bio-Rad) and normalized with the respective β-actin band density.

## 11. Caspase-3-like activity assay

Caspase-3-like activity was measured spectrophotometrically through the detection of p-nitro-aniline (pNA), the yellow product from the cleavage of caspase-3-substrate (Ac-DEVD-pNA).

Briefly, 5 µL of total protein extract was incubated overnight at 37 ºC with assay buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT) and 200 µM of Ac-DEVD-pNA. Next, the absorbance was measured at 405 nm using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). The amount of pNA produced was calculated by extrapolation using a standard curve with known amounts of pNA. All measurements were normalized to the total amount (µg) of protein in each sample.

## 12. Quantification of glucose and lactate

The concentrations of glucose and lactate in the cell culture medium of si-RGN and si-scramble-transfected PNT1A cells was determined through spectrophotometric analysis using commercial kits (Spinreact, Girona, Spain) following the manufacturer's instructions. Glucose consumption and lactate production were determined relatively to the initial concentration of these metabolites at 0 h of treatment and normalized to the total amount (µg) of protein in each sample.

## 13. Statistical analysis

Statistically significant differences between the experimental groups were evaluated by unpaired T-test with Welch's correction using GraphPad Prism v8.00 (GraphPad Software, San Diego, CA, USA). Statistical analysis provided by CancerTool included ANOVA,

student t-test, Mantel-Cox test and Pearson and Spearman correlations. $P< 0.05$ was considered statistically significant. All experimental data are shown as mean ± S.E.M.

# IV. Results

# 1. Regucalcin expression is down-regulated in metastatic prostate cancer and related with poor survival outcomes

The expression levels of RGN were analysed in non-tumoral and tumoral conditions in five patients datasets using the CancerTool resource [363]. Data analysis in four (Grasso *et al.* [365], Varambally *et al.* [366], Taylor *et al.* [104] and Lapointe *et al.* [368]) of the five datasets considered demonstrated that RGN expression is significantly decreased in PCa cases (Figure IV.1). Furthermore, the deregulated expression of RGN with the appearance of primary prostate tumours continues with the progression of disease to metastatic PCa. RGN expression in metastatic tumours was significantly decreased in three (Grasso *et al.* [365], Taylor *et al.* [104] and Lapointe *et al.* [368]) of the five datasets analysed (Figure IV.2). Nevertheless, in the Varambally *et al.* dataset [366] although RGN expression did not change between non-tumour and primary-tumour conditions, it decreased severely when PCa progresses to the metastatic stage.

No relationship was found between RGN expression and the GS of PCa (Figure IV.3). However, for one (Glinsky *et al.* [364]) of the three datasets evaluated RGN expression levels were correlated with disease free-survival. The group of patients with RGN expression greater than or equal to the mean expression levels had higher disease-free survival compared to patients with lower RGN expression (Figure IV.4). RGN expression levels correlated with the recurrence of PCa were also analysed. None of the three datasets analysed showed any difference between disease-free and recurred patients (Figure IV.5).

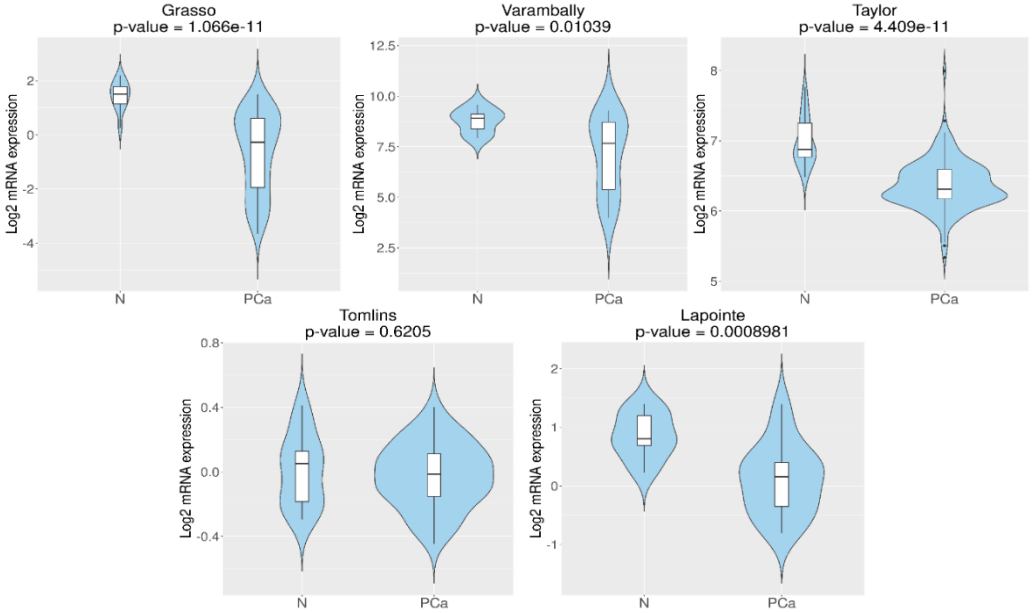


**Figure IV.1.** Regucalcin (RGN) expression in human prostate cancer (PCa) cases determined using the CancerTool resource [363]. Violin plots show the expression of RGN in non-tumoral (N) and PCa tissues for each of the datasets used, Grasso *et al.* [365], Varambally *et al.* [366], Taylor *et al.* [104], Tomlins *et al.* [367] and Lapointe *et al.* [368]. Mean gene expression between two groups was compared with a Student T-test.

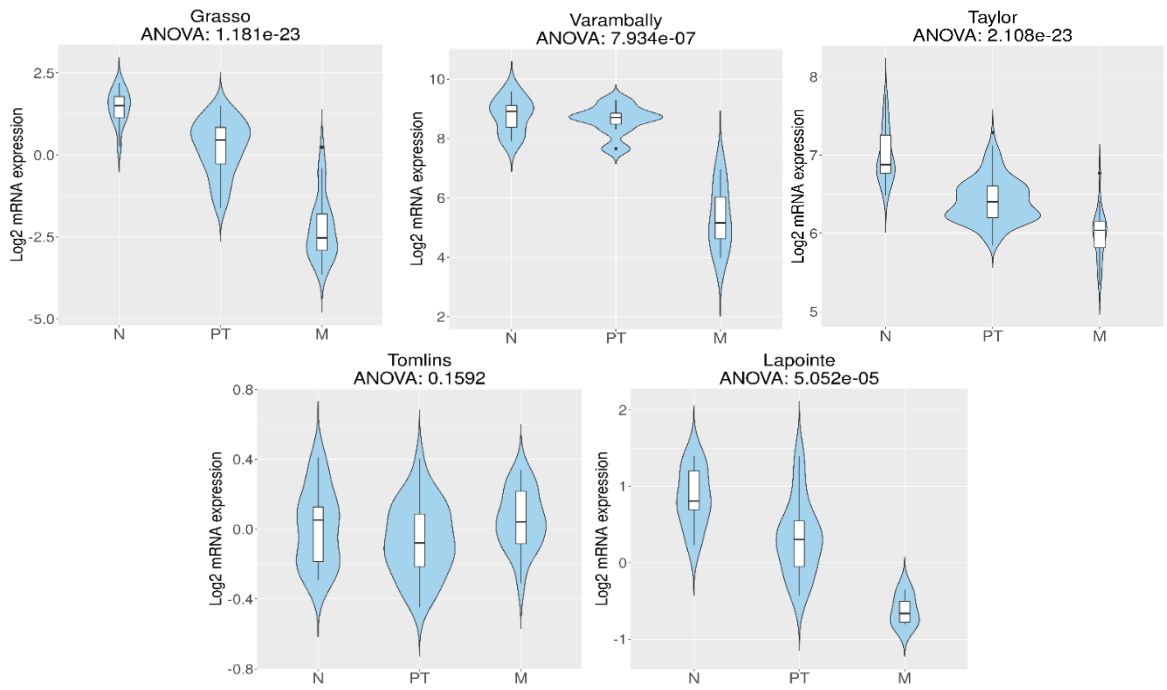


**Figure IV.2.** Regucalcin (RGN) expression with the progression of human prostate cancer (PCa) determined using the CancerTool resource [363]. Violin plots show the expression of RGN in non-tumoral tissue (N), primary tumours (PT) and metastatic PCa (M) for each datasets used, Grasso *et al.* [365], Varambally *et al.* [366], Taylor *et al.* [104], Tomlins *et al.* [367] and Lapointe *et al.* [368]. Mean gene expression between the three groups was compared with an ANOVA test.

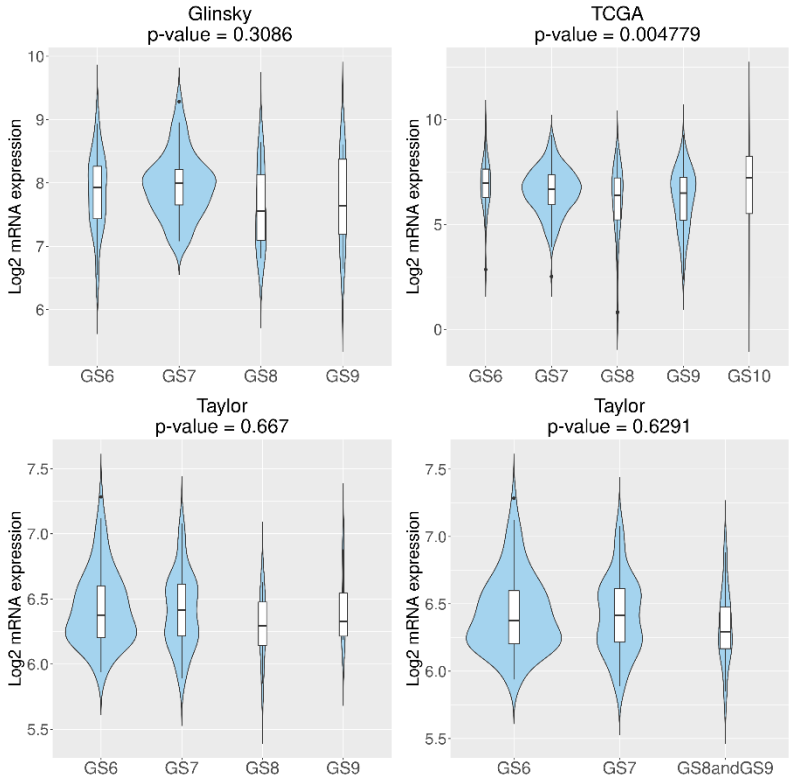


**Figure IV.3.** Regucalcin (RGN) expression accordingly with prostate cancer (PCa) Gleason score (GS) determined using the CancerTool resource [363]. Violin plots show the expression of RGN in PCa samples with GS ranging from 6 (GS6) to 10 (GS10) for each of the datasets used, Glinsky *et al.* [364], TCGA [369] and Taylor *et al.* [104]. Mean gene expression between the groups was compared with an ANOVA test.

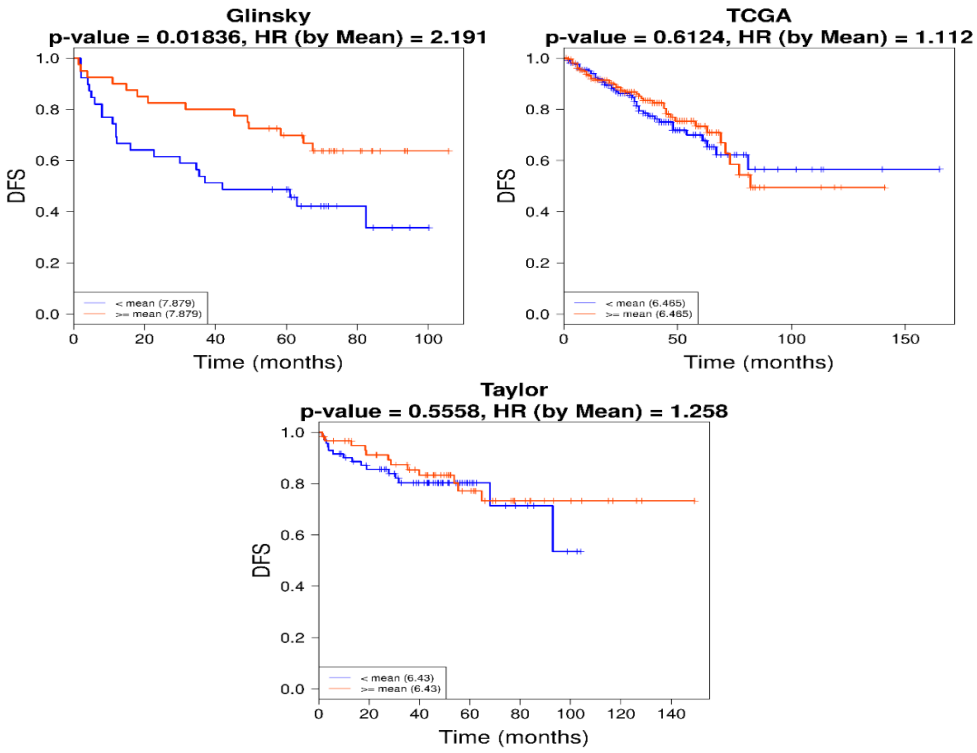


**Figure IV.4.** Regucalcin (RGN) expression and prostate cancer (PCa) patients disease free-survival (DFS) determined using the CancerTool resource [363]. Kaplan-Meier curves represent the DFS of patient groups with distinct RGN expression levels. Each curve represents the percentage of the PCa patients that exhibit DFS over time (months) when RGN expression is lower (blue curve), higher or equal (red curve) to the mean. Data from Glinsky *et al.* [364], TCGA [369] and Taylor *et al.*[104] datasets are shown. Vertical ticks indicate censored patients. Differences between groups were compared with a Mantel-Cox test, and a Cox proportional hazards regression model was performed to calculate de Hazard Ratio (HR).

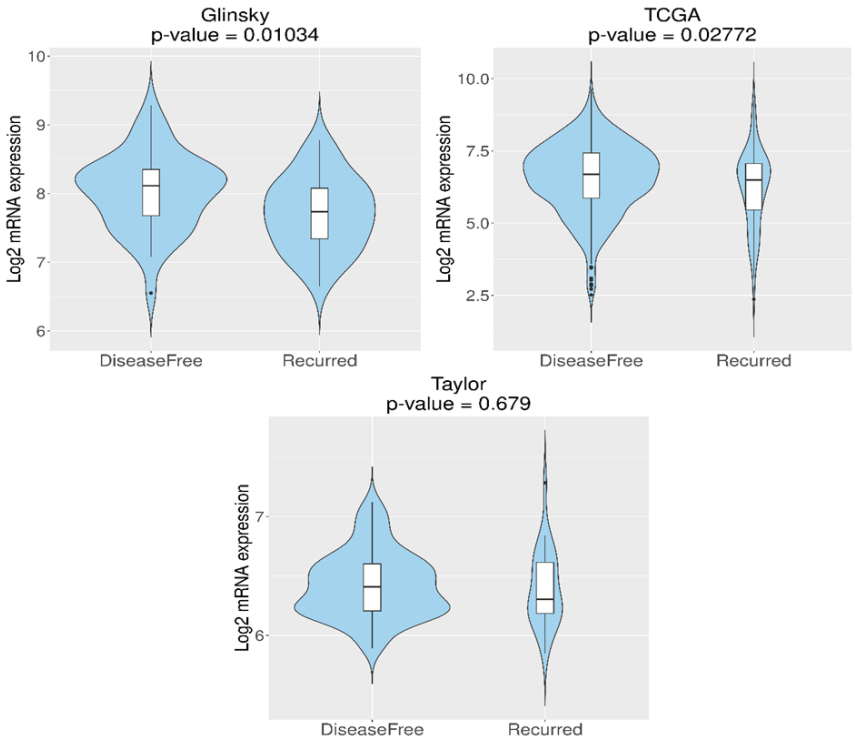


**Figure IV.5.** Regucalcin (RGN) expression and prostate cancer recurrence determined using the CancerTool resource [363]. Violin plots show the expression of RGN in disease-free and recurred patients for each of the datasets used, Glinsky *et al.* [364], TCGA [369] and Taylor *et al.* [104]. Mean gene expression between two groups was compared with a Student T-test.

## 2. Regucalcin expression correlates with CDKN1A and IL6 in prostate cancer cases

In order to identify the RGN' protein network in PCa, RGN expression was correlated with that of oncogenes and target regulators of cell cycle, and apoptotic and survival pathways (Table IV.1).

**Table IV.1.** Oncogenes and regulators of cell cycle, survival and apoptosis used for expression analysis correlated with regucalcin in human prostate cancer cases.

| Gene | Protein | Function | References |
|---|---|---|---|
| *Cyclin dependent kinase inhibitor 1A* | p21 | Cell cycle inhibitor; induces cell cycle arrest and cellular senescence; plays an important role in DNA repair and inhibition of apoptosis. | [375] |
| *Tumour Protein P53* | p53 | Transcription factor activated by several genotoxic and cellular stress signals; regulates transcription of proteins that control cell cycle arrest, apoptosis, senescence, differentiation and DNA repair. | [153] |
| *IL6* | Interleukin-6 | Pro-inflammatory cytokine that controls the acute phase response at the beginning of acute inflammation; stimulates cell survival, proliferation, invasion and metastization, and the angiogenic process. | [124] |
| *AKT1* | AKT | Oncogenic protein at the downstream activation of PI3K that induces cell survival, growth, proliferation, migration and angiogenesis. | [108] |
| *Mitogen Activated Protein Kinase 1* | Mitogen Activated Protein Kinase | Oncogenic protein in the MAPK/ERK pathway that promotes cell survival, proliferation, differentiation, migration and metabolic reprogramming. | [376, 377] |
| *MYC* | c-myc | Transcription factor that regulates the expression of genes related with cell proliferation, differentiation, apoptosis and metabolism. | [378, 379] |
| *Nuclear Factor Kappa B Subunit 1* | Nuclear Factor Kappa-light-chain-enhancer of activated B cells | Transcription factor that regulates the expression of proteins that promote inflammation, cell growth, survival and proliferation. | [380] |

Using the CancerTool resource [363], gene-to-gene correlation analysis of *RGN* expression with that *cyclin dependent kinase inhibitor 1A (CDKN1A), tumour protein P53 (TP53), IL6, AKT1, MAPK1, MYC and nuclear factor kappa B subunit 1 (NFKB1)* genes in non-tumour and tumour conditions was performed in seven patients datasets (Glinsky *et al.* [364], Grasso *et al.* [365], Lapointe *et al.* [368], Taylor *et al.* [104], TCGA [369], Tomlins *et al.* [367] and Varambally *et al.* [366] ) (Figure IV.6 and Figure IV.7).

In primary PCa tumours, *RGN* gene expression was directly correlated with *CDKN1A* and *IL6*, which was confirmed by the correlation analysis employed (Pearson, Figure IV.6B and Spearman, Figure IV.7B). No correlation was found between the *RGN* gene expression and that of *CDKN1A, TP53, IL6, AKT1, MAPK1, MYC and NFKB1*, both in non-tumour tissue (Figure IV.6A) and metastatic PCa (Figure IV.6C). Moreover, these results were consistent for both Pearson (Figure IV.6A and Figure IV.6C) and Spearman (Figure IV.7A and Figure IV.7C) correlation analysis.

