Evaluation of regulator of G-protein signaling 2 (RGS2) at different stages of prostate cancer

Significance and clinical potential

Anna Linder

2019

Department of Urology
Institute of Clinical Sciences
Sahlgrenska Academy, University of Gothenburg
Sweden

UNIVERSITY OF GOTHENBURG

Cover illustration: Immunofluorecent staining of LNCaP prostate cancer cells labeled for RGS2

Evaluation of regulator of G-protein signaling 2 (RGS2) at different stages of prostate cancer

Significance and clinical potential

© Anna Linder 2019

anna.linder@gu.se

ISBN 978-91-7833-470-4 (PRINT)
ISBN 978-91-7833-471-1 (PDF)
http://hdl.handle.net/2077/59541

Printed in Gothenburg, Sweden 2019
Printed by BrandFactory

“All you really need to know for the moment is that the universe is a lot more complicated than you might think, even if you start from a position of thinking it's pretty damn complicated in the first place.”

Douglas Adams

Evaluation of regulator of G-protein signaling 2 (RGS2) at different stages of prostate cancer

Significance and clinical potential

Anna Linder

Department of Urology, Institute of Clinical Sciences, Sahlgrenska Academy,
University of Gothenburg, Gothenburg, Sweden

ABSTRACT

Prostate cancer (PC) is often a slow-growing and symptom-free disease with good prognosis. However, a substantial number will progress, ultimately metastasize if left untreated and finally kill the patient. The standard treatment for these stages of PC is androgen deprivation therapy (ADT), which generally has an initially good clinical response. However, ADT drives development of highly aggressive forms of castration-resistant PC (CRPC) and promote development of bone metastases. Thus, early detection of resistance is invaluable considering the incurability of these stages once they are established.

The purpose of the present thesis was to assess the regulation and significance of regulator of G-protein signaling (RGS2) in PC; with focus on PC progression, and development of CRPC and bone metastases. Furthermore, evaluate its potential as a prognostic biomarker for hormone-naïve prostate cancer (HNPC) and in association to development and progress of CRPC. This, as the new era of treatment options calls for stable reliable biomarkers for adequate treatment decisions.

The principal findings from this work suggest that, RGS2 was highly expressed in both advanced HNPC and CRPC. The significance of this was reflected by the association between high levels of RGS2 and poor clinical outcome in both of these stages. Moreover, experimental data suggest that RGS2 expression is regulated by hypoxia and HIF1. The implication of different levels of RGS2 was assessed with RGS2 knockdown in the PC cell line LNCaP. The results show that low and high RGS2 expressing PC cells have distinct PC phenotypes, resembling early low-risk tumors and advanced PC, respectively. Furthermore, the data suggests that by mediating the effect of hypoxia, RGS2 has significant tumor promoting roles in HNPC. Additionally, induced RGS2 expression, in response to ADT, was found predictive of decreased time to relapse in association with resumed androgen-receptor (AR) signaling. The stromal expression of RGS2 display a contrasting expression pattern compared to the epithelial, with decreased expression in association with more advanced disease, the relevance of this was suggested by a prognostic property of stromal RGS2 expression. Finally, high RGS2 expression levels were noticed in human PC bone metastases, and found to be essential for the tumor cells ability to establish in the bone, as well as endorsing of the sclerotic phenotype that is associated with PC bone metastases.

In conclusion, the present thesis suggests a tumor-promoting function for RGS2, associated with PC progress and development of CRPC and PC bone metastases. Furthermore, the results suggest that RGS2 has potential as a prognostic and treatment-predictive biomarker in PC.

Keywords: Prostate cancer, regulator of G-protein signaling 2, castration-resistance, androgen receptor, prostate cancer bone metastases

ISBN 978-91-7833-470-4 (PRINT)
ISBN 978-91-7833-471-1 (PDF)
http://hdl.handle.net/2077/59541