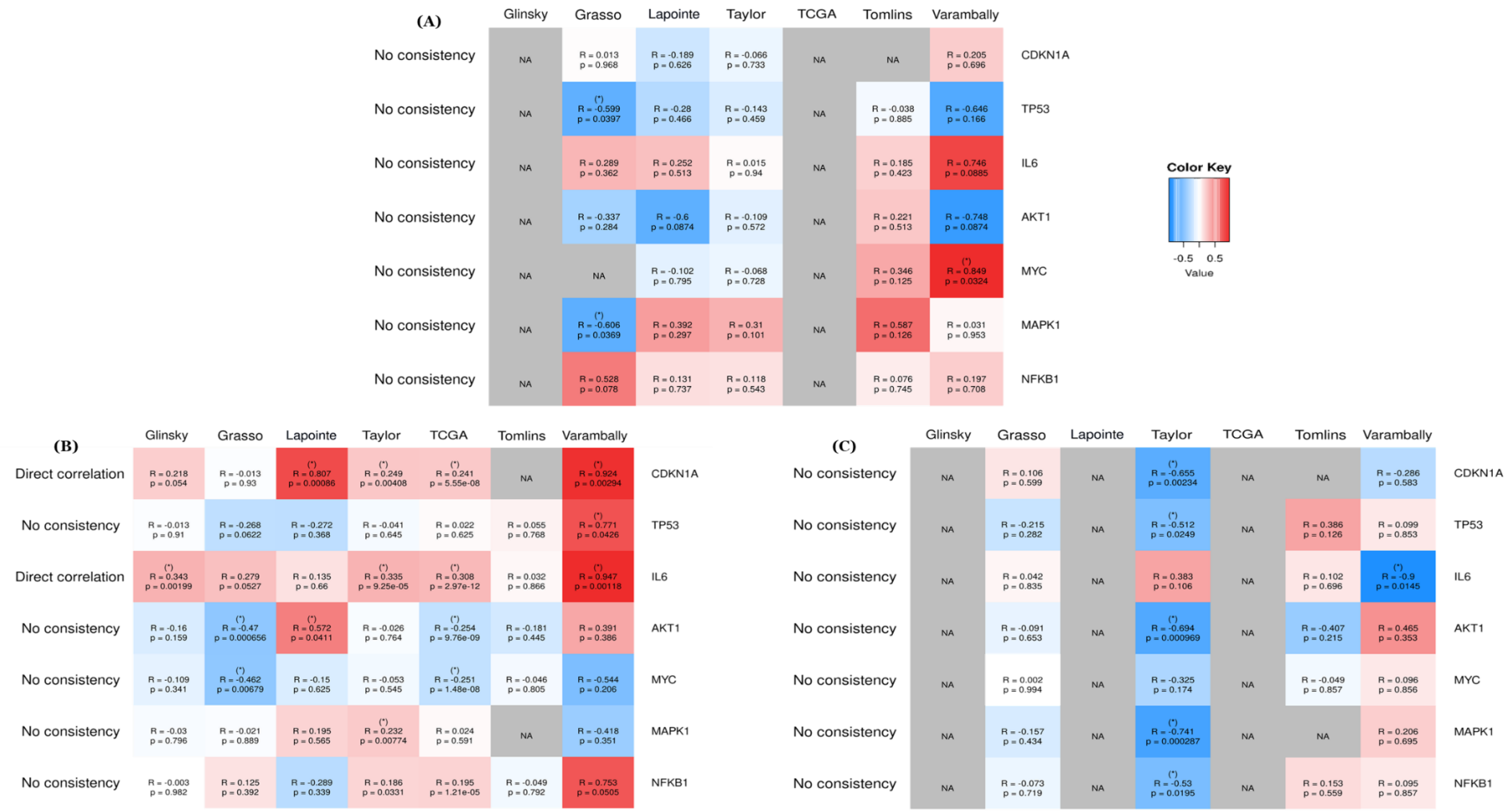


**Figure IV.6.** Representative heatmaps for Pearson correlation analyses of *regucalcin* gene expression with that of *cyclin dependent kinase inhibitor 1A* (*CDKN1A*), *tumour protein P53* (*TP53*), *IL6*, *AKT1*, *mitogen activated protein kinase 1* (*MAPK1*), *MYC* and *nuclear factor kappa B subunit 1* (*NFKB1*) genes in non-tumour tissues **(A)** primary prostate tumours **(B)** and metastatic prostate cancer **(C)** obtained using the CancerTool resource [363] and the Glinsky *et al.* [364], Grasso *et al.* [365], Lapointe *et al.* [368], Taylor *et al.* [104], TCGA [369], Tomlins *et al.* [367] and Varambally *et al.* [366] datasets. The colour of each cell represents the correlation R value towards 1 (direct correlation, red) or -1 (inverse correlation, blue). In the case of datasets contain insufficient number of samples the correlation is annotated as not applicable (NA) and the cell is grey with no data. The correlations with $p \leq 0.05$ and correlation coefficient greater than 0.2 for the direct and lower than 0.2 for inverse correlations are indicated with (*). On the left side, the coherence among datasets is shown for each pair of genes (direct correlation is considered when in more than 50% of datasets the $p$ is equal or lower than 0.05 and correlation coefficient is greater or lower than 0.2).

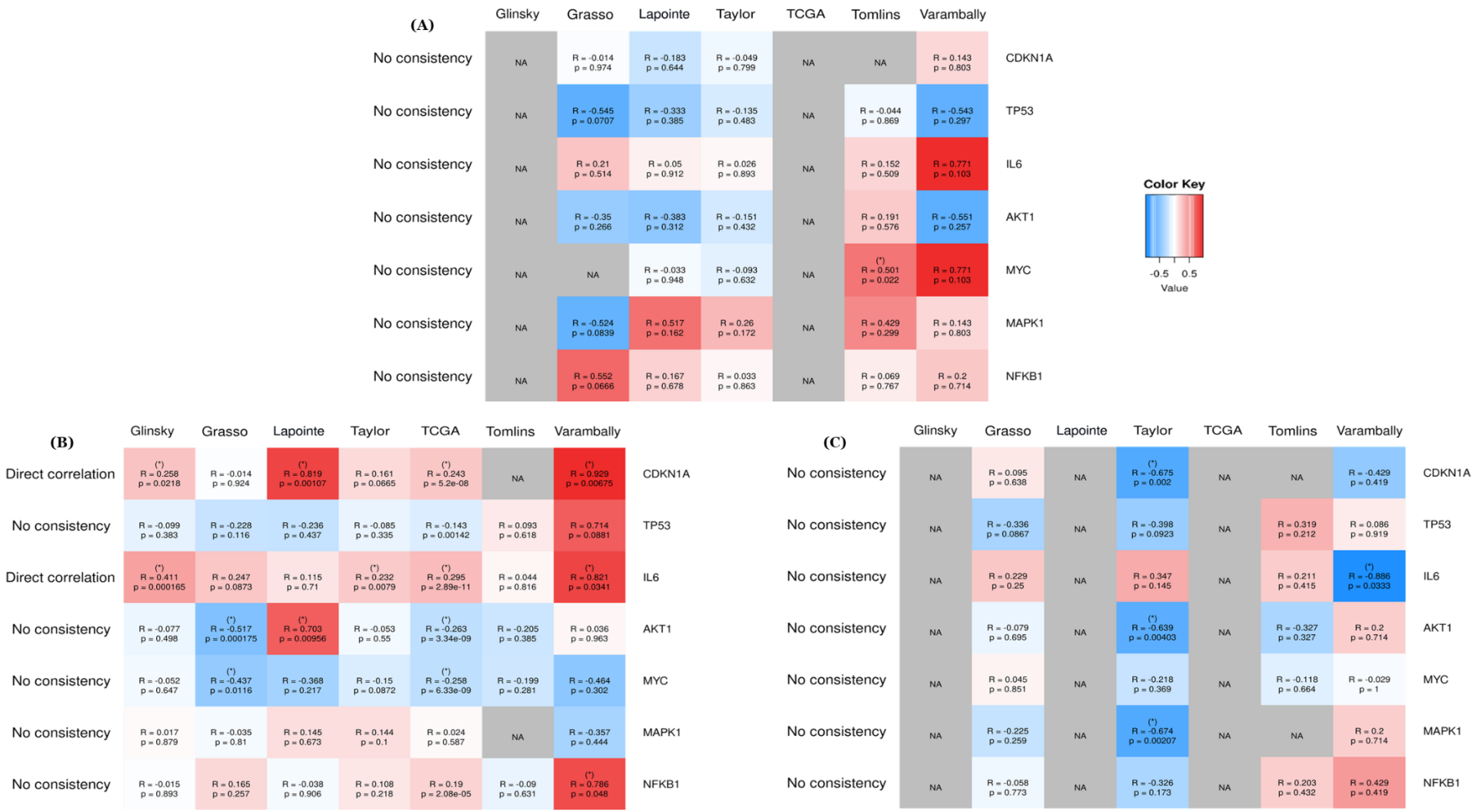


**Figure IV.7.** Representative heatmaps for Spearman correlation analyses of *regucalcin* gene expression with that of *cyclin dependent kinase inhibitor 1A* (*CDKN1A*), *tumour protein P53* (*TP53*), *IL6*, *AKT1*, *mitogen activated protein kinase 1* (*MAPK1*), *MYC* and *nuclear factor kappa B subunit 1* (*NFKB1*) genes in non-tumour tissues **(A)** primary prostate tumours **(B)** and metastatic prostate cancer **(C)** obtained using the CancerTool resource [363] and Glinsky *et al.* [364], Grasso *et al.* [365] , Lapointe *et al.* [368], Taylor *et al.* [104], TCGA [369], Tomlins *et al.* [367] and Varambally *et al.* [366] datasets. The colour of each cell represents the correlation R value towards 1 (direct correlation, red) or -1 (inverse correlation, blue). In the case of datasets contain insufficient number of samples the correlation is annotated as not applicable (NA) and the cell is grey and with no data. The correlations with $p \leq 0.05$ and correlation coefficient greater than 0.2 for the direct and lower than 0.2 for inverse correlations are indicated with (*). On the left side, the coherence among datasets is shown for each pair of genes (direct correlation is considered when in more than 50% of datasets the $p$ is equal or lower than 0.05 and correlation coefficient is greater or lower than 0.2).

# 3. Regucalcin gene silencing in human PNT1A prostate cells

*RGN* gene expression in PNT1A cells was silenced using the si-RGN. Gene silencing was evaluated for 24 and 48 h after transfection through qPCR (Figure IV.8). RGN mRNA expression levels were significantly lower in the si-RGN group, which was confirmed in different independent assays. Figure IV.8 shows a representative result of RGN silencing for 24 and 48 h of transfection; 88.92% and 83.03% reduction, respectively.

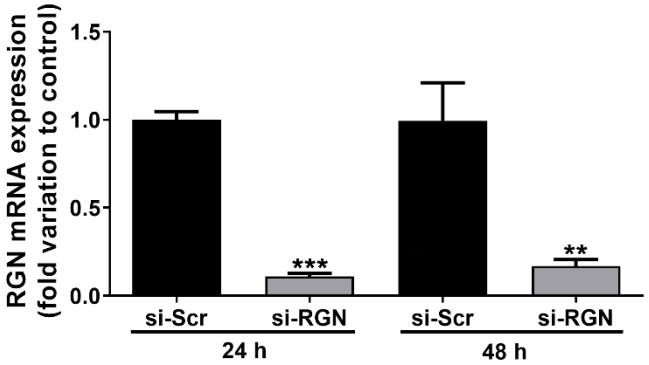


**Figure IV.8.** Regucalcin (RGN) mRNA expression levels in non-neoplastic human PNT1A prostate cells after transfection with the small interfering RNA (si-RNA) targeting RGN (si-RGN) or si-scramble (si-Scr) for 24 and 48 h of transfection. Relative RGN expression was determinated by qPCR after normalization with the β2M housekeeping gene. Results are expressed as fold-variation relative to the si-Scr control group. Error bars indicate mean ± SEM (n=6). $^{**}p< 0.01$; $^{***}p< 0.001$.

# 4. Down-regulation of regucalcin gene expression increased PNT1A cell viability

To determinate the effect of *RGN* gene KD in PNT1A cell viability an MTT assay was performed for 48, 72 and 96 h. PNT1A cells treated with the si-RGN displayed increased viability compared with the si-Scr control group (Figure IV.9). Although PNT1A cells viability did not change at 48 h, it significantly increased for 72 (110.44% ± 4.59, $p< 0.05$) and 96 h (122.73% ± 6.83, $p< 0.01$) after transfection. Moreover, the effect enhanced over time, as PNT1A cells viability was significantly higher at 96 h relative to 72 h ($^{\#}p< 0.05$, Figure IV.9).

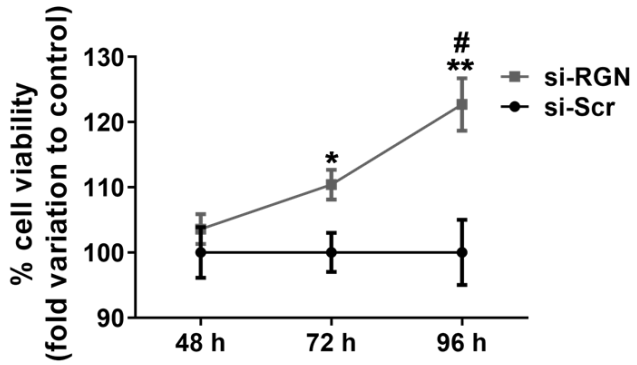


**Figure IV.9.** Percentage of PNT1A viable cells (MTT assay) after transfection with the small interfering RNA (si-RNA) targeting regucalcin (si-RGN) or si-scramble (si-Scr) for 48, 72 and 96 h of transfection. Results are expressed as fold-variation relative to the si-Scr control group. Error bars indicate mean ± S.E.M (n=6). $^{*}p< 0.05$; $^{**}p< 0.01$. $^{\#}p< 0.05$ compared with 72 h.

# 5. Regucalcin gene silencing promoted the proliferative activity of PNT1A cells

The effect of *RGN* gene silencing on PNT1A cell proliferation was evaluated by the SRB assay (Figure IV.10A) and immune-fluorescent labelling of Ki-67 (Figure IV.10B and Figure IV.10C). Ki-67 is a nuclear proliferation marker and its staining is one of the most widespread methods to evaluate cell proliferation [381]. The SRB assay evaluates cell mass being also indicative of the proliferation index [373].

The percentage of PNT1A proliferating cells determined by the SRB assay was significantly augmented at 48 (124.76% ± 9.58 $p< 0.05$) and 96 h (119.41% ± 6.86, $p< 0.05$) after transfection (Figure IV.10A). However, no effects were observed for 72 h.

The results of Ki-67 fluorescent immunocytochemistry for 48 h showed that the number of Ki-67-positive cells relative to total cells was significantly increased in the si-RGN (1.07 ± 0.02 $p< 0.01$) compared to the control group (Figure IV.10B). Figure IV.10C shows representative fluorescent immunocytochemistry images of Ki-67-labelled PNT1A cells.

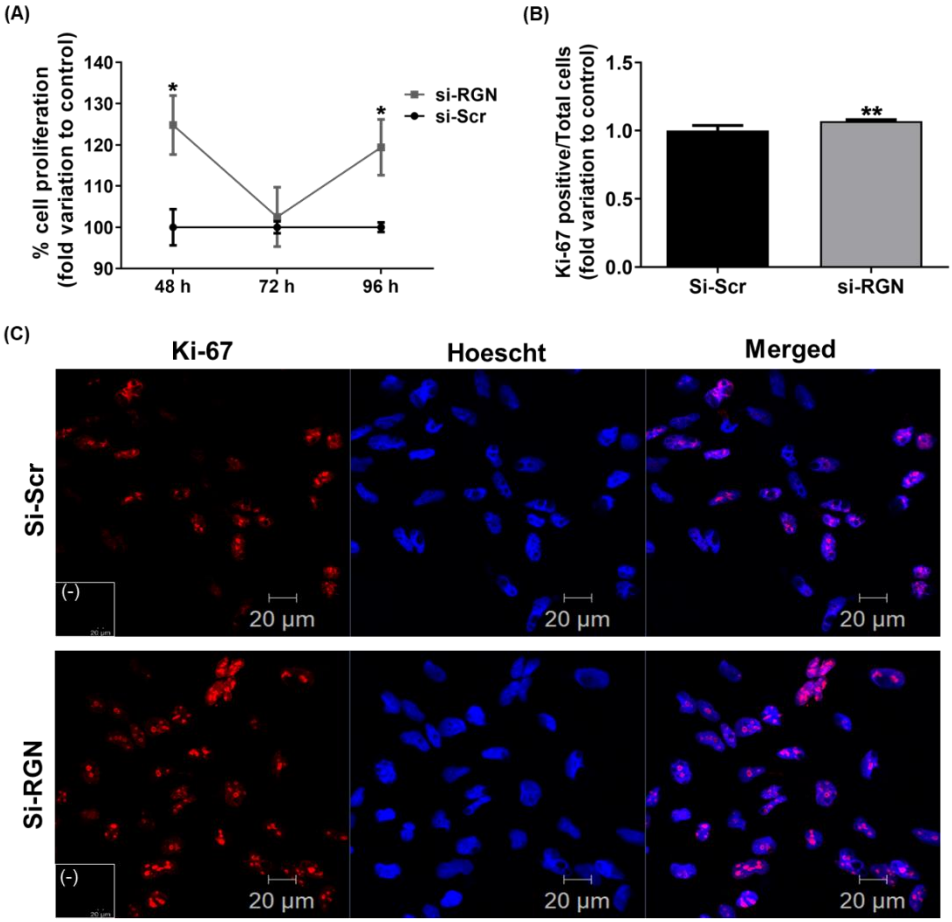


**Figure IV.10.** Proliferation of PNT1A cells after transfection with the small interfering RNA (si-RNA) targeting regucalcin (si-RGN) or si-scramble (si-Scr). **(A)** Percentage of proliferating cells determined by the SRB assay for 48, 72 and 96 h of transfection. **(B)** Ki-67 positive cells relative to the total cell number after 48 h of transfection. 15 randomly selected fields per microscope cover glass were assessed. **(C)** Representative fluorescent immunohistochemistry images of Ki-67 labelled cells (red) and Hoechst 33342 stained nuclei (blue) were obtained in the Zeiss LSM 710 laser scanning confocal microscope under 400x magnification. Negative controls obtained by omission of the primary antibody are provided as insert panels (-). Results are expressed as fold-variation relative to the si-Scr control group. Error bars indicate mean ± S.E.M (n=6). *$p< 0.05$; **$p< 0.01$.