POPULÄRVETENSKAPLIG SAMMANFATTNING

Prostata cancer (PC) är den vanligaste cancerformen hos män i Sverige och även den cancerform som står för flest cancer-relaterade dödsfall. PC har ofta ett långsamt symptomfritt förlopp som är begränsat till prostatan, dessa tumörer har en mycket god prognos och upptäcks främst via rutinmässiga PSA kontroller. I vissa fall utvecklar dock PC ett aggressivt tillväxt mönster och växer utanför prostatan i den omkringliggande vävnaden, eller sprider sig vidare till andra organ som metastaser. PC är beroende av manligt könshormon (androgener) för tillväxt och överlevnad, följaktligen behandlas patienter med lokalt aggressiv eller metastaserande sjukdom med medicinsk eller kirurgisk kastration, så kallad "androgendeprivations terapi" (ADT). Behandlingen bromsar tumörtillväxten genom att blockera produktionen av manligt könshormon från testiklarna. ADT har initialt en mycket god effekt hos de flesta patienter, dock är denna effekt begränsad och cancern återupptar sin tillväxt i kastrations-resistent form (CRPC). Då det idag inte finns någon botande terapi för CRPC är prognosen för dessa patienter mycket dålig och överlevnaden är generellt kortare än tre år. Livslängden kan för en del patienter förlängas med några år vid insats av tillgängliga livsförlängande behandlingsalternativ. Övergången till CRPC är oftast relaterad till fortsatt signalering via androgen receptorn (AR) trots pågående ADT. Kastrationsresistens kan cancercellerna uppnå genom flertalet mekanismer exempelvis via AR- associerade mekanismer där cellen blir oberoende av hormonstimulans för AR aktivitet eller mekanismer då även små mängder av androgener är tillräckligt för fortsatt signalering. Ytterligare kan tumörcellerna själva börja producera androgener.
PC metastaserar främst till skelettet och när tumören väl etablerat sig i ben är den obotbar. Benmetastaser är förknippade med kraftigt reducerad livskvalité till följd av benmetastasernas aggressiva tillväxt och modulering av benet.

Mekanismerna bakom utvecklingen av avancerad sjukdom, kastrations-resistens och benmetastaser är till stor del okända. För att bättre kunna behandla alla stadier av PC är det viktigt att förstå de biologiska processer som driver denna utveckling. I detta avseende har vi studerat proteinet "regulator of G-protein signalling (RGS2)", för att bestämma dess relevans för PC utveckling vid de olika stadierna av sjukdomen, samt för att utvärdera det kliniska värdet av RGS2 som prognostiskmarkör.

RGS2s roll i cancer har inte studerats nämnvärt, och det är relativt få och motstridiga rapporter om dess kliniska relevans. I våra studier av obehandlad PC, har vi experimentellt visat att vid låga nivåer av RGS2 får PC cellerna karaktärsdrag påminnande om de långsamt växande tidiga tumörerna, medan

högre nivåer var associerade med en snabbt växande och metastaserande tumörtyp. I linje med dessa fynd visade data från kliniska material att höga nivåer av RGS2 i tumörvävnaden var associerad med en försämrad överlevnad. Förekomsten av RGS2 i tumörangränsande celler uppvisade ett motsatt förhållande, och låg förekomst var associerat med lägre överlevnad.
Vidare studerades RGS2 i kliniska material bestående av CRPC och obehandlade tumörer, samt i tumörer från patienter som under kort tid behandlats med ADT. Dessa studier visade att nivåerna av RGS2 generellt var högre i CRPC jämfört med obehandlade tumörer, dessutom var en hög nivå i CRPC associerat med en förkortad överlevnad hos dessa patienter. En hög nivå av RGS2 efter påbörjad ADT var associerat med en snabb kastrations-resistent återväxt hos dessa patienter. Experimentellt konstaterades RGS2s prognostiska egenskaper vara associerade med fortsatt AR signalering. Vidare studier i ett patientmaterial bestående av benmetastaser från både obehandlade och CRPC patienter, visade generellt höga nivåer av RGS2 i tumörerna, och särskilt höga nivåer i CRPC. Experimentella studier visades att RGS2 har stor påverkan på PC cellernas förmåga att bilda tumör i ben. RGS2 visades även bidra till tumörcellernas stimulerande effekt på osteoblaster, de benbyggande cellerna i benet. På detta vis bidrar RGS2 till den generella ben tillväxt som är vanlig hos benmetastaser vid PC.

Sammanfattningsvis beskriver denna avhandling att RGS2 har en relevant tumör-främjande roll vid utveckling av avancerad PC och benmetastaser. Vidare visades, att höga nivåer av RGS2 i tumör cellerna är associerat med dålig prognos i både obehandlad och kastrations-resistent PC, samt att RGS2 i ett tidigt skede under behandling med ADT kan prediktera snabb utveckling till CRPC. Resultaten visar att RGS2 har potential som prognostisk och behandlingsprediktiv biomarkör vid PC.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their roman numerals.