# 6. Regucalcin gene silencing activated cell survival pathways

To understand the increased proliferative activity of PNT1A cells in response *RGN* gene KD, the expression of proteins associated with cell survival pathways was evaluated. The results of WB analysis demonstrated that AKT (1.46 ± 0.15 fold-variation, $p< 0.05$, Figure IV.11A), pAKT (1.32 ± 0.07 fold-variation, $p< 0.01$, Figure IV.11B) and ERK (1.37 ± 0.07 fold-variation, $p< 0.01$, Figure IV.11D) expression levels were increased in si-RGN PNT1A cells compared to control. Contrarily, pERK (0.78 ± 0.09 fold-variation, $p< 0.05$, Figure IV.11E) and phosphorylated c-myc (0.70 ± 0.09 fold-variation, $p< 0.05$, Figure IV.11C) levels were decreased in the si-RGN group compared to the si-Scr control. However, no statistically significant difference was found in the pERK/ERK protein ratio between the treated (si-RGN) and control group (Figure IV.11F).

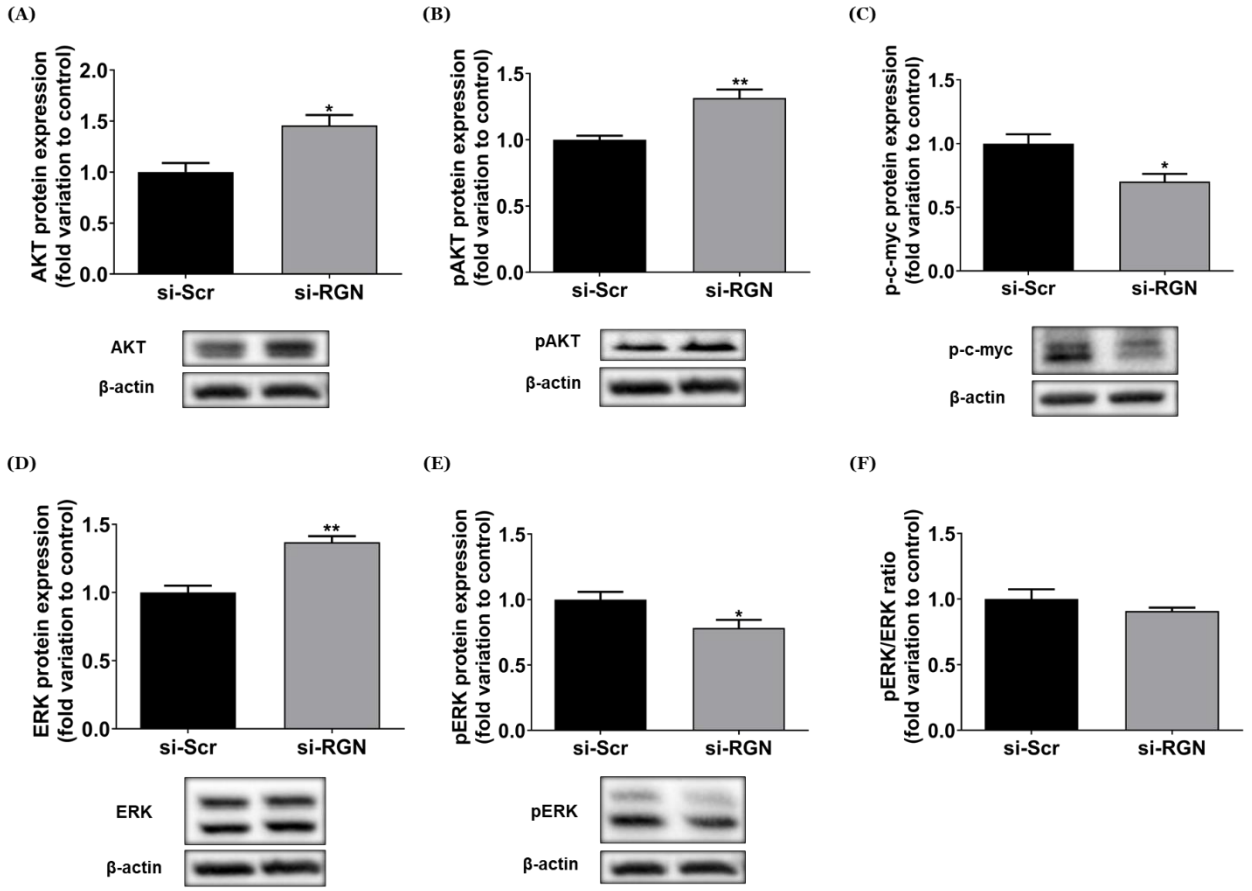


**Figure IV.11.** Expression of proteins associated with the PI3K/AKT and MAPK/ERK cell survival pathways in PNT1A cells transfected with the small interfering RNA (si-RNA) targeting regucalcin (si-RGN) or si-scramble (si-Scr) for 48 h. **(A)** AKT. **(B)** pAKT. **(C)** phosphorylated-c-myc (p-c-myc). **(D)** ERK. **(E)** pERK. **(F)** pERK/ERK ratio. Relative protein expression was determinated by WB after normalization to β-actin. Results are expressed as fold-variation relative to the si-Scr control group. Error bars indicate mean ± S.E.M (n=6). *$p< 0.05$; **$p< 0.01$.

## 7. Regucalcin gene knockdown inhibited the extrinsic pathway of apoptosis

Caspase-3-like activity and WB analysis were used to evaluate the apoptotic process in PNT1A cells in response to *RGN* gene silencing. Caspase-3-like activity was significantly lower in si-RGN PNT1A cells compared to the control group (0.71 ± 0.09 fold-variation, $p< 0.05$, Figure IV.12A). WB results showed that the expression of FasL (0.80 ± 0.07 fold-variation, $p< 0.05$, Figure IV.12B), FasR (0.78 ± 0.08 fold-variation, $p< 0.05$, Figure IV.12C) and caspase-8 (0.80 ± 0.06 fold-variation, $p< 0.05$, Figure IV.12D) were significantly decreased in the si-RGN group compared to the si-Scr control. *RGN* gene KD did not altered significantly the expression levels of p53 (Figure IV.12E) and caspase-9 (Figure IV.12F).

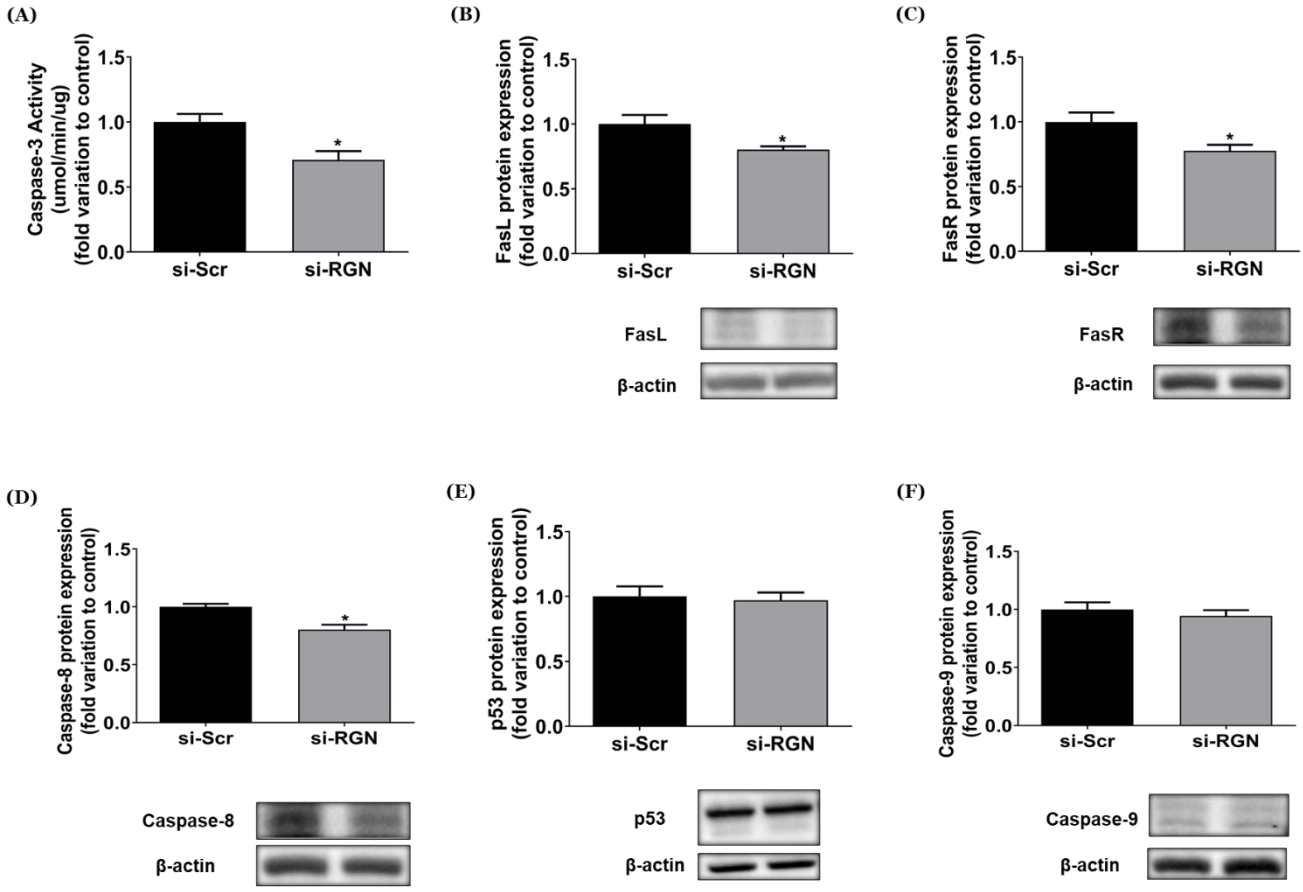


**Figure IV.12.** Evaluation of the apoptotic process of PNT1A cells after transfection with the small interfering RNA (si-RNA) targeting regucalcin (si-RGN) or si-scramble (si-Scr) for 48 h. **(A)** Caspase-3-like activity. Expression of proteins associated with the apoptotic pathways: **(B)** FasL; **(C)** FasR; (**D)** Caspase-8; **(E)** p53; **(F)** Caspase-9. Relative protein expression was determinated by WB after normalization to β-actin. Results are expressed as fold-variation relative to the si-Scr control group. Error bars indicate mean ± S.E.M (n=6). *$p< 0.05$.

# 8.Glycolytic metabolism was not altered by regucalcin gene knockdown

High glucose consumption and lactate production are key features of highly glycolytic cells, as is the case of cancer cells [347]. Therefore, and based on the reported RGN actions in regulating cell metabolism, we decided to investigate whether *RGN* gene KD can modify the glycolytic metabolism of PNT1A cells. No statistically significant differences were found in glucose consumption (Figure IV.13A) and lactate production (Figure IV.13B) between the si-RGN and the control group.

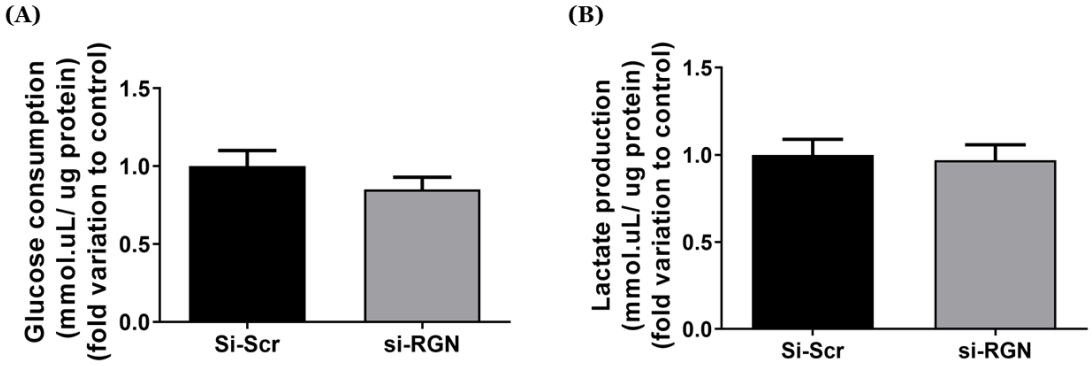


**Figure IV.13.** Glucose consumption **(A)** and lactate production **(B)** in PNT1A cells transfected with the small interfering RNA (si-RNA) targeting regucalcin (si-RGN) or si-scramble (si-Scr) for 48 h. Results are expressed as fold-variation relative to the si-Scr control group. Error bars indicate mean ± S.E.M (n=6).

# V. Discussion

In the last few years, several pieces of evidence have related the $Ca^{2+}$-binding protein RGN with the onset and development of several human cancers [198, 201, 291-295, 307, 308, 310, 312, 314]. Also, its clinical usefulness predicting the outcome of cancer patients has been suggested [307-310, 312, 314, 382, 383]. Our research group demonstrated the effect of RGN in suppressing cell survival and growth in the rat prostate [228, 271, 354], and established the relationship between the loss of RGN expression and the cellular differentiation of prostate adenocarcinoma [198]. However, the importance of RGN protein in the onset, development and progression of PCa is not entirely understood. Moreover, it is unknown if is the RGN loss that concurs to the emergence of malignancy.

The first part of the present dissertation investigated whether the expression levels of RGN in human PCa correlate with patients' clinicopathological data, PCa progression, GS, disease-free survival and recurrence. Bioinformatics analysis using the CancerTool software confirmed the deregulated expression of RGN in prostate tumour tissues, which was related to the appearance of primary tumours and the evolution of disease to the metastatic stage (Figure IV.1 and Figure IV.2). These findings corroborate the outcomes of a previous report linking the downregulated expression of RGN with the progression of disease and the cellular differentiation of prostate adenocarcinoma [198]. Moreover, they suggest that the loss of RGN may be used as a biomarker of PCa aggressiveness. However, no direct relationship was found between RGN expression levels and the GS. This could be explained by the variability between the datasets (Appendix; Table VIII.1) or the use of different datasets for the analysis of RGN expression levels with GS, though no definitive conclusion can be drawn at this stage.

*In silico* bioinformatics analysis also suggested that PCa patients with higher expression levels of RGN display higher disease-free survival (Figure IV.4), further indicating that the loss of RGN contributes to the progression of the disease and is a bad prognosis indicator. Nonetheless, more research is needed to confirm this assumption, as, for example, RGN expression levels did not influence the recurrence of PCa.

Importantly, the association found between the loss of RGN protein and cancer development is not exclusive of PCa. Previous studies of our research group also reported that the loss of RGN expression accompanies breast cancer progression, being correlated with the histological grade of infiltrating ductal breast carcinoma [198, 293]. These findings were followed by other similar in different human tissues. The down-regulated expression of RGN was associated with the development of lung, pancreatic, colorectal, hepatocellular and renal carcinomas [307, 308, 310, 312, 314]. These studies also have been proposing the use of RGN in the prediction of patients' survival and clinical outcomes. The existent reports in different cancer types and biological models highly support the usefulness of RGN as a prognostic marker. This is of utmost relevance considering that RGN was identified as a

secreted protein found in the serum of hepatocellular carcinoma patients, which was associated with the neoplastic status [294, 383].

Moreover, a cDNA microarray analysis in the rat liver demonstrated that the decrease of RGN expression occurs in pre-neoplastic foci anticipating the appearance of neoplastic lesions [296], which supports that is the loss of RGN that precedes malignant transformation, and this protein role as a tumour suppressor. Altogether, the existing knowledge also allows speculating that the maintenance of RGN expression levels may prevent cancer onset and disease progression.

Secondly, it was hypothesized that silencing RGN expression in non-neoplastic PNT1A human prostate cells, could be a driving force to change their proliferative ability and response to apoptosis. Deregulated cell proliferation is a well-established cancer hallmark and one of the first deregulated mechanisms underlying the establishment of neoplasia [162]. *RGN* gene silencing increased the proliferative capacity of PNT1A cells, as indicated by the estimated cell proliferation index assessed by the SRB assay and Ki-67 fluorescent immunocytochemistry (Figure IV.10). Also, PNT1A cells viability was increased over time with the suppression of RGN expression (Figure IV.9). This is the first report demonstrating that the loss of RGN can shape the behaviour of human non-neoplastic prostate cells increasing their proliferation rate. Accordingly, *in vivo* and *in vitro* overexpression of RGN was shown to suppress proliferation in rat prostate [228, 271, 354], liver H4-II-E and kidney NRK52E rat cancer cells [205, 315, 316], and in human lung, pancreatic, colorectal, hepatocellular, renal, cervical, breast and osteosarcoma cell lines [295, 307-310, 312-314, 317]. Moreover, non-invasive mammary gland tumours of Tg-RGN rats displayed lower proliferation rates compared with tumours of wild-type counterparts [293], which further supports the anti-proliferative effects of RGN.

Mechanistically, it was investigated whether the RGN' role suppressing proliferation would be related with the activity of PI3K/AKT and MAPK/ERK pathways, by assessing the expression of total and p-AKT and –ERK proteins, as well as the expression of TF c-myc. The activation of PNT1A cells proliferation was further confirmed by the increased levels of AKT, p-AKT (Figure IV.11A and Figure IV.11B) and decreased levels of phosphorylated c-myc (Figure IV.11C). The PI3K/AKT pathway and the c-myc TF are important molecular players in PCa, which strongly supports the relevance RGN would have in prostate tissue homeostasis. The mechanisms underlying the anti-proliferative actions of RGN would be further disclosed, but studies in human hepatocellular, pancreatic, breast, lung, colorectal, cervical, renal and osteosarcoma cell lines also reported that RGN overexpression inhibited the expression of AKT and its phosphorylated form [307-310, 312, 314, 317, 331].

The TF c-myc is a master regulator of the transcriptional program that controls cell survival and proliferation [111]. In non-neoplastic cells, c-myc activity is controlled by its phosphorylation and rapid degradation by the ubiquitin proteasome pathway [111].

Previous studies have analysed the c-myc mRNA expression under RGN overexpression in the rat mammary gland [293], rat prostate [228], and in rat and human hepatoma cells [293, 307, 318]. However, this is the first report looking at phosphorylated c-myc, which indicates that RGN may play a role in the proteasomal degradation of c-myc. Further investigation is needed to completely confirm this hypothesis. Nevertheless, a role for RGN in the control of the expression and/or activity of several protein kinases, such as GSK3-β, has been reported [295, 307-310, 312-314, 317, 331]. RGN was shown to increase GSK3-β levels [295, 313]. Moreover, GSK3-β phosphorylates c-myc targeting it to proteasomal degradation [110], which further sustains our assumption.

Concerning the MAPK/ERK pathway, *RGN* KD increased the ERK levels whereas decreasing those of its phosphorylated form (Figure IV.11D and Figure IV.11E). Therefore, no change was observed on the pERK/ERK ratio, which indicates that this pathway is not being responsible for PNT1A cells proliferation. However, and in line with our findings, RGN overexpression inhibited ERK expression in human hepatocellular, pancreatic, breast, lung, colorectal, cervical, renal and osteosarcoma cells [307-310, 312, 314, 317]. Contrarily, existent reports also demonstrated that the overexpression of RGN decreases the levels of p-ERK [307-309, 312, 314, 317]. Further research is needed to completely understand the liaison of RGN with the MAPK/ERK pathway. Nevertheless, consistent knowledge supports the RGN' role in controlling prostate cell proliferation and viability.

To further have an insight on the RGN proteins interacting network, the expression of *RGN* gene was correlated with that of *CDKN1A, TP53, IL6, AKT1, MAPK1, MYC* and *NFKB1* genes. Bioinformatics analysis using the CancerTool software and several PCa patients' datasets demonstrated that *RGN* expression did not correlate with the expression of *AKT1*, *MYC* and *MAPK1* genes, both in the prostate of healthy subjects and PCa patients (Figure IV.6 and Figure IV.7). This was contradictory to the *in vitro* findings and may be explained by the complexity of the *in vivo* systems, which include an intricacy of regulatory processes that are absent in simple *in vitro* cell systems. The role of RGN in controlling the expression of these cell cycle regulators needs further research to draw more precise conclusions.

However, *RGN* expression correlated directly with the expression of *IL6* and *CDKN1A* genes in the prostate of primary tumour patients, though that was not observed in healthy subjects and metastatic PCa patients (Figure IV.6 and Figure IV.7), which was consistent for different correlation analysis. This relationship is quite interesting as the *CDKN1A* gene product (Table IV.1), the cell cycle inhibitor p21 has been described as an important tumour suppressor in prostate [384]. Consistently with our results, RGN overexpression was linked with the increased levels of p21 in the rat prostate [228, 271] and in human hepatocellular, colorectal, renal and osteosarcoma cell lines [307, 312, 314, 317]. Further research will completely disclose if the RGN anti-proliferative actions in human prostate cells strictly depend on p21.

Concerning IL-6 protein, it has been shown to induce cell survival and angiogenesis, stimulating the progression of PCa metastization [124]. No other previous study has identified a connection of RGN with IL-6 in cancer cells or tissues. However, in mouse embryo 3T3-L1 pre-adipocytes cells RGN overexpression was shown to decrease IL-6 levels in response to TNF-α induced inflammation [385].

Noteworthy, a liaison between p21 and IL-6 upregulation and cell cycle progression of human PCa cells has been reported. In PC3 and DU145 cells, it was demonstrated that the upregulation of p21 and IL-6 induces cell cycle arrest in the G0/G1 phase [384]. However, it remains unknown if the upregulation of the expression of *CDKN1A* and *IL6* genes with the increase of *RGN* gene expression produces a similar effect in human primary prostate tumours.

The NF-kβ signalling pathway is deeply related to the onset and progression of PCa [140], regulating cell proliferation and invasion. In human renal, colorectal, hepatocellular and breast cancer cell lines it was reported that the overexpression of RGN decreased NF-kβ levels [307, 309, 312, 314]. However, the bioinformatic analysis performed in the present dissertation did not find a correlation between the *RGN* and *NFKB1* gene expression either in non-tumoral and tumoral conditions (Figure IV.6 and Figure IV.7). *In vitro* and *in vivo* studies may confirm the relationship of RGN with the NF-kβ pathway in prostate cells. However, in human pancreatic and breast cancer bone metastatic cells it was demonstrated that RGN suppresses the NF-kβ pathway [308, 319, 320], which reinforces the possible relationship between RGN and NF-kβ pathway in PCa.

Resistance to apoptosis is another hallmark of cancer that, besides playing a role in the onset and progression of disease, is strongly implicated in tumour's aggressiveness and therapy failure [162]. Reduced activity of the executioner of apoptosis caspase-3 was found in si-RGN transfected PNT1A cells (Figure IV.12A). Caspase-3 is an end point of apoptosis and its increased activity is indicative of higher apoptotic rates [150]. Therefore, silencing of RGN seems to be related with a lower apoptotic response of PNT1A cells. Accordingly, our research group showed that RGN overexpression in rat mammary gland and old-rats prostate is accompanied by the increased activity of caspase-3 [271, 293]. Curiously, several *in vivo* and *in vitro* experiments also have demonstrated that the overexpression of RGN inhibits apoptosis in response to cell damage [295, 307-314, 317, 324-330]. The findings obtained herein suggest that RGN can suppress the development of the apoptosis resistance typical of cancer cells.

Apoptosis can be triggered by both the intrinsic and extrinsic pathways, which converge at the activation of caspase-3. To understand which of the apoptotic pathways was inhibited, the expression levels of target apoptotic regulators was analysed. The *RGN* KD was followed by the decreased expression FasL and FasR, which trigger the activation of the extrinsic pathway, and caspase-8 that is the initiator caspase in this pathway [148, 156, 160, 161]

(Figure IV.12B to Figure IV.12F). Accordingly, increased apoptosis was found in the prostate of old Tg-RGN rats through the upregulation of the extrinsic pathway [271]. Also, it is relevant that Fas-mediated apoptosis was highly activated in PCa cells [177, 178]. Gathering all these data, the RGN role in increasing Fas-mediated apoptosis and avoiding apoptosis resistance of PCa cells may be suggested.

The intrinsic pathway can be activated by the tumour suppressor protein p53 [153] that regulates the expression and activity of mitochondrial regulators of apoptosis, which in turn contributes to the activation of the initiator caspase, caspase-9 [148, 158, 159]. p53 expression levels remained unchanged when *RGN* expression was silenced. This finding was corroborated by the *in silico* correlation analysis, as no correlation was found between the *RGN* and *TP53* gene expression, regardless of the tumour or non-tumour status (Figure IV.6 and Figure IV.7). Curiously, in the prostate of young rats, RGN overexpression decreased p53 levels [228, 271]. However, in the prostate of old rats, rat mammary gland [293] and in human breast, hepatocellular, pancreatic, colorectal and renal cancer cell lines [307-309, 312, 314] RGN overexpression was followed by the augmented levels of p53. These discrepant reports may be related to tissue-specific effects or cell physiological status that cannot be foreseen at the moment.

Besides p53, also caspase-9 expression was not affected by the manipulation of RGN expression levels, which indicates that the extrinsic pathway is the major route mediating RGN regulation of human prostate cells' apoptosis.

Reprogramming of glycolytic metabolism is one of the new cancer hallmarks proposed in 2011 by Hanahan and Weinberg [162]. Cancer cells undergo a metabolic switch, and preferentially use glycolysis as the main ATP source, relying on increased glucose consumption rates and consequent high lactate production [343]. This adaptation sacrifices the efficiency of ATP production to obtain energy faster, supporting the continuous cell proliferation and tumour growth [342, 345]. Also, the intense lactate production and its transport to the extracellular medium induce an acidification of the tumour microenvironment that has been related with cell migration, invasion, suppression of immune system, and, tumours aggressiveness [386]. Despite the fact that PCa has unique metabolic features, the advanced stages of disease, similarly to other cancers, display an hyperglycolytic profile [347]. The liaison of RGN with the regulation of this cancer hallmark has not been much investigated. However, RGN overexpression in the rat prostate was shown to suppress the glycolytic metabolism, with reduced tissue content of both glucose and lactate, diminished expression of GLUT3 and PFK, and reduced LDH activity [354]. Surprisingly, *RGN* gene silencing did not induce alterations in the glycolytic metabolism of PNT1A cells (Figure IV.13). These contradictory findings might be explained by the fact that *in vivo* systems display complex regulatory processes that are absent in simple *in vitro* cell systems. For example, the ERK/MAPK signalling pathway that has been demonstrated to

be involved in the control of glycolytic metabolism [377], was not affected by the *in vitro* down-regulation of RGN. These findings raise the curiosity about analysing the activity of the ERK/MAPK pathway in rat prostate, and the potential of RGN suppressing glycolytic metabolism in PCa cells.

# VI. Conclusions and Future Perspectives

The present dissertation showed that the diminished RGN expression levels, besides correlating with prostate malignancy, are associated with the development of metastatic disease. Also, it was reported that PCa patients with higher RGN expression levels display higher disease-free survival. These findings highly suggest the usefulness of using RGN as a prognosis biomarker and a molecular target for the better management of PCa. To completely establish this RGN's role, immunohistochemistry analysis of RGN expression in a larger number of PCa patients and an enzyme-linked immunosorbent assay assessing the RGN levels in the serum of healthy subjects vs. PCa patients will be needed.

Herein, we also first demonstrated that altering RGN expression levels has an impact on human prostate cell fate. *RGN* gene silencing increased PNT1A cells proliferation and viability, whereas diminishing their apoptotic activity. Additionally, this study reported that the RGN actions in regulating prostate cell survival might rely on the activation of the PI3K/AKT pathway and c-myc phosphorylation. Future research is needed to fully understand the mechanisms and the molecular targets involved in the RGN actions controlling the proliferation of prostate cells. However, considering its importance in prostate carcinogenesis and the existing knowledge linking RGN with the activation of Wnt/β-catenin and NF-kβ pathways, the expression of these molecular targets in si-RGN transfected PNT1A cells should be evaluated. Also, the inhibition of the PI3k/AKT pathway in PNT1A cells with and without decreased RGN expression will be crucial to implicate this pathway in the anti-proliferative actions of RGN. Future experimental approaches could also envisage the study of the ERK/MAPK pathway and analyse the glycolytic metabolism in human PCa tissues. Moreover, it might be interesting to analyse ERK/MAPK pathway activity in the prostate of rats overexpressing RGN compared with controls. These strategies are of major interest to draw precise conclusions regarding the effect of RGN in these molecular pathways.

Not least, it will be interesting to disclose the role of RGN in the c-myc phosphorylation and its proteasomal degradation, which could be assessed through the evaluation of GSK3-β expression levels and activity together with the inhibition of the proteasomal degradation in si-RGN transfected PNT1A cells *vs*. controls.

Concerning the suppression of apoptotic activity, *RGN* gene KD in non-neoplastic PNT1A cells was related to the altered expression of key molecular targets of the extrinsic pathway of apoptosis, namely, FasR, FasL and caspase-8. The resistance to apoptosis found in si-RGN transfected PNT1A cells could be confirmed by future experiments evaluating the apoptotic activity in the presence of apoptosis inducers.

Overall, the present findings demonstrate that the loss of RGN expression alters the behaviour of human prostate cells, and that reduced RGN expression levels favour the aggressiveness and progression of disease. Therefore, supporting the RGN role as a tumour suppressor, On the other hand, the outcomes of this thesis allow proposing that the

maintenance of RGN expression levels may prevent the carcinogenic process and slow-down PCa progression. Also, they open new avenues of research aiming to explore possible mechanisms that may upregulate RGN expression in human prostate cells.

# VII. References

1. Ittmann, M., *Anatomy and histology of the human and murine prostate*. Cold Spring Harbor Perspectives in Medicine, 2018. **8**(5): p. a030346.
2. Lee, C.H., O. Akin-Olugbade, and A. Kirschenbaum, *Overview of prostate anatomy, histology, and pathology*. Endocrinology and Metabolism Clinics, 2011. **40**(3): p. 565-575.
3. Toivanen, R. and M.M. Shen, *Prostate organogenesis: tissue induction, hormonal regulation and cell type specification*. Development, 2017. **144**(8): p. 1382-1398.
4. Timms, B.G., *Prostate development: a historical perspective*. Differentiation, 2008. **76**(6): p. 565-577.
5. Kumar, V. and P. Majumder, *Prostate gland: structure, functions and regulation*. International urology and nephrology, 1995. **27**(3): p. 231-243.
6. Hayward, S.W. and G.R. Cunha, *The prostate: development and physiology*. Radiologic Clinics of North America, 2000. **38**(1): p. 1-14.
7. Hershel Raff, Eric P. Widmaier, and K.T. Strang, *Vander's Human Physiology: The Mechanisms of Body Function*. 23 ed. Vander's Human Physiology. 2013: McGraw-Hill. 800.
8. McNeal, J.E., *The zonal anatomy of the prostate*. The Prostate, 1981. **2**(1): p. 35-49.
9. Burkitt, H.G., et al., *Wheater's functional histology: a text and colour atlas*. 1993: Churchill Livingstone.
10. Franklin, R.B., et al., *Zinc and zinc transporters in normal prostate function and the pathogenesis of prostate cancer*. Frontiers in bioscience: a journal and virtual library, 2005. **10**: p. 2230.
11. Vaz, C.A.V., *Androgens and calcium-binding protein regucalcin in the control of prostate cells metabolism and survival: implications in carcinogenesis*. 2015.
12. Hall, J.E., *Reproductive and hormonal functions of the male (and function of the pineal gland)*. Textbook of medical physiology. 12th ed. Philadelphia: Saunders Elsevier, 2011: p. 973-986.
13. Motrich, R.D., et al., *Implications of prostate inflammation on male fertility*. Andrologia, 2018. **50**(11): p. e13093.
14. Robertson, S.A. and D.J. Sharkey, *Seminal fluid and fertility in women*. Fertility and Sterility, 2016. **106**(3): p. 511-519.
15. Couñago, F., et al., *Clinical Applications of Molecular Biomarkers in Prostate Cancer*. Cancers, 2020. **12**(6): p. 1550.
16. *World Health Organization data about cancer Incidence and Mortality in Portugal in 2020*. 2020 [cited 2020 16/12/2020]; Available from: https://gco.iarc.fr/today/data/factsheets/populations/620-portugal-fact-sheets.pdf.

17. Ferlay, J., et al., *Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018*. European Journal of Cancer, 2018. **103**: p. 356-387.
18. Pina, F., et al., *Prostate cancer incidence and mortality in Portugal: trends, projections and regional differences*. European Journal of Cancer Prevention, 2017. **26**(5): p. 404-410.
19. Zuniga, K.B., et al. *Diet and lifestyle considerations for patients with prostate cancer*. in *Urologic Oncology: Seminars and Original Investigations*. 2019. Elsevier.
20. Perdana, N.R., et al., *The risk factors of prostate cancer and its prevention: a literature review*. Acta Med Indones, 2016. **48**(3): p. 228-38.
21. Daniyal, M., et al., *Epidemiology, etiology, diagnosis and treatment of prostate cancer*. Asian Pac J Cancer Prev, 2014. **15**(22): p. 9575-8.
22. Rebbeck, T.R., *Prostate Cancer Genetics: Variation by Race, Ethnicity, and Geography*. Seminars in Radiation Oncology, 2017. **27**(1): p. 3-10.
23. Attard, G., et al., *Prostate cancer*. The Lancet, 2016. **387**(10013): p. 70-82.
24. Benafif, S. and R. Eeles, *Genetic predisposition to prostate cancer*. British Medical Bulletin, 2016. **120**(1): p. 75-89.
25. Castro, E. and R. Eeles, *The role of BRCA1 and BRCA2 in prostate cancer*. Asian journal of andrology, 2012. **14**(3): p. 409-414.
26. Tulinius, H., et al., *Risk of prostate, ovarian, and endometrial cancer among relatives of women with breast cancer*. BMJ (Clinical research ed.), 1992. **305**(6858): p. 855-857.
27. Messina, C., et al., *BRCA Mutations in Prostate Cancer: Prognostic and Predictive Implications*. Journal of Oncology, 2020. **2020**: p. 4986365.
28. Pilarski, R., *The Role of BRCA Testing in Hereditary Pancreatic and Prostate Cancer Families*. American Society of Clinical Oncology Educational Book, 2019(39): p. 79-86.
29. Castro, E., et al., *Germline BRCA mutations are associated with higher risk of nodal involvement, distant metastasis, and poor survival outcomes in prostate cancer*. Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2013. **31**(14): p. 1748-1757.
30. Edwards, S.M., et al., *Prostate cancer in BRCA2 germline mutation carriers is associated with poorer prognosis*. British Journal of Cancer, 2010. **103**(6): p. 918-924.
31. John, E.M., et al., *Meat consumption, cooking practices, meat mutagens, and risk of prostate cancer*. Nutrition and cancer, 2011. **63**(4): p. 525-537.

32. Michaud, D.S., et al., *A prospective study on intake of animal products and risk of prostate cancer*. Cancer Causes & Control, 2001. **12**(6): p. 557-567.
33. Cross, A.J., et al., *A prospective study of meat and meat mutagens and prostate cancer risk*. Cancer research, 2005. **65**(24): p. 11779-11784.
34. Rohrmann, S., et al., *Meat and dairy consumption and subsequent risk of prostate cancer in a US cohort study*. Cancer Causes & Control, 2007. **18**(1): p. 41-50.
35. Chan, J.M., P.H. Gann, and E.L. Giovannucci, *Role of Diet in Prostate Cancer Development and Progression*. Journal of Clinical Oncology, 2005. **23**(32): p. 8152-8160.
36. Lophatananon, A., et al., *Dietary fat and early-onset prostate cancer risk*. British journal of nutrition, 2010. **103**(9): p. 1375-1380.
37. Wright, S.M. and L.J. Aronne, *Causes of obesity*. Abdom Imaging, 2012. **37**(5): p. 730-2.
38. Bandini, M., G. Gandaglia, and A. Briganti, *Obesity and prostate cancer*. Current Opinion in Urology, 2017. **27**(5).
39. Venkateswaran, V. and L.H. Klotz, *Diet and prostate cancer: mechanisms of action and implications for chemoprevention*. Nature Reviews Urology, 2010. **7**(8): p. 442.
40. Maly, I. and W. Hofmann, *Fatty Acids and Calcium Regulation in Prostate Cancer*. Nutrients, 2018. **10**(6): p. 788.
41. Zaichick, V., S. Zaichick, and M. Rossmann, *Intracellular calcium excess as one of the main factors in the etiology of prostate cancer*. AIMS Mol Sci, 2016. **3**(4): p. 635-647.
42. Brookman-May, S.D., et al., *Latest Evidence on the Impact of Smoking, Sports, and Sexual Activity as Modifiable Lifestyle Risk Factors for Prostate Cancer Incidence, Recurrence, and Progression: A Systematic Review of the Literature by the European Association of Urology Section of Oncological Urology (ESOU)*. European Urology Focus, 2019. **5**(5): p. 756-787.
43. De Nunzio, C., et al., *Smoking and Prostate Cancer: A Systematic Review*. European Urology Focus, 2015. **1**(1): p. 28-38.
44. Keogh, J.W. and R.D. MacLeod, *Body composition, physical fitness, functional performance, quality of life, and fatigue benefits of exercise for prostate cancer patients: a systematic review*. Journal of pain and symptom management, 2012. **43**(1): p. 96-110.
45. Bonn, S.E., et al., *Physical activity and survival among men diagnosed with prostate cancer*. Cancer Epidemiology and Prevention Biomarkers, 2015. **24**(1): p. 57-64.