I. Linder A, Hagberg Thulin M, Damber JE, Welén K. Analysis of regulator of G-protein signalling 2 (RGS2) expression and function during prostate cancer progression. Scientific reports. 2018; **8:** 1-14

II. Linder A, Larsson K, Welén K, Damber JE, RGS2 is prognostic for development of castration-resistance and cancer-specific survival in CRPC. *Manuscript*

III. Linder A, Spyratou V, Stattin P, Granfors T, Egevad L, Linxweiler J, Jung V, Junker K, Saar M, Hammarsten P, Bergh A, Welén K, Damber JE, Josefsson A. Prognostic value of stromal expression of regulator of G protein signaling 2 (RGS2) and androgen receptor (AR) for men with prostate cancer followed with expectancy management. *Manuscript*

IV. Linder A, Hagberg Thulin M, Welén K, Damber JE. Importance of RGS2 in prostate cancer bone metastases. *Manuscript*

CONTENT

ABBREVIATIONS vi
1 INTRODUCTION 1
1.1 Cancer 1
1.2 Prostate cancer 1
1.2.1 The prostate gland and cancer 1
1.2.2 Androgens and AR signaling 4
1.2.3 Prostate cancer pathology and diagnosis 5
1.2.4 Treatment of Prostate cancer 7
1.3 Castration-resistant PC (CRPC) 8
1.3.1 Mechanisms of castration resistance 8
1.4 Progression of PC 10
1.4.1 Epithelial-mesenchymal transition (EMT) 10
1.4.2 Hypoxia and hypoxia inducible factor 1 (HIF1) 11
1.4.3 PI3K/AKT and IL-6/STAT3 pathways 12
1.5 Prostate cancer bone metastases 13
1.6 Regulator of G-protein signaling 2 (RGS2) 15
1.6.3 Other roles for RGS2 17
2 AIMS 20
3 MATERIAL AND METHODS 21
3.1 Clinical Specimens 21
3.2 Experimental *in vitro* studies 23
3.2.1 Cell lines and culture 23
3.2.2 Induction of hypoxia and stabilization of HIF1α in vitro 25
3.2.3 Knockdown of RGS2 25
3.2.4 Assessment of RGS2 knockdown 26
3.2.5 RGS2 expression in association to AR activity 27
3.2.5.1 Hormone starvation 27
3.2.5.2 AR inhibition by enzalutamide 27

3.3 Experimental *In vivo* studies 28
3.3.1 Subcutaneous implantation 28
3.3.2 Orthotopic implantation 28
3.3.3 Intratibial implantation 29
3.4 Assessment of gene expression 30
3.4.1 RNA extraction and cDNA preparation 30
3.4.2 Quantitative real-time polymerase chain reaction (qPCR) 30
3.4.3 Gene expression profile 30
3.5 Assessment of protein expression 30
3.5.1 Immunohistochemistry (IHC) 30
3.5.1.1 Quantification of IHC staining in clinical specimens (scoring) 31
3.5.1.2 Quantification of IHC in in vivo material 32
3.5.2 Immunocytochemistry (ICC) 32
3.5.3 Western blot (WB) 32
3.5.4 Enzyme-linked immunosorbent assay (ELISA) 33
3.6 Statistics 33
4 RESULTS AND COMMENTS 35
4.1 (Paper I) Analysis of regulator of G-protein signalling 2 (RGS2) expression and function during prostate cancer progression 35
4.2 (Paper II) RGS2 is prognostic for development of castration-resistance and cancer-specific survival in CRPC 38
4.3 (Paper III) Prognostic value of stromal expression of regulator of G-protein signaling 2 (RGS2) and androgen receptor (AR) for men with prostate cancer followed with expectancy management 42
4.4 (Paper IV) Importance of RGS2 in prostate cancer bone metastases. 44
5 GENERAL DISCUSSION 47
5.1 Lack of biomarkers 47
5.2 The multifaceted role of RGS2 47
5.2.1 The impact of a hypoxic environment 48
5.2.2 PI3K/AKT 48
5.2.3 STAT3 49

5.2.4 RGS2 and proliferation 49
5.2.5 AR and RGS2 49
5.2.6 RGS2 and Stress 50
5.2.7 RGS2 in bone metastases 50
5.2.8 RGS2 as a biomarker 51
5.2.9 Future perspectives and concluding remarks 52
6 CONCLUSIONS 54
ACKNOWLEDGEMENT 55
REFERENCES 57

ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AD	Androstenedione
ADT	Androgen deprivation therapy
AKT	AKT serine/threonine kinase 1
AR	Androgen receptor
ARE	Androgen response elements
BAD	BCL2 associated agonist of cell death
BAX	BCL2 associated X, apoptosis regulator
BCL-2	B-cell lymphoma 2
BMP	Bone morphogenic proteins
BPH	Benign prostatic hyperplasia
CDH1	Cadherin 1
CRH	Corticotrophin
CRPC	Castration-resistant prostate cancer
CSS	Cancer-specific survival
Cyp17	Steroid 17-alpha-hydroxylase/17,20 lyase
DCC	Dextrane charcoal striped - fetal bovine serum
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DTC	Disseminating tumor cells
EGF	Epidermal growth factor
eIF2b	Eukaryotic initiation factor 2B
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial–mesenchymal transition
ERK	Extracellular regulated MAP kinase
FBS	Fetal bovine serum
FOXO	Forkhead box, sub-group O
GAP	GTPase activating protein
GG	Gleason grade
GNRH	Gonadotropin releasing hormone
GPCR	G-protein coupled receptor
GS	Gleason score
HIF1	Hypoxia inducible factor 1
HNPC	Hormone-naïve prostate cancer
HR	Hazard ratio
HSP	Heat shock protein
ICC	Immuno cytochemistry

IGF-1	Insulin like growth factor 1
IHC	Immunohistochemistry
IL-6	Interleukin 6
JAK	Janus kinase
KGF	Keratinocyte growth factor
LBD	Ligand binding domain
LH	Luteinzing hormone
LNCaP	Lymphnode carcinoma of the prostate
M stage	Metastatic stage
MAPK	Mitogen-activated protein kinase
MDSC	Myeloid derived suppressor cells
MET	Mesenchymal-epithelial transitions
MMP	Matrix metalloproteinase
mPC	Metastatic PC
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cells
mTOR	Mammalian target of rapamycin
PC	Prostate cancer
PI3K	Phosphoinositide 3-kinase
PIN	Intraepithelial neoplasia
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
PTH	Parathyroid hormone
PTH1R	Parathyroid hormone receptor 1
PTHrP	Parathyroid hormone-related protein
RANKL	Receptor activator of nuclear factor ĸβ ligand
RGS2	Regulator of G-protein signaling 2
RUNX2	Runt-related transcription factor 2
SRDA1	Steroid-5α-reductase isoenzyme-1
STAT3	Signal transducer and activator of transcription 3
T stage	Tumor stage
TGFβ	Transforming growth factor beta
TSC2	TSC complex subunit 2
TURP	Transurethral resection of the prostate
VEGF	Vascular endothelial growth factor
ZEB	Zinc-finger E-box-binding
α-SMA	Alpha-smooth muscle actin

1 INTRODUCTION

1.1 Cancer

Cancer is a global cause of death and a universal health problem [1-4]. The term cancer is an assemblage of related diseases that originates from different cell types of the human body. The majorities of cancers arise in epithelial cells, and are designated carcinomas, or adenocarcinoma, when the epithelial cell is originating from glandular tissue [5, 6] as in the case of prostate cancer (PC).

The major dissimilarities between a normal cell and the cancer cell are an increased growth rate, loss of differentiation, and ability to escape from programmed cell death (apoptosis) and senescence [5, 6].
Carcinogenesis, the development of malignant tumors, is a multistep process involving not only the tumor epithelial cells but also the surrounding stroma [7-9]. The longevity and life style of the modern human, permits the accumulation of mutations and genomic instability that together leads to the development of cancer [10, 11].

1.2 Prostate cancer

Prostate cancer is the most prevalent diagnosed cancer and the second leading cause of cancer-associated death in males around the world [2, 3].
Like most cancers, PC development is associated with the accumulation of genetic and epigenetic aberrations over time, thus PC is a disease of the elderly, with an average time of diagnosis around the age of 65 years. Although, most PC is sporadic, there is a significant increased risk of developing the disease with a family history of PC [12-14].
In addition to genetic predisposition [15], several life style factors have been associated with the development of PC [16, 17]. Consistent with multifactorial diseases, familial PC has an early onset and often more aggressive course compared to sporadic cases [18, 19].
PC incidence has dramatically increased over the past decades, while PC mortality has remained fairly constant, although a small decrease has been observed in most countries the last decade. This is most likely mainly associated with the introduction of PSA testing, introduced in the late 1980s and now widely used in clinical practice [20]. Additionally, with an aging population these trends could be expected.

1.2.1 *The prostate gland and cancer*

The prostate is an exocrine gland of the male mammalian reproductive system. It can be anatomically divided into three distinct glandular zones, the

peripheral, central and transitional zone - the peripheral zone being the largest and the predominant