46. Richman, E.L., et al., *Physical activity after diagnosis and risk of prostate cancer progression: data from the cancer of the prostate strategic urologic research endeavor*. Cancer research, 2011. **71**(11): p. 3889-3895.
47. Kenfield, S.A., et al., *Physical activity and survival after prostate cancer diagnosis in the health professionals follow-up study*. Journal of Clinical Oncology, 2011. **29**(6): p. 726.
48. Powell, I.J. and A. Bollig-Fischer, *Minireview: the molecular and genomic basis for prostate cancer health disparities*. Molecular endocrinology, 2013. **27**(6): p. 879-891.
49. Wu, I. and C.S. Modlin, *Disparities in prostate cancer in African American men: what primary care physicians can do*. Cleve Clin J Med, 2012. **79**(5): p. 313-320.
50. Akaza, H., et al., *Efficacy and safety of dutasteride on prostate cancer risk reduction in Asian men: the results from the REDUCE study*. Japanese journal of clinical oncology, 2011. **41**(3): p. 417-423.
51. Denis, L., M.S. Morton, and K. Griffiths, *Diet and its preventive role in prostatic disease*. Eur Urol, 1999. **35**(5-6): p. 377-87.
52. Ito, K., *Prostate cancer in Asian men*. Nature Reviews Urology, 2014. **11**(4): p. 197-212.
53. Packer, J.R. and N.J. Maitland, *The molecular and cellular origin of human prostate cancer*. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2016. **1863**(6, Part A): p. 1238-1260.
54. Wang, X., et al., *A luminal epithelial stem cell that is a cell of origin for prostate cancer*. Nature, 2009. **461**(7263): p. 495-500.
55. Lawson, D.A., et al., *Basal epithelial stem cells are efficient targets for prostate cancer initiation*. Proceedings of the National Academy of Sciences, 2010. **107**(6): p. 2610-2615.
56. Wang, Z.A., et al., *Lineage analysis of basal epithelial cells reveals their unexpected plasticity and supports a cell-of-origin model for prostate cancer heterogeneity*. Nature cell biology, 2013. **15**(3): p. 274-283.
57. Maitland, N.J. and A.T. Collins, *Inflammation as the primary aetiological agent of human prostate cancer: A stem cell connection?* Journal of Cellular Biochemistry, 2008. **105**(4): p. 931-939.
58. Shafi, A.A., A.E. Yen, and N.L. Weigel, *Androgen receptors in hormone-dependent and castration-resistant prostate cancer*. Pharmacology & therapeutics, 2013. **140**(3): p. 223-238.
59. Cai, Z., et al., *Androgen receptor: what we know and what we expect in castration-resistant prostate cancer*. International urology and nephrology, 2018. **50**(10): p. 1753-1764.

60. Rybak, A.P., R.G. Bristow, and A. Kapoor, *Prostate cancer stem cells: deciphering the origins and pathways involved in prostate tumorigenesis and aggression*. Oncotarget, 2015. **6**(4): p. 1900.
61. Feldman, B.J. and D. Feldman, *The development of androgen-independent prostate cancer*. Nature Reviews Cancer, 2001. **1**(1): p. 34-45.
62. Hoang, D.T., et al., *Androgen receptor-dependent and-independent mechanisms driving prostate cancer progression: Opportunities for therapeutic targeting from multiple angles*. Oncotarget, 2017. **8**(2): p. 3724.
63. Mostaghel, E.A. and P.S. Nelson, *Intracrine androgen metabolism in prostate cancer progression: mechanisms of castration resistance and therapeutic implications*. Best practice & research Clinical endocrinology & metabolism, 2008. **22**(2): p. 243-258.
64. Cai, C. and S.P. Balk, *Intratumoral androgen biosynthesis in prostate cancer pathogenesis and response to therapy*. Endocrine-related cancer, 2011. **18**(5): p. R175-R182.
65. Koivisto, P., et al., *Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer*. Cancer research, 1997. **57**(2): p. 314-319.
66. Visakorpi, T., et al., *In vivo amplification of the androgen receptor gene and progression of human prostate cancer*. Nature genetics, 1995. **9**(4): p. 401-406.
67. Titus, M.A., et al., *Steroid 5α-reductase isozymes I and II in recurrent prostate cancer*. Clinical Cancer Research, 2005. **11**(12): p. 4365-4371.
68. Penning, T.M. and M.C. Byrns, *Steroid hormone transforming aldo-keto reductases and cancer*. Annals of the New York Academy of Sciences, 2009. **1155**: p. 33.
69. Zhou, Y., E.C. Bolton, and J.O. Jones, *Androgens and androgen receptor signaling in prostate tumorigenesis*. J Mol Endocrinol, 2015. **54**(1): p. R15-R29.
70. Saraon, P., et al., *Mechanisms of Androgen-Independent Prostate Cancer*. EJIFCC, 2014. **25**(1): p. 42-54.
71. Nemes, A., C. Tomuleasa, and G. Kacso, *The androgen receptor remains a key player in metastatic hormone-refractory prostate cancer. Implications for new treatments*. J BUON, 2014. **19**(2): p. 357-364.
72. Culig, Z., et al., *Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor*. Cancer research, 1994. **54**(20): p. 5474-5478.
73. Wu, J.D., et al., *Interaction of IGF signaling and the androgen receptor in prostate cancer progression*. Journal of cellular biochemistry, 2006. **99**(2): p. 392-401.
74. Yeh, S., et al., *From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through*

*MAP kinase in prostate cancer cells*. Proceedings of the National Academy of Sciences, 1999. **96**(10): p. 5458-5463.

75. Craft, N., et al., *A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase*. Nature medicine, 1999. **5**(3): p. 280-285.
76. Saraon, P., K. Jarvi, and E.P. Diamandis, *Molecular alterations during progression of prostate cancer to androgen independence*. Clinical chemistry, 2011. **57**(10): p. 1366-1375.
77. Edwards, J. and J.M. Bartlett, *The androgen receptor and signal-transduction pathways in hormone-refractory prostate cancer. Part 2: androgen-receptor cofactors and bypass pathways*. BJU international, 2005. **95**(9): p. 1327-1335.
78. Colombel, M., et al., *Detection of the apoptosis-suppressing oncoprotein bc1-2 in hormone-refractory human prostate cancers*. The American journal of pathology, 1993. **143**(2): p. 390.
79. Furuya, Y., et al., *Expression of bcl-2 and the progression of human and rodent prostatic cancers*. Clinical Cancer Research, 1996. **2**(2): p. 389-398.
80. Gleave, M., et al., *Progression to Androgen Independence Is Delayed by Adjuvant Treatment with Antisense Bcl-2 Oligodeoxynucleotides after Castration in the LNCaP Prostate Tumor Model*. Clinical Cancer Research, 1999. **5**(10): p. 2891.
81. Puhr, M., et al., *The glucocorticoid receptor is a key player for prostate cancer cell survival and a target for improved antiandrogen therapy*. Clinical Cancer Research, 2018. **24**(4): p. 927-938.
82. Isaacs, J.T., *The biology of hormone refractory prostate cancer: Why does it develop?* Urologic Clinics of North America, 1999. **26**(2): p. 263-273.
83. Craft, N., et al., *Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumors through a two-step process*. Cancer research, 1999. **59**(19): p. 5030-5036.
84. Heinlein, C.A. and C. Chang, *The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions*. Molecular endocrinology, 2002. **16**(10): p. 2181-2187.
85. Griffin, J.E., *Androgen resistance—the clinical and molecular spectrum*. New England Journal of Medicine, 1992. **326**(9): p. 611-618.
86. Melmed, S., et al., *Williams textbook of endocrinology*. 2015: Elsevier Health Sciences.
87. Radmayr, C., et al., *5-alpha-reductase and the development of the human prostate*. Indian journal of urology: IJU: journal of the Urological Society of India, 2008. **24**(3): p. 309.

88. Dunn, J.F., B.C. Nisula, and D. Rodbard, *Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma*. The Journal of Clinical Endocrinology & Metabolism, 1981. **53**(1): p. 58-68.

89. Rosner, W., et al., *Sex hormone-binding globulin: anatomy and physiology of a new regulatory system*. The Journal of steroid biochemistry and molecular biology, 1991. **40**(4-6): p. 813-820.

90. Brinkmann, A., et al., *Mechanisms of androgen receptor activation and function*. The Journal of steroid biochemistry and molecular biology, 1999. **69**(1-6): p. 307-313.

91. Dai, C., H. Heemers, and N. Sharifi, *Androgen signaling in prostate cancer*. Cold Spring Harbor perspectives in medicine, 2017. **7**(9): p. a030452.

92. Lu, S., S.Y. Tsai, and M.-J. Tsai, *Regulation of androgen-dependent prostatic cancer cell growth: androgen regulation of CDK2, CDK4, and CKI p16 genes*. Cancer research, 1997. **57**(20): p. 4511-4516.

93. Wong, Y., X. Wang, and M. Ling, *Prostate development and carcinogenesis*. International review of cytology, 2003. **227**: p. 65-130.

94. Kimura, K., et al., *Androgen blocks apoptosis of hormone-dependent prostate cancer cells*. Cancer research, 2001. **61**(14): p. 5611-5618.

95. Liao, R.S., et al., *Androgen receptor-mediated non-genomic regulation of prostate cancer cell proliferation*. Translational andrology and urology, 2013. **2**(3): p. 187.

96. Peterziel, H., et al., *Rapid signalling by androgen receptor in prostate cancer cells*. Oncogene, 1999. **18**(46): p. 6322-6329.

97. Sun, Y.-H., et al., *Androgens Induce Increases in Intracellular Calcium Via a G Protein—Coupled Receptor in LNCaP Prostate Cancer Cells*. Journal of Andrology, 2006. **27**(5): p. 671-678.

98. Newton, A.C., *Protein kinase C: structure, function, and regulation*. Journal of biological chemistry, 1995. **270**(48): p. 28495-28498.

99. Gille, H., et al., *ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation*. The EMBO journal, 1995. **14**(5): p. 951-962.

100. Marais, R., J. Wynne, and R. Treisman, *The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain*. Cell, 1993. **73**(2): p. 381-393.

101. Yang, S.-H., et al., *The Elk-1 ETS-domain transcription factor contains a mitogen-activated protein kinase targeting motif*. Molecular and cellular biology, 1998. **18**(2): p. 710-720.

102. Unni, E., et al., *Changes in androgen receptor nongenotropic signaling correlate with transition of LNCaP cells to androgen independence*. Cancer research, 2004. **64**(19): p. 7156-7168.
103. El Sheikh, S.S., et al., *Predictive value of PTEN and AR coexpression of sustained responsiveness to hormonal therapy in prostate cancer - A pilot study*. Neoplasia, 2008. **10**(9): p. 949-953.
104. Taylor, B.S., et al., *Integrative genomic profiling of human prostate cancer*. Cancer cell, 2010. **18**(1): p. 11-22.
105. Reid, A.H.M., et al., *Molecular characterisation of ERG, ETV1 and PTEN gene loci identifies patients at low and high risk of death from prostate cancer*. British Journal of Cancer, 2010. **102**(4): p. 678-684.
106. Sun, M., et al., *Activation of phosphatidylinositol 3-kinase/Akt pathway by androgen through interaction of p85α, androgen receptor, and Src*. Journal of Biological Chemistry, 2003. **278**(44): p. 42992-43000.
107. Chen, H., et al., *The PI3K/AKT pathway in the pathogenesis of prostate cancer*. Front Biosci (Landmark Ed), 2016. **21**: p. 1084-1091.
108. Edlind, M. and A. Hsieh, *PI3K-AKT-mTOR signaling in prostate cancer progression and androgen deprivation therapy resistance*. Asian Journal of Andrology, 2014. **16**(3): p. 378-386.
109. Labbé, D.P. and M. Brown, *Transcriptional Regulation in Prostate Cancer*. Cold Spring Harb Perspect Med, 2018. **8**(11).
110. Sears, R.C., *The Life Cycle of C-Myc: From Synthesis to Degradation*. Cell Cycle, 2004. **3**(9): p. 1131-1135.
111. Farrell, A.S. and R.C. Sears, *MYC degradation*. Cold Spring Harbor perspectives in medicine, 2014. **4**(3): p. a014365.
112. Heinlein, C.A. and C. Chang, *Androgen Receptor in Prostate Cancer*. Endocrine Reviews, 2004. **25**(2): p. 276-308.
113. Migliaccio, A., et al., *Steroid-induced androgen receptor–oestradiol receptor β–Src complex triggers prostate cancer cell proliferation*. The EMBO Journal, 2000. **19**(20): p. 5406-5417.
114. Leung, J.K. and M.D. Sadar, *Non-Genomic Actions of the Androgen Receptor in Prostate Cancer*. Frontiers in Endocrinology, 2017. **8**(2).
115. Pandini, G., et al., *Androgens up-regulate the insulin-like growth factor-I receptor in prostate cancer cells*. Cancer research, 2005. **65**(5): p. 1849-1857.
116. Bonkhoff, H., *Estrogen receptor signaling in prostate cancer: Implications for carcinogenesis and tumor progression*. The Prostate, 2018. **78**(1): p. 2-10.

117. Bonkhoff, H. and R. Berges, *The Evolving Role of Oestrogens and Their Receptors in the Development and Progression of Prostate Cancer*. European Urology, 2009. **55**(3): p. 533-542.
118. Ricke, W.A., et al., *Prostatic hormonal carcinogenesis is mediated by in situ estrogen production and estrogen receptor alpha signaling*. The FASEB journal, 2008. **22**(5): p. 1512-1520.
119. Yeap, B.B., et al., *Differential associations of testosterone, dihydrotestosterone and oestradiol with physical, metabolic and health-related factors in community-dwelling men aged 17–97 years from the Busselton Health Survey*. Clinical Endocrinology, 2014. **81**(1): p. 100-108.
120. Araujo, A.B., et al., *Prevalence and Incidence of Androgen Deficiency in Middle-Aged and Older Men: Estimates from the Massachusetts Male Aging Study*. The Journal of Clinical Endocrinology & Metabolism, 2004. **89**(12): p. 5920-5926.
121. Vermeulen, A., et al., *Estradiol in elderly men*. The Aging Male, 2002. **5**(2): p. 98-102.
122. Ross, R., et al., *Serum testosterone levels in healthy young black and white men*. Journal of the National Cancer Institute, 1986. **76**(1): p. 45-48.
123. Leung, Y.K. and S.M. Ho, *Estrogen receptor β: switching to a new partner and escaping from estrogen*. Science Signaling, 2011. **4**(168): p. pe19.
124. Nguyen, D.P., J. Li, and A.K. Tewari, *Inflammation and prostate cancer: the role of interleukin 6 (IL-6)*. BJU international, 2014. **113**(6): p. 986-992.
125. Sfanos, K.S. and A.M. De Marzo, *Prostate cancer and inflammation: the evidence*. Histopathology, 2012. **60**(1): p. 199-215.
126. Rani, A., P. Dasgupta, and J.J. Murphy, *Prostate Cancer: The Role of Inflammation and Chemokines*. The American Journal of Pathology, 2019. **189**(11): p. 2119-2137.
127. Gueron, G., A. De Siervi, and E. Vazquez, *Advanced prostate cancer: reinforcing the strings between inflammation and the metastatic behavior*. Prostate Cancer and Prostatic Diseases, 2012. **15**(3): p. 213-221.
128. Wise, G.J., et al., *Cytokine variations in patients with hormone treated prostate cancer*. The Journal of urology, 2000. **164**(3): p. 722-725.
129. Adler, H.L., et al., *Elevated levels of circulating interleukin-6 and transforming growth factor-beta 1 in patients with metastatic prostatic carcinoma*. The Journal of urology, 1999. **161**(1): p. 182-187.
130. Shariat, S.F., et al., *Plasma levels of interleukin-6 and its soluble receptor are associated with prostate cancer progression and metastasis*. Urology, 2001. **58**(6): p. 1008-1015.

131. Giri, D., M. Ozen, and M. Ittmann, *Interleukin-6 Is an Autocrine Growth Factor in Human Prostate Cancer*. The American Journal of Pathology, 2001. **159**(6): p. 2159-2165.
132. Lee, S.O., et al., *Interleukin-6 promotes androgen-independent growth in LNCaP human prostate cancer cells*. Clinical cancer research : an official journal of the American Association for Cancer Research, 2003. **9**(1): p. 370-376.
133. Wegiel, B., et al., *Interleukin-6 activates PI3K/Akt pathway and regulates cyclin A1 to promote prostate cancer cell survival*. International Journal of Cancer, 2008. **122**(7): p. 1521-1529.
134. Steiner, H., et al., *An autocrine loop for vascular endothelial growth factor is established in prostate cancer cells generated after prolonged treatment with interleukin 6*. European Journal of Cancer, 2004. **40**(7): p. 1066-1072.
135. Shiota, M., et al., *Hsp27 Regulates Epithelial Mesenchymal Transition, Metastasis, and Circulating Tumor Cells in Prostate Cancer*. Cancer Research, 2013. **73**(10): p. 3109-3119.
136. Culig, Z. and M. Puhr, *Interleukin-6 and prostate cancer: Current developments and unsolved questions*. Molecular and cellular endocrinology, 2018. **462**: p. 25-30.
137. Xie, K., *Interleukin-8 and human cancer biology*. Cytokine & growth factor reviews, 2001. **12**(4): p. 375-391.
138. Araki, S., et al., *Interleukin-8 is a molecular determinant of androgen independence and progression in prostate cancer*. Cancer research, 2007. **67**(14): p. 6854-6862.
139. Waugh, D.J. and C. Wilson, *The interleukin-8 pathway in cancer*. Clinical cancer research, 2008. **14**(21): p. 6735-6741.
140. Guo, Y., et al., *IL-8 promotes proliferation and inhibition of apoptosis via STAT3/AKT/NF-κB pathway in prostate cancer Corrigendum in/10.3892/mmr. 2019.9942*. Molecular medicine reports, 2017. **16**(6): p. 9035-9042.
141. Krušlin, B., M. Ulamec, and D. Tomas, *Prostate cancer stroma: an important factor in cancer growth and progression*. Bosnian journal of basic medical sciences, 2015. **15**(2): p. 1-8.
142. Djakiew, D., *Dysregulated expression of growth factors and their receptors in the development of prostate cancer*. The Prostate, 2000. **42**(2): p. 150-160.
143. Levesque, C. and P.S. Nelson, *Cellular constituents of the prostate stroma: Key contributors to prostate cancer progression and therapy resistance*. Cold Spring Harbor perspectives in medicine, 2017: p. a030510.
144. Tuxhorn, J.A., G.E. Ayala, and D.R. Rowley, *Reactive stroma in prostate cancer progression*. The Journal of urology, 2001. **166**(6): p. 2472-2483.

145. Tuxhorn, J.A., et al., *Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling*. Clinical Cancer Research, 2002. **8**(9): p. 2912-2923.
146. Ayala, G., et al., *Reactive stroma as a predictor of biochemical-free recurrence in prostate cancer*. Clinical cancer research, 2003. **9**(13): p. 4792-4801.
147. Palumbo Jr, A., et al., *Extracellular matrix secreted by reactive stroma is a main inducer of pro-tumorigenic features on LNCaP prostate cancer cells*. Cancer letters, 2012. **321**(1): p. 55-64.
148. Jan, R., *Understanding apoptosis and apoptotic pathways targeted cancer therapeutics*. Advanced pharmaceutical bulletin, 2019. **9**(2): p. 205.
149. Zielinski, R.R., B.J. Eigl, and K.N. Chi, *Targeting the apoptosis pathway in prostate cancer*. The Cancer Journal, 2013. **19**(1): p. 79-89.
150. Porter, A.G. and R.U. Jänicke, *Emerging roles of caspase-3 in apoptosis*. Cell death and differentiation, 1999. **6**(2): p. 99.
151. Huang, X., et al., *Novel targeted pro-apoptotic agents for the treatment of prostate cancer*. The Journal of urology, 2007. **178**(5): p. 1846-1854.
152. Khan, N., V.M. Adhami, and H. Mukhtar, *Apoptosis by dietary agents for prevention and treatment of prostate cancer*. Endocrine-related cancer, 2010. **17**(1): p. R39-R52.
153. Liu, D. and Y. Xu, *p53, oxidative stress, and aging*. Antioxidants & redox signaling, 2011. **15**(6): p. 1669-1678.
154. Demir, Ö., et al., *An integrated view of p53 dynamics, function, and reactivation*. Current Opinion in Structural Biology, 2021. **67**: p. 187-194.
155. Westphal, D., et al., *Molecular biology of Bax and Bak activation and action*. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2011. **1813**(4): p. 521-531.
156. Naoum, G.E., et al., *Role of nanotechnology and gene delivery systems in TRAIL-based therapies*. ecancermedicalscience, 2016. **10**.
157. Ferreira, K.S., et al., *Caspase-3 feeds back on caspase-8, Bid and XIAP in type I Fas signaling in primary mouse hepatocytes*. Apoptosis, 2012. **17**(5): p. 503-515.
158. Pan, J., G. Xu, and S.-C.J. Yeung, *Cytochrome c release is upstream to activation of caspase-9, caspase-8, and caspase-3 in the enhanced apoptosis of anaplastic thyroid cancer cells induced by manumycin and paclitaxel*. The Journal of Clinical Endocrinology & Metabolism, 2001. **86**(10): p. 4731-4740.
159. Pop, C., et al., *The apoptosome activates caspase-9 by dimerization*. Molecular cell, 2006. **22**(2): p. 269-275.
160. Elmore, S., *Apoptosis: a review of programmed cell death*. Toxicologic pathology, 2007. **35**(4): p. 495-516.

161. Hassan, M., et al., *Apoptosis and molecular targeting therapy in cancer*. BioMed research international, 2014. **2014**.
162. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation*. cell, 2011. **144**(5): p. 646-674.
163. Sun, A., et al., *Androgen receptor-dependent regulation of Bcl-xL expression: Implication in prostate cancer progression*. The Prostate, 2008. **68**(4): p. 453-461.
164. Van Delft, M.F. and D.C. Huang, *How the Bcl-2 family of proteins interact to regulate apoptosis*. Cell research, 2006. **16**(2): p. 203.
165. Sternberg, C.N., et al., *Docetaxel plus oblimersen sodium (Bcl-2 antisense oligonucleotide): an EORTC multicenter, randomized phase II study in patients with castration-resistant prostate cancer*. Annals of Oncology, 2009. **20**(7): p. 1264-1269.
166. Liu, G., et al., *An open-label, multicenter, phase I/II study of single-agent AT-101 in men with castrate-resistant prostate cancer*. Clinical cancer research, 2009. **15**(9): p. 3172-3176.
167. Sonpavde, G., et al., *Randomized phase II trial of docetaxel plus prednisone in combination with placebo or AT-101, an oral small molecule Bcl-2 family antagonist, as first-line therapy for metastatic castration-resistant prostate cancer*. Annals of Oncology, 2011. **23**(7): p. 1803-1808.
168. Nguyen, M., et al., *Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis*. Proceedings of the National Academy of Sciences, 2007. **104**(49): p. 19512-19517.
169. Dash, R., et al., *Apogossypol derivative BI-97C1 (Sabutoclax) targeting Mcl-1 sensitizes prostate cancer cells to mda-7/IL-24–mediated toxicity*. Proceedings of the National Academy of Sciences, 2011. **108**(21): p. 8785-8790.
170. Krajewska, M., et al., *Immunohistochemical analysis of bcl-2, bax, bcl-X, and mcl-1 expression in prostate cancers*. The American journal of pathology, 1996. **148**(5): p. 1567.
171. Zellweger, T., et al., *Expression patterns of potential therapeutic targets in prostate cancer*. International journal of cancer, 2005. **113**(4): p. 619-628.
172. Yoshino, T., et al., *Bcl-2 expression as a predictive marker of hormone-refractory prostate cancer treated with taxane-based chemotherapy*. Clinical Cancer Research, 2006. **12**(20): p. 6116-6124.
173. Khor, L.Y., et al., *Bcl-2 and Bax expression predict prostate cancer outcome in men treated with androgen deprivation and radiotherapy on radiation therapy oncology group protocol 92-02*. Clinical cancer research, 2007. **13**(12): p. 3585-3590.

174. Beltran, H., et al., *Targeted Next-generation Sequencing of Advanced Prostate Cancer Identifies Potential Therapeutic Targets and Disease Heterogeneity*. European Urology, 2013. **63**(5): p. 920-926.
175. Bova, G.S., et al., *Physical mapping of chromosome 8p22 markers and their homozygous deletion in a metastatic prostate cancer*. Genomics, 1996. **35**(1): p. 46-54.
176. Sanlioglu, A.D., et al., *Differential expression of TRAIL and its receptors in benign and malignant prostate tissues*. The Journal of urology, 2007. **177**(1): p. 359-364.
177. Hyer, M.L., et al., *Intracellular Fas ligand expression causes Fas-mediated apoptosis in human prostate cancer cells resistant to monoclonal antibody-induced apoptosis*. Molecular Therapy, 2000. **2**(4): p. 348-358.
178. Symes, J.C., et al., *Fas-mediated killing of primary prostate cancer cells is increased by mitoxantrone and docetaxel*. Molecular cancer therapeutics, 2008. **7**(9): p. 3018-3028.
179. Schatten, H., *Brief Overview of Prostate Cancer Statistics, Grading, Diagnosis and Treatment Strategies*, in *Cell & Molecular Biology of Prostate Cancer: Updates, Insights and New Frontiers*, H. Schatten, Editor. 2018, Springer International Publishing: Cham. p. 1-14.
180. Litwin, M.S. and H.-J. Tan, *The diagnosis and treatment of prostate cancer: a review*. Jama, 2017. **317**(24): p. 2532-2542.
181. Nguyen-Nielsen, M. and M. Borre, *Diagnostic and Therapeutic Strategies for Prostate Cancer*. Seminars in Nuclear Medicine, 2016. **46**(6): p. 484-490.
182. Lopez-Beltran, A., et al., *Current practice of Gleason grading of prostate carcinoma*. Virchows Archiv, 2006. **448**(2): p. 111-118.
183. Giannico, G.A. and O. Hameed, *Evaluation of Prostate Needle Biopsies*, in *Molecular & Diagnostic Imaging in Prostate Cancer: Clinical Applications and Treatment Strategies*, H. Schatten, Editor. 2018, Springer International Publishing: Cham. p. 69-86.
184. Holm, H.V., et al., *Modern treatment of metastatic prostate cancer*. Tidsskr Nor Laegeforen, 2017. **137**(11): p. 803-805.
185. *Hormone Therapy for Prostate Cancer*. [cited 2021 07/01/2021]; Available from: https://www.cancer.org/cancer/prostate-cancer/treating/hormone-therapy.html.
186. Gamat, M. and D.G. McNeel, *Androgen deprivation and immunotherapy for the treatment of prostate cancer*. Endocr Relat Cancer, 2017. **24**(12): p. T297-t310.
187. Perner, S., et al., *Adaptive responses of androgen receptor signaling in castration-resistant prostate cancer*. Oncotarget, 2015. **6**(34): p. 35542.
188. Galletti, G., et al., *Mechanisms of resistance to systemic therapy in metastatic castration-resistant prostate cancer*. Cancer treatment reviews, 2017. **57**: p. 16-27.

189. Mimeault, M. and S.K. Batra, *Recent advances on multiple tumorigenic cascades involved in prostatic cancer progression and targeting therapies*. Carcinogenesis, 2005. **27**(1): p. 1-22.
190. Shaffer, D.R. and H.I. Scher, *Prostate cancer: a dynamic illness with shifting targets*. The Lancet Oncology, 2003. **4**(7): p. 407-414.
191. Hotte, S.J. and F. Saad, *Current management of castrate-resistant prostate cancer*. Current oncology, 2010. **17**(Suppl 2): p. S72.
192. Teo, M.Y., D.E. Rathkopf, and P. Kantoff, *Treatment of Advanced Prostate Cancer*. Annu Rev Med, 2019. **70**: p. 479-499.
193. *Chemotherapy for Prostate Cancer*. [cited 2021 07/01/2021]; Available from: https://www.cancer.org/cancer/prostate-cancer/treating/chemotherapy.html?fbclid=IwAR0ho_mhAu1klsiptQjhXu81baO4fmF1wd91fMMjN2Ptkp6lBZ9CVbng5-I.
194. Yamaguchi, M. and T. Yamamoto, *Purification of calcium binding substance from soluble fraction of normal rat liver*. Chemical and Pharmaceutical Bulletin, 1978. **26**(6): p. 1915-1918.
195. Fujita, T., et al., *Isolation of cDNA clone encoding human homologue of senescence marker protein-30 (SMP30) and its location on the X chromosome*. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression, 1995. **1263**(3): p. 249-252.
196. Shimokawa, N., Y. Matsuda, and M. Yamaguchi, *Genomic cloning and chromosomal assignment of rat regucalcin gene*. Molecular and cellular biochemistry, 1995. **151**(2): p. 157-163.
197. Yamaguchi, M., R. Makino, and N. Shimokawa, *The 5′ end sequences and exon organization in rat regucalcin gene*. Molecular and cellular biochemistry, 1996. **165**(2): p. 145-150.
198. Maia, C., et al., *Regucalcin is under-expressed in human breast and prostate cancers: Effect of sex steroid hormones*. Journal of cellular biochemistry, 2009. **107**(4): p. 667-676.
199. Misawa, H. and M. Yamaguchi, *Transcript heterogeneity of the human gene for Ca2+-binding protein regucalcin*. International journal of molecular medicine, 2000. **5**(3): p. 283-290.
200. Laurentino, S.S., et al., *Regucalcin, a calcium-binding protein with a role in male reproduction?* Molecular human reproduction, 2011. **18**(4): p. 161-170.
201. Murata, T. and M. Yamaguchi, *Alternatively spliced variants of the regucalcin gene in various human normal and tumor tissues*. International journal of molecular medicine, 2014. **34**(4): p. 1141-1146.

202. Arun, P., et al., *Senescence Marker Protein 30 (SMP30) Expression in Eukaryotic Cells: Existence of Multiple Species and Membrane Localization*. PLOS ONE, 2011. **6**(2): p. e16545.
203. MURATA, T. and M. YAMAGUCHI, *Ca2+ administration stimulates the binding of AP-1 factor to the 5′-flanking region of the rat gene for the Ca2+-binding protein regucalcin*. Biochemical Journal, 1998. **329**(1): p. 157-163.
204. Misawa, H. and M. Yamaguchi, *Intracellular signaling factors—enhanced hepatic nuclear protein binding to TTGGC sequence in the rat regucalcin gene promoter: involvement of protein phosphorylation*. Biochemical and biophysical research communications, 2000. **279**(1): p. 275-281.
205. Misawa, H., S. Inagaki, and M. Yamaguchi, *Suppression of cell proliferation and deoxyribonucleic acid synthesis in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin*. Journal of cellular biochemistry, 2002. **84**(1): p. 143-149.
206. Yamaguchi, M., *Novel protein RGPR-p117: its role as the regucalcin gene transcription factor*. Molecular and cellular biochemistry, 2009. **327**(1-2): p. 53-63.
207. Yamaguchi, M., *The transcriptional regulation of regucalcin gene expression*. Molecular and Cellular Biochemistry, 2011. **346**(1): p. 147-171.
208. Akhter, T., et al., *Suppression of regucalcin mRNA expression in the hearts of rats administered with free radical compound: The administration-induced death is accelerated in regucalcin transgenic rats*. International journal of molecular medicine, 2007. **19**(4): p. 653-658.
209. Jung, K.J., et al., *The redox-sensitive DNA binding sites responsible for age-related downregulation of SMP30 by ERK pathway and reversal by calorie restriction*. Antioxid Redox Signal, 2006. **8**(3-4): p. 671-80.
210. Ishigami, T., et al., *Regulatory effects of senescence marker protein 30 on the proliferation of hepatocytes*. Pathology international, 2001. **51**(7): p. 491-497.
211. Jung, K.J., et al., *Modulation of gene expression of SMP-30 by LPS and calorie restriction during aging process*. Experimental gerontology, 2004. **39**(8): p. 1169-1177.
212. Nakajima, M., T. Murata, and M. Yamaguchi, *Expression of calcium-binding protein regucalcin mRNA in the cloned rat hepatoma cells (Hr-II-E) is stimulated through Ca2+ signaling factors: Involvement of protein kinase C*. Molecular and cellular biochemistry, 1999. **198**(1-2): p. 101-107.
213. Yamaguchi, M., K. Oishi, and M. Isogai, *Expression of hepatic calcium-binding protein regucalcin mRNA is elevated by refeeding of fasted rats: Involvement of glucose, insulin and calcium as stimulating factors*. Molecular and cellular biochemistry, 1995. **142**(1): p. 35-41.

214. Shimokawa, N. and M. Yamaguchi, *Calcium administration stimulates the expression of calcium-binding protein regucalcin mRNA in rat liver*. FEBS letters, 1992. **305**(2): p. 151-154.
215. Murata, T. and M. Yamaguchi, *Molecular cloning of the cDNA coding for regucalcin and its mRNA expression in mouse liver: the expression is stimulated by calcium administration*. Molecular and cellular biochemistry, 1997. **173**(1-2): p. 127-133.
216. Isogai, M. and M. Yamaguchi, *Calcium administration increases calcium-binding protein regucalcin concentration in the liver of rats*. Molecular and cellular biochemistry, 1995. **143**(1): p. 53-58.
217. Yamaguchi, M. and H. Kurota, *Expression of calcium-binding protein regucalcin mRNA in the kidney cortex of rats: The stimulation by calcium administration*. Molecular and Cellular Biochemistry, 1995. **146**(1): p. 71-77.
218. Yamaguchi, M. and S. Ueoka, *Expression of calcium-binding protein regucalcin mRNA in fetal rat liver is stimulated by calcium administration*. Molecular and cellular biochemistry, 1998. **178**(1-2): p. 283-287.
219. Yamaguchi, M. and M. Nakajima, *Involvement of intracellular signaling factors in the serum-enhanced Ca2+-binding protein regucalcin mRNA expression in the cloned rat hepatoma cells (H4-II-E)*. Journal of cellular biochemistry, 1999. **74**(1): p. 81-89.
220. Shimokawa, N. and M. Yamaguchi, *Expression of hepatic calcium-binding protein regucalcin mRNA is mediated through Ca2+/calmodulin in rat liver*. FEBS letters, 1993. **316**(1): p. 79-84.
221. V Vaz, C., et al., *The emerging role of regucalcin as a tumor suppressor: facts and views*. Current molecular medicine, 2016. **16**(7): p. 607-619.
222. Yamaguchi, M. and K. Oishi, *17β-Estradiol stimulates the expression of hepatic calcium-binding protein regucalcin mRNA in rats*. Molecular and cellular biochemistry, 1995. **143**(2): p. 137-141.
223. Correia, S.C.d.L., *Estrogens and regucalcin in testicular apoptosis and sperm function:"a matter of life and death"*. 2014.
224. Kurota, H. and M. Yamaguchi, *Steroid hormonal regulation of calcium-binding protein regucalcin mRNA expression in the kidney cortex of rats*. Molecular and cellular biochemistry, 1996. **155**(2): p. 105-111.
225. Maia, C.J.B., et al., *Regucalcin is expressed in rat mammary gland and prostate and down-regulated by 17β-estradiol*. Molecular and Cellular Biochemistry, 2008. **311**(1): p. 81-86.
226. Cucuzza, L.S., et al., *Regucalcin expression in bovine tissues and its regulation by sex steroid hormones in accessory sex glands*. PloS one, 2014. **9**(11): p. e113950.

227. Lin, P.-H., et al., *Induction of renal senescence marker protein-30 (SMP30) expression by testosterone and its contribution to urinary calcium absorption in male rats*. Scientific reports, 2016. **6**: p. 32085.
228. Vaz, C.V., et al., *Regucalcin is an androgen-target gene in the rat prostate modulating cell-cycle and apoptotic pathways*. The Prostate, 2014. **74**(12): p. 1189-1198.
229. Laurentino, S.S., et al., *Regucalcin is broadly expressed in male reproductive tissues and is a new androgen-target gene in mammalian testis*. Reproduction, 2011. **142**(3): p. 447-456.
230. Shinya, N. and M. Yamaguchi, *Stimulatory effect of calcium administration on regucalcin mRNA expression is attenuated in the kidney cortex of rats ingested with saline*. Molecular and cellular biochemistry, 1998. **178**(1-2): p. 275-281.
231. Shinya, N., H. Kurota, and M. Yamaguchi, *Calcium-binding protein regucalcin mRNA expression in the kidney cortex is suppressed by saline ingestion in rats*. Molecular and cellular biochemistry, 1996. **162**(2): p. 139-144.
232. Nakagawa, T. and M. Yamaguchi, *Hormonal regulation on regucalcin mRNA expression in cloned normal rat kidney proximal tubular epithelial NRK52E cells*. Journal of cellular biochemistry, 2005. **95**(3): p. 589-597.
233. Yamaguchi, M., et al., *Hormonal regulation of regucalcin mRNA expression in osteoblastic MC3T3-E1 cells*. International journal of molecular medicine, 2008. **21**(6): p. 771-775.
234. Yamaguchi, M., Y. Kanayama, and N. Shimokawa, *Expression of calcium-binding protein regucalcin mRNA in rat liver is stimulated by calcitonin: the hormonal effect is mediated through calcium*. Molecular and cellular biochemistry, 1994. **136**(1): p. 43-48.
235. Sar, P., et al., *Alterations in expression of senescence marker protein-30 gene by 3, 3′, 5-triiodo-l-thyronine (T 3)*. Molecular and cellular biochemistry, 2007. **303**(1-2): p. 239-242.
236. Chakraborti, S. and B.J. Bahnson, *Crystal structure of human senescence marker protein 30: insights linking structural, enzymatic, and physiological functions*. Biochemistry, 2010. **49**(16): p. 3436-3444.
237. Harikrishna, P., et al., *Calcium Binding Ability of Recombinant Buffalo Regucalcin: A Study Using Circular Dichroism Spectroscopy*. Protein J, 2017. **36**(2): p. 108-111.
238. Dutta, R.K., et al., *Comparative analysis of the metal-dependent structural and functional properties of mouse and human SMP30*. PloS one, 2019. **14**(6): p. e0218629-e0218629.
239. Yamaguchi, M., *Role of regucalcin in calcium signaling*. Life sciences, 2000. **66**(19): p. 1769-1780.

240. Yamaguchi, M., *Role of regucalcin in maintaining cell homeostasis and function*. International journal of molecular medicine, 2005. **15**(3): p. 371-389.
241. Nakagawa, T. and M. Yamaguchi, *Overexpression of regucalcin enhances its nuclear localization and suppresses L-type Ca2+ channel and calcium-sensing receptor mRNA expressions in cloned normal rat kidney proximal tubular epithelial NRK52E cells*. Journal of cellular biochemistry, 2006. **99**(4): p. 1064-1077.
242. Vaz, C.V., et al., *Effect of extracellular calcium on regucalcin expression and cell viability in neoplastic and non-neoplastic human prostate cells*. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2015. **1853**(10): p. 2621-2628.
243. Fujita, T., et al., *Senescence marker protein-30 (SMP30) rescues cell death by enhancing plasma membrane Ca2+-pumping activity in Hep G2 cells*. Biochemical and biophysical research communications, 1998. **250**(2): p. 374-380.
244. YAMAGUCHI, M., S. MORI, and S. KATO, *Calcium-binding protein regucalcin is an activator of (Ca2+-Mg2+)-adenosine triphosphatase in the plasma membranes of rat liver*. Chemical and pharmaceutical bulletin, 1988. **36**(9): p. 3532-3539.
245. Takahashi, H. and M. Yamaguchi, *Regucalcin modulates hormonal effect on (Ca 2+– Mg 2+)-ATPase activity in rat liver plasma membranes*. Molecular and cellular biochemistry, 1993. **125**(2): p. 171-177.
246. Takahashi, H. and M. Yamaguchi, *Increase of (Ca 2+– Mg 2+)-ATPase activity in hepatic plasma membranes of rats administered orally calcium: The endogenous role of regucalcin*. Molecular and cellular biochemistry, 1995. **144**(1): p. 1-6.
247. Takahasi, H. and M. Yamaguchi, *Enhancement of plasma membrane (Ca 2+-Mg 2+)-ATPase activity in regenerating rat liver: involvement of endogenous activating protein regucalcin*. Molecular and cellular biochemistry, 1996. **162**(2): p. 133-138.
248. Takahashi, H. and M. Yamaguchi, *Activating effect of regucalcin on (Ca 2+-Mg 2+)-ATPase in rat liver plasma membranes: relation to sulfhydryl group*. Molecular and cellular biochemistry, 1994. **136**(1): p. 71-76.
249. Takahashi, H. and M. Yamaguchi, *Stimulatory effect of regucalcin on ATP-dependent calcium transport in rat liver plasma membranes*. Molecular and cellular biochemistry, 1997. **168**(1-2): p. 149-153.
250. Kurota, H. and M. Yamaguchi, *Activatory effect of calcium-binding protein regucalcin on ATP-dependent calcium transport in the basolateral membranes of rat kidney cortex*. Molecular and cellular biochemistry, 1997. **169**(1-2): p. 149-156.
251. Yamaguchi, M. and R. Nakajima, *Role of regucalcin as an activator of sarcoplasmic reticulum Ca2+-ATPase activity in rat heart muscle*. Journal of cellular biochemistry, 2002. **86**(1): p. 184-193.

252. Takahashi, H. and M. Yamaguchi, *Role of regucalcin as an activator of Ca2+-ATPase activity in rat liver microsomes.* Journal of cellular biochemistry, 1999. **74**(4): p. 663-669.
253. Tsurusaki, Y. and M. Yamaguchi, *Role of endogenous regucalcin in the regulation of Ca2+-ATPase activity in rat liver nuclei.* Journal of cellular biochemistry, 2000. **78**(4): p. 541-549.
254. Mori, S. and M. Yamaguchi, *Calcium-binding protein regucalcin stimulates the uptake of Ca2+ by rat liver mitochondria.* Chemical & pharmaceutical bulletin, 1991. **39**(1): p. 224-226.
255. Takahashi, H. and M. Yamaguchi, *Stimulatory effect of regucalcin on ATP-dependent Ca(2+) uptake activity in rat liver mitochondria.* Journal of cellular biochemistry, 2000. **78**(1): p. 121-130.
256. Xue, J.H., H. Takahashi, and M. Yamaguchi, *Stimulatory effect of regucalcin on mitochondrial ATP-dependent calcium uptake activity in rat kidney cortex.* Journal of cellular biochemistry, 2000. **80**(2): p. 285-292.
257. Yamaguchi, M., Y. Takakura, and T. Nakagawa, *Regucalcin increases Ca2+-ATPase activity in the mitochondria of brain tissues of normal and transgenic rats.* Journal of cellular biochemistry, 2008. **104**(3): p. 795-804.
258. Akhter, T., N. Sawada, and M. Yamaguchi, *Regucalcin increases Ca2+-ATPase activity in the heart mitochondria of normal and regucalcin transgenic rats.* International journal of molecular medicine, 2006. **18**(1): p. 171-176.
259. Ishigami, A., et al., *Nuclear localization of senescence marker protein-30, SMP30, in cultured mouse hepatocytes and its similarity to RNA polymerase.* Bioscience, biotechnology, and biochemistry, 2003. **67**(1): p. 158-160.
260. Tsurusaki, Y., H. Misawa, and M. Yamaguchi, *Translocation of regucalcin to rat liver nucleus: involvement of nuclear protein kinase and protein phosphatase regulation.* International journal of molecular medicine, 2000. **6**(6): p. 655-715.
261. Morooka, Y. and M. Yamaguchi, *Endogenous regucalcin suppresses the enhancement of protein phosphatase activity in the cytosol and nucleus of kidney cortex in calcium-administered rats.* Journal of cellular biochemistry, 2002. **85**(3): p. 553-560.
262. Tobisawa, M. and M. Yamaguchi, *Role of endogenous regucalcin in brain function: suppression of cytosolic nitric oxide synthase and nuclear protein tyrosine phosphatase activities in brain tissue of transgenic rats.* International journal of molecular medicine, 2003. **12**(4): p. 581-585.
263. Omura, M. and M. Yamaguchi, *Regulation of protein phosphatase activity by regucalcin localization in rat liver nuclei.* Journal of cellular biochemistry, 1999. **75**(3): p. 437-445.

264. Nakagawa, T. and M. Yamaguchi, *Nuclear localization of regucalcin is enhanced in culture with protein kinase C activation in cloned normal rat kidney proximal tubular epithelial NRK52E cells*. International journal of molecular medicine, 2008. **21**(5): p. 605-610.

265. Yamaguchi, M., ed. *Regucalcin: Metabolic Regulation and Disease*. The horizons of regucalcin in male reproduction. , ed. P.H. Correia S, Silva AMS, Santos VM and Socorro S. 2019

Nova Science Publishers, Inc, New York, USA.

266. Sato, T., et al., *Senescence marker protein-30 protects mice lungs from oxidative stress, aging, and smoking*. American journal of respiratory and critical care medicine, 2006. **174**(5): p. 530-537.

267. Son, T.G., et al., *SMP30 deficiency causes increased oxidative stress in brain*. Mechanisms of ageing and development, 2006. **127**(5): p. 451-457.

268. Fukaya, Y. and M. Yamaguchi, *Regucalcin increases superoxide dismutase activity in rat liver cytosol*. Biological and Pharmaceutical Bulletin, 2004. **27**(9): p. 1444-1446.

269. Ichikawa, E. and M. Yamaguchi, *Regucalcin increases superoxide dismutase activity in the heart cytosol of normal and regucalcin transgenic rats*. International journal of molecular medicine, 2004. **14**(4): p. 691-696.

270. Handa, S., N. Maruyama, and A. Ishigami, *Over-expression of Senescence Marker Protein-30 decreases reactive oxygen species in human hepatic carcinoma Hep G2 cells*. Biological and Pharmaceutical Bulletin, 2009. **32**(10): p. 1645-1648.

271. Vaz, C.V., et al., *Aging-associated changes in oxidative stress, cell proliferation, and apoptosis are prevented in the prostate of transgenic rats overexpressing regucalcin*. Translational Research, 2015. **166**(6): p. 693-705.

272. Ma, Z.J. and M. Yamaguchi, *Suppressive role of endogenous regucalcin in the regulation of nitric oxide synthase activity in heart muscle cytosol of normal and regucalcin transgenic rats*. International journal of molecular medicine, 2002. **10**(6): p. 761-766.

273. Tobisawa, M. and M. Yamaguchi, *Inhibitory role of regucalcin in the regulation of nitric oxide synthase activity in rat brain cytosol: involvement of aging*. Journal of the neurological sciences, 2003. **209**(1-2): p. 47-54.

274. Izumi, T., Y. Tsurusaki, and M. Yamaguchi, *Suppressive effect of endogenous regucalcin on nitric oxide synthase activity in cloned rat hepatoma H4-II-E cells overexpressing regucalcin*. Journal of cellular biochemistry, 2003. **89**(4): p. 800-807.

275. Ma, Z.J. and M. Yamaguchi, *Regulatory effect of regucalcin on nitric oxide synthase activity in rat kidney cortex cytosol: Role of endogenous regucalcin in transgenic rats*. International journal of molecular medicine, 2003. **12**(2): p. 201-206.

276. Yamaguchi, M., H. Takahashi, and Y. Tsurusaki, *Suppressive role of endogenous regucalcin in the enhancement of nitric oxide synthase activity in liver cytosol of normal and regucalcin transgenic rats*. Journal of cellular biochemistry, 2003. **88**(6): p. 1226-1234.

277. Kondo, Y., et al., *Senescence marker protein 30 functions as gluconolactonase in L-ascorbic acid biosynthesis, and its knockout mice are prone to scurvy*. Proceedings of the National Academy of Sciences, 2006. **103**(15): p. 5723-5728.

278. Amano, A., et al., *Effects of ascorbic acid deficiency on protein and lipid oxidation in livers from SMP30/GNL knockout mice*. Journal of nutritional science and vitaminology, 2013. **59**(6): p. 489-495.

279. Koike, K., et al., *Complete lack of vitamin C intake generates pulmonary emphysema in senescence marker protein-30 knockout mice*. American Journal of Physiology-Lung Cellular and Molecular Physiology, 2010. **298**(6): p. L784-L792.

280. Kondo, Y., et al., *Senescence marker protein-30/superoxide dismutase 1 double knockout mice exhibit increased oxidative stress and hepatic steatosis*. FEBS open bio, 2014. **4**: p. 522-532.

281. Kondo, Y., et al., *Vitamin C depletion increases superoxide generation in brains of SMP30/GNL knockout mice*. Biochemical and biophysical research communications, 2008. **377**(1): p. 291-296.

282. Kondo, Y., et al., *Senescence marker protein-30 is a unique enzyme that hydrolyzes diisopropyl phosphorofluoridate in the liver*. FEBS letters, 2004. **570**(1-3): p. 57-62.

283. Marques, R., et al., *The diverse roles of calcium-binding protein regucalcin in cell biology: from tissue expression and signalling to disease*. Cellular and molecular life sciences, 2014. **71**(1): p. 93-111.

284. Shimokawa, N. and M. Yamaguchi, *Molecular cloning and sequencing of the cDNA coding for a calcium-binding protein regucalcin from rat liver*. FEBS letters, 1993. **327**(3): p. 251-255.

285. Fujita, T., et al., *Isolation of cDNA clone encoding rat senescence marker protein-30 (SMP30) and its tissue distribution*. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression, 1992. **1132**(3): p. 297-305.

286. Yamaguchi, M. and M. Isogai, *Tissue concentration of calcium-binding protein regucalcin in rats by enzyme-linked immunoadsorbent assay*. Molecular and Cellular Biochemistry, 1993. **122**(1): p. 65-68.

287. Ishii, K., et al., *Immunohistochemical localization of senescence marker protein-30 (SMP30) in the submandibular gland and ultrastructural changes of the granular duct cells in SMP30 knockout mice*. Histology and histopathology, 2005.
288. Yamaguchi, M., Y. Hanahisa, and T. Murata, *Expression of calcium-binding protein regucalcin and microsomal Ca2+-ATPase regulation in rat brain: attenuation with increasing age*. Molecular and cellular biochemistry, 1999. **200**(1-2): p. 43-49.
289. Yamaguchi, M., et al., *Role of endogenous regucalcin in bone metabolism: bone loss is induced in regucalcin transgenic rats*. International journal of molecular medicine, 2002. **10**(4): p. 377-383.
290. Yamaguchi, M., et al., *Expression of regucalcin in rat bone marrow cells: involvement of osteoclastic bone resorption in regucalcin transgenic rats*. International journal of molecular medicine, 2004. **13**(3): p. 437-443.
291. Blanc, J.F., et al., *Proteomic analysis of differentially expressed proteins in hepatocellular carcinoma developed in patients with chronic viral hepatitis C*. Proteomics, 2005. **5**(14): p. 3778-3789.
292. Li, C., et al., *Proteome analysis of human lung squamous carcinoma*. PROTEOMICS, 2006. **6**(2): p. 547-558.
293. Marques, R., et al., *Histopathological and in vivo evidence of regucalcin as a protective molecule in mammary gland carcinogenesis*. Experimental cell research, 2015. **330**(2): p. 325-335.
294. Zhou, S.-F., et al., *Serum immunoreactivity of SMP30 and its tissues expression in hepatocellular carcinoma*. Clinical biochemistry, 2011. **44**(4): p. 331-336.
295. Li, X., et al., *Exogenous regucalcin negatively regulates the progression of cervical adenocarcinoma*. Oncology Letters, 2019. **18**(1): p. 609-616.
296. Suzuki, S., et al., *Specific differences in gene expression profile revealed by cDNA microarray analysis of glutathione S -transferase placental form (GST-P) immunohistochemically positive rat liver foci and surrounding tissue*. Carcinogenesis, 2004. **25**(3): p. 439-443.
297. Fujita, T., et al., *Gene regulation of senescence marker protein-30 (SMP30): coordinated up-regulation with tissue maturation and gradual down-regulation with aging*. Mechanisms of Ageing and Development, 1996. **87**(3): p. 219-229.
298. Maniyadath, B., N. Shukla, and U. Kolthur-Seetharam, *Gene Expression, Epigenetics and Ageing*. Sub-cellular biochemistry, 2018. **90**: p. 471-504.
299. Weinberg, R. and D. Hanahan, *The hallmarks of cancer*. Cell, 2000. **100**(1): p. 57-70.
300. Fouad, Y.A. and C. Aanei, *Revisiting the hallmarks of cancer*. American journal of cancer research, 2017. **7**(5): p. 1016.

301. Yamaguchi, M., *Role of regucalcin in cell nuclear regulation: involvement as a transcription factor*. Cell and Tissue Research, 2013. **354**(2): p. 331-341.
302. Tsurusaki, Y. and M. Yamaguchi, *Role of regucalcin in liver nuclear function: binding of regucalcin to nuclear protein or DNA and modulation of tumor-related gene expression*. Int J Mol Med, 2004. **14**(2): p. 277-81.
303. Yamaguchi, M. and T. Sakurai, *Inhibitory effect of calcium-binding protein regucalcin on Ca2+-activated DNA fragmentation in rat liver nuclei*. FEBS letters, 1991. **279**(2): p. 281-284.
304. Jones, D.P., et al., *Calcium-activated DNA fragmentation in rat liver nuclei*. Journal of Biological Chemistry, 1989. **264**(11): p. 6398-6403.
305. Tsurusaki, Y. and M. Yamaguchi, *Suppressive effect of endogenous regucalcin on guanosine triphosphatase activity in rat liver nucleus*. Biological and Pharmaceutical Bulletin, 2001. **24**(8): p. 958-961.
306. Yamaguchi, M., *Involvement of Regucalcin in Human Carcinogenesis Prevention*. Cancer Studies, 2017. **1**(1): p. 2.
307. Yamaguchi, M., et al., *Prolonged survival in hepatocarcinoma patients with increased regucalcin gene expression: HepG2 cell proliferation is suppressed by overexpression of regucalcin in vitro*. International journal of oncology, 2016. **49**(4): p. 1686-1694.
308. Yamaguchi, M., et al., *Prolonged survival in pancreatic cancer patients with increased regucalcin gene expression: Overexpression of regucalcin suppresses the proliferation in human pancreatic cancer MIA PaCa-2 cells in vitro*. International journal of oncology, 2016. **48**(5): p. 1955-1964.
309. Yamaguchi, M., et al., *Increased regucalcin gene expression extends survival in breast cancer patients: Overexpression of regucalcin suppresses the proliferation and metastatic bone activity in MDA-MB-231 human breast cancer cells in vitro*. International journal of oncology, 2016. **49**(2): p. 812-822.
310. Yamaguchi, M., et al., *Survival of lung cancer patients is prolonged with higher regucalcin gene expression: Suppressed proliferation of lung adenocarcinoma A549 cells in vitro*. Molecular and cellular biochemistry, 2017. **430**(1-2): p. 37-46.
311. Yamaguchi, M. and T. Murata, *Exogenous regucalcin suppresses the growth of human liver cancer HepG2 cells in vitro*. Oncology reports, 2018. **39**(6): p. 2924-2930.
312. Yamaguchi, M., S. Osuka, and T. Murata, *Prolonged survival of patients with colorectal cancer is associated with a higher regucalcin gene expression: Overexpression of regucalcin suppresses the growth of human colorectal carcinoma cells in vitro*. International journal of oncology, 2018. **53**(3): p. 1313-1322.

313. Li, X., et al., *A mechanism of regucalcin knock-down in the promotion of proliferation and movement of human cervical cancer HeLa cells.* Translational Cancer Research, 2019. **8**(2): p. 402-409.
314. Yamaguchi, M., et al., *Prolonged survival of renal cancer patients is concomitant with a higher regucalcin gene expression in tumor tissues: Overexpression of regucalcin suppresses the growth of human renal cell carcinoma cells in vitro.* International journal of oncology, 2019. **54**(1): p. 188-198.
315. Yamaguchi, M. and Y. Daimon, *Overexpression of regucalcin suppresses cell proliferation in cloned rat hepatoma H4-II-E cells: involvement of intracellular signaling factors and cell cycle-related genes.* Journal of cellular biochemistry, 2005. **95**(6): p. 1169-1177.
316. Nakagawa, T., N. Sawada, and M. Yamaguchi, *Overexpression of regucalcin suppresses cell proliferation of cloned normal rat kidney proximal tubular epithelial NRK52E cells.* International journal of molecular medicine, 2005. **16**(4): p. 637-643.
317. Yamaguchi, M. and T. Murata, *Overexpression of Regucalcin Suppresses the Growth of Human Osteosarcoma Cells in Vitro: Repressive Effect of Extracellular Regucalcin.* Cancer investigation, 2020. **38**(1): p. 37-51.
318. Tsurusaki, Y. and M. Yamaguchi, *Overexpression of regucalcin modulates tumor-related gene expression in cloned rat hepatoma H4-II-E cells.* Journal of Cellular Biochemistry, 2003. **90**(3): p. 619-626.
319. Yamaguchi, M. and T. Murata, *Suppressive effects of exogenous regucalcin on the proliferation of human pancreatic cancer MIA PaCa-2 cells in vitro.* Int J Mol Med, 2015. **35**(6): p. 1773-8.
320. Yamaguchi, M. and T. Murata, *Exogenous regucalcin suppresses the proliferation of human breast cancer MDA-MB-231 bone metastatic cells in vitro.* Mol Med Rep, 2015. **12**(5): p. 7801-5.
321. Yamaguchi, M., *Suppressive role of regucalcin in liver cell proliferation: involvement in carcinogenesis.* Cell Proliferation, 2013. **46**(3): p. 243-253.
322. Yamaguchi, M. and S. Mori, *Effect of calcium-binding protein regucalcin on hepatic protein synthesis: inhibition of aminoacyl-tRNA synthetase activity.* Molecular and cellular biochemistry, 1990. **99**(1): p. 25-32.
323. Yamaguchi, M. and N. Nishina, *Characterization of regucalcin effect on proteolytic activity in rat liver cytosol: relation to cysteinyl-proteases.* Molecular and cellular biochemistry, 1995. **148**(1): p. 67-72.
324. Yamaguchi, M., *The anti-apoptotic effect of regucalcin is mediated through multisignaling pathways.* Apoptosis, 2013. **18**(10): p. 1145-1153.

325. Izumi, T. and M. Yamaguchi, *Overexpression of regucalcin suppresses cell death in cloned rat hepatoma H4-II-E cells induced by tumor necrosis factor-α or thapsigargin*. Journal of cellular biochemistry, 2004. **92**(2): p. 296-306.
326. Izumi, T. and M. Yamaguchi, *Overexpression of regucalcin suppresses cell death and apoptosis in cloned rat hepatoma H4-II-E cells induced by lipopolysaccharide, PD 98059, dibucaine, or Bay K 8644*. Journal of cellular biochemistry, 2004. **93**(3): p. 598-608.
327. Lim, S., et al., *Differential expression of regucalcin (SMP30) and its function in hypoxic cardiomyocytes*. Tissue Engineering and Regenerative Medicine, 2009. **6**(13): p. 1273-1281.
328. Correia, S., et al., *Transgenic overexpression of regucalcin leads to suppression of thapsigargin-and actinomycin D-induced apoptosis in the testis by modulation of apoptotic pathways*. Andrology, 2014. **2**(2): p. 290-298.
329. Nakagawa, T. and M. Yamaguchi, *Overexpression of regucalcin suppresses apoptotic cell death in cloned normal rat kidney proximal tubular epithelial NRK52E cells: Change in apoptosis-related gene expression*. Journal of cellular biochemistry, 2005. **96**(6): p. 1274-1285.
330. Fukaya, Y. and M. Yamaguchi, *Overexpression of regucalcin suppresses cell death and apoptosis in cloned rat hepatoma H4-II-E cells induced by insulin or insulin-like growth factor-I*. Journal of cellular biochemistry, 2005. **96**(1): p. 145-154.
331. Matsuyama, S., et al., *Senescence marker protein-30 regulates Akt activity and contributes to cell survival in Hep G2 cells*. Biochemical and biophysical research communications, 2004. **321**(2): p. 386-390.
332. Ishigami, A., et al., *Senescence marker protein-30 knockout mouse liver is highly susceptible to tumor necrosis factor-α-and Fas-mediated apoptosis*. The American journal of pathology, 2002. **161**(4): p. 1273-1281.
333. Oudin, M.J. and V.M. Weaver, *Physical and Chemical Gradients in the Tumor Microenvironment Regulate Tumor Cell Invasion, Migration, and Metastasis*. Cold Spring Harb Symp Quant Biol, 2016. **81**: p. 189-205.
334. Shang, S., F. Hua, and Z.W. Hu, *The regulation of β-catenin activity and function in cancer: therapeutic opportunities*. Oncotarget, 2017. **8**(20): p. 33972-33989.
335. Lei, Y., et al., *MicroRNAs target the Wnt/β-catenin signaling pathway to regulate epithelial-mesenchymal transition in cancer (Review)*. Oncol Rep, 2020. **44**(4): p. 1299-1313.
336. Soleimani, A., et al., *The potential role of regulatory microRNAs of RAS/MAPK signaling pathway in the pathogenesis of colorectal cancer*. Journal of cellular biochemistry, 2019. **120**(12): p. 19245-19253.

337. Campbell, P.M. and C.J. Der. *Oncogenic Ras and its role in tumor cell invasion and metastasis*. in *Seminars in cancer biology*. 2004. Elsevier.
338. Toker, A. and M. Yoeli-Lerner, *Akt signaling and cancer: surviving but not moving on*. Cancer research, 2006. **66**(8): p. 3963-3966.
339. Mali, S.B., *Review of STAT3 (Signal Transducers and Activators of Transcription) in head and neck cancer*. Oral oncology, 2015. **51**(6): p. 565-569.
340. Yu, H., et al., *Revisiting STAT3 signalling in cancer: new and unexpected biological functions*. Nature reviews cancer, 2014. **14**(11): p. 736-746.
341. Dolcet, X., et al., *NF-kB in development and progression of human cancer*. Virchows archiv, 2005. **446**(5): p. 475-482.
342. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation*. Cell, 2011. **144**(5): p. 646-74.
343. Warburg, O., F. Wind, and E. Negelein, *The metabolism of tumors in the body*. The Journal of general physiology, 1927. **8**(6): p. 519.
344. Koppenol, W.H., P.L. Bounds, and C.V. Dang, *Otto Warburg's contributions to current concepts of cancer metabolism*. Nat Rev Cancer, 2011. **11**(5): p. 325-37.
345. Potter, M., E. Newport, and K.J. Morten, *The Warburg effect: 80 years on*. Biochemical Society Transactions, 2016. **44**(5): p. 1499-1505.
346. Li, X.b., J.d. Gu, and Q.h. Zhou, *Review of aerobic glycolysis and its key enzymes–new targets for lung cancer therapy*. Thoracic cancer, 2015. **6**(1): p. 17-24.
347. Cardoso, H.J., et al., *Revisiting prostate cancer metabolism: From metabolites to disease and therapy*. Med Res Rev, 2020.
348. Vaz, C.V., et al., *Androgen-responsive and nonresponsive prostate cancer cells present a distinct glycolytic metabolism profile*. The international journal of biochemistry & cell biology, 2012. **44**(11): p. 2077-2084.
349. Vaz, C.V., et al., *Androgens enhance the glycolytic metabolism and lactate export in prostate cancer cells by modulating the expression of GLUT1, GLUT3, PFK, LDH and MCT4 genes*. Journal of Cancer Research and Clinical Oncology, 2016. **142**(1): p. 5-16.
350. YAMAGUCHI, M. and H. SHIBANO, *Calcium-binding protein isolated from rat liver cytosol reverses activation of pyruvate kinase by Ca2+*. Chemical and pharmaceutical bulletin, 1987. **35**(5): p. 2025-2029.
351. YAMAGUCHI, M. and H. YOSHIDA, *Regulatory effect of calcium-binding protein isolated from rat liver cytosol on activation of fructose 1, 6-diphosphatase by Ca2+-calmodulin*. Chemical and pharmaceutical bulletin, 1985. **33**(10): p. 4489-4493.

352. YAMAGUCHI, M., S. MORI, and Y. SUKETA, *Effects of Ca2+ and V5+ on glucose-6-phosphatase activity in rat liver microsomes: the Ca2+ effect is reversed by regucalcin*. Chemical and pharmaceutical bulletin, 1989. **37**(2): p. 388-390.
353. YAMAGUCHI, M. and H. SHIBANO, *Reversible Effect of Calcium-Binding Protein on the Ca2+-Induced Activation of Succinate Dehydrogenase in Rat Liver Mitochondoria*. Chemical and pharmaceutical bulletin, 1987. **35**(9): p. 3766-3770.
354. Vaz, C.V., et al., *Suppressed glycolytic metabolism in the prostate of transgenic rats overexpressing calcium-binding protein regucalcin underpins reduced cell proliferation*. Transgenic research, 2016. **25**(2): p. 139-148.
355. Nakashima, C. and M. Yamaguchi, *Overexpression of regucalcin enhances glucose utilization and lipid production in cloned rat hepatoma H4-II-E cells: Involvement of insulin resistance*. Journal of cellular biochemistry, 2006. **99**(6): p. 1582-1592.
356. Yamaguchi, M. and S. Uchiyama, *Regucalcin stimulates osteoclast-like cell formation in mouse marrow cultures*. Journal of cellular biochemistry, 2005. **94**(4): p. 794-803.
357. YAMAGUCHI, M. and H. SHIBANO, *Effect of Calcium-Binding Protein on the Activation of Phosphorylase a in Rat Hepatic Particulate Glycogen by Ca2+*. Chemical and pharmaceutical bulletin, 1987. **35**(6): p. 2581-2584.
358. Exton, J., *Gluconeogenesis*. Metabolism, 1972. **21**(10): p. 945-990.
359. Liao, J., et al., *Extracellular Calcium as a Candidate Mediator of Prostate Cancer Skeletal Metastasis*. Cancer Research, 2006. **66**(18): p. 9065-9073.
360. Brown, E.M. and R.J. MacLeod, *Extracellular Calcium Sensing and Extracellular Calcium Signaling*. Physiological Reviews, 2001. **81**(1): p. 239-297.
361. Liguori, I., et al., *Oxidative stress, aging, and diseases*. Clin Interv Aging, 2018. **13**: p. 757-772.
362. Moldogazieva, N.T. and I.M. Mokhosoev, *Oxidative Stress and Advanced Lipoxidation and Glycation End Products (ALEs and AGEs) in Aging and Age-Related Diseases*. 2019. **2019**: p. 3085756.
363. Cortazar, A.R., et al., *CANCERTOOL: a visualization and representation interface to exploit cancer datasets*. Cancer research, 2018. **78**(21): p. 6320-6328.
364. Glinsky, G.V., et al., *Gene expression profiling predicts clinical outcome of prostate cancer*. J Clin Invest, 2004. **113**(6): p. 913-23.
365. Grasso, C.S., et al., *The mutational landscape of lethal castration-resistant prostate cancer*. Nature, 2012. **487**(7406): p. 239-43.
366. Varambally, S., et al., *Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression*. Cancer Cell, 2005. **8**(5): p. 393-406.

367. Tomlins, S.A., et al., *Integrative molecular concept modeling of prostate cancer progression*. Nat Genet, 2007. **39**(1): p. 41-51.
368. Lapointe, J., et al., *Gene expression profiling identifies clinically relevant subtypes of prostate cancer*. Proc Natl Acad Sci U S A, 2004. **101**(3): p. 811-6.
369. *The Cancer Genome Atlas Program*. [cited 2020 28/08/2020]; Available from: https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga.
370. Mitchell, S., et al., *Phenotypic and genotypic characterization of commonly used human prostatic cell lines*. BJU Int, 2000. **85**(7): p. 932-44.
371. Avancès, C., et al., *Human prostatic cell line PNT1A, a useful tool for studying androgen receptor transcriptional activity and its differential subnuclear localization in the presence of androgens and antiandrogens*. Molecular and Cellular Endocrinology, 2001. **184**(1): p. 13-24.
372. Van Meerloo, J., G.J. Kaspers, and J. Cloos, *Cell sensitivity assays: the MTT assay*, in *Cancer cell culture*. 2011, Springer. p. 237-245.
373. Vichai, V. and K. Kirtikara, *Sulforhodamine B colorimetric assay for cytotoxicity screening*. Nature protocols, 2006. **1**(3): p. 1112.
374. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT–PCR*. Nucleic acids research, 2001. **29**(9): p. e45-e45.
375. Karimian, A., Y. Ahmadi, and B. Yousefi, *Multiple functions of p21 in cell cycle, apoptosis and transcriptional regulation after DNA damage*. DNA Repair, 2016. **42**: p. 63-71.
376. Wagner, E.F. and Á.R. Nebreda, *Signal integration by JNK and p38 MAPK pathways in cancer development*. Nature Reviews Cancer, 2009. **9**(8): p. 537-549.
377. Papa, S., P.M. Choy, and C. Bubici, *The ERK and JNK pathways in the regulation of metabolic reprogramming*. Oncogene, 2019. **38**(13): p. 2223-2240.
378. Murai, N., et al., *Novel ubiquitin-independent nucleolar c-Myc degradation pathway mediated by antizyme 2*. Scientific Reports, 2018. **8**(1): p. 3005.
379. Gurel, B., et al., *Nuclear MYC protein overexpression is an early alteration in human prostate carcinogenesis*. Modern Pathology, 2008. **21**(9): p. 1156-1167.
380. Khan, H., et al., *Targeting NF-κB signaling pathway in cancer by dietary polyphenols*. Critical reviews in food science and nutrition, 2020. **60**(16): p. 2790-2800.
381. Gerdes, J., et al., *Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67*. The journal of immunology, 1984. **133**(4): p. 1710-1715.

382. Mo, Z., et al., *Senescence marker protein 30 (SMP30) serves as a potential prognostic indicator in hepatocellular carcinoma*. Scientific reports, 2016. **6**: p. 39376.
383. Zheng, S.-X., et al., *Diagnostic Value of Serum SMP30 and Anti-SMP30 Antibody in Hepatocellular Carcinoma*. Laboratory medicine, 2018. **49**(3): p. 203-210.
384. Zhang, D., et al., *Polyphyllin I induces cell cycle arrest in prostate cancer cells via the upregulation of IL6 and P21 expression*. Medicine, 2019. **98**(44): p. e17743.
385. Murata, T., et al., *Regucalcin enhances adipocyte differentiation and attenuates inflammation in 3T3-L1 cells*. FEBS Open Bio, 2020. **10**(10): p. 1967-1984.
386. Baltazar, F., et al., *Lactate Beyond a Waste Metabolite: Metabolic Affairs and Signaling in Malignancy*. Frontiers in Oncology, 2020. **10**: p. 231.

# VIII. Appendix

**Table VIII.1.** Number of samples in each dataset and conditions analysed in the bioinformatic analysis using the CancerTool software [363].

| Parameters analysed | | | Datasets | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Glinsky [364] | Grasso [365] | Lapointe [368] | Taylor [104] | TCGA [369] | Tomlins [367] | Varambally [366] |
| RGN expression in human prostate cancer cases | Normal | | NA | 12 | 9 | 29 | NA | 21 | 6 |
| | Prostate cancer | | NA | 76 | 17 | 150 | NA | 48 | 13 |
| RGN expression with the progression of human prostate cancer | Non-tumoral | | NA | 12 | 9 | 29 | NA | 21 | 6 |
| | Primary tumours | | NA | 49 | 13 | 130 | NA | 31 | 7 |
| | Metastatic prostate cancer | | NA | 27 | 4 | 19 | NA | 17 | 6 |
| Regucalcin expression accordingly with prostate cancer gleason score | Gleason score 6 | | 15 | NA | NA | 76 | 44 | NA | NA |
| | Gleason score 7 | | 44 | NA | NA | 42 | 247 | NA | NA |
| | Gleason score 8 | | 10 | NA | NA | 7 | 64 | NA | NA |
| | Gleason score 9 | | 8 | NA | NA | 4 | 137 | NA | NA |
| | Gleason score 10 | | NA | NA | NA | NA | 4 | NA | NA |
| Regucalcin expression and prostate cancer patient's disease free-survival | Lower than the mean | | 39 | NA | NA | 73 | 219 | NA | NA |
| | Higher than the mean | | 40 | NA | NA | 58 | 271 | NA | NA |
| Regucalcin expression and prostate cancer recurrence | Disease-free | | 42 | NA | NA | 103 | 397 | NA | NA |
| | Recurred | | 37 | NA | NA | 27 | 91 | NA | NA |
| Correlation analyses of *regucalcin* gene expression with that of *cyclin dependent kinase inhibitor 1A*, *tumour protein P53*, *IL6*, *AKT1*, *mitogen activated protein kinase 1*, *MYC* and *nuclear factor kappa B subunit 1* genes | *Cyclin dependent kinase inhibitor 1A* | Non-tumoral | 0 | 12 | 9 | 29 | 0 | 0 | 6 |
| | | Primary tumours | 79 | 49 | 13 | 131 | 494 | 0 | 7 |
| | | Metastatic prostate cancer | 0 | 27 | 4 | 19 | 0 | 0 | 6 |
| | *Tumour protein P53* | Non-tumoral | 0 | 12 | 9 | 29 | 0 | 17 | 6 |
| | | Primary tumours | 79 | 49 | 13 | 131 | 495 | 31 | 7 |
| | | Metastatic prostate cancer | 0 | 27 | 4 | 19 | 0 | 17 | 6 |
| | *IL6* | Non-tumoral | 0 | 12 | 9 | 29 | 0 | 21 | 6 |
| | | Primary tumours | 79 | 49 | 13 | 131 | 492 | 30 | 7 |
| | | Metastatic prostate cancer | 0 | 27 | 4 | 19 | 0 | 17 | 6 |
| | *AKT1* | Non-tumoral | 0 | 12 | 9 | 29 | 0 | 11 | 6 |
| | | Primary tumours | 79 | 49 | 13 | 131 | 495 | 20 | 7 |
| | | Metastatic prostate cancer | 0 | 27 | 4 | 19 | 0 | 11 | 6 |
| | *Mitogen activated protein kinase 1* | Non-tumoral | 0 | 12 | 9 | 29 | 0 | 8 | 6 |
| | | Primary tumours | 79 | 49 | 11 | 131 | 495 | 4 | 7 |
| | | Metastatic prostate cancer | 0 | 27 | 4 | 19 | 0 | 3 | 6 |
| | *MYC* | Non-tumoral | 0 | 4 | 9 | 29 | 0 | 21 | 6 |
| | | Primary tumours | 79 | 33 | 13 | 131 | 495 | 31 | 7 |
| | | Metastatic prostate cancer | 0 | 20 | 4 | 19 | 0 | 16 | 6 |
| | *Nuclear factor kappa B subunit 1* | Non-tumoral | 0 | 12 | 9 | 29 | 0 | 21 | 6 |
| | | Primary tumours | 79 | 49 | 13 | 131 | 495 | 31 | 7 |
| | | Metastatic prostate cancer | 0 | 27 | 4 | 19 | 0 | 17 | 6 |

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# CERTIFICATION

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The Editors

Pedro M Guerreiro and João Carlos Cardoso

Faro, October 23, 2020

1) THE RELATIONSHIP OF CALCIUM-BINDING PROTEIN REGUCALCIN WITH CANCER AND MAMMALIAN REPRODUCTION, by L. Fonseca, S. Correia, C.V. Vaz, S. Socorro.

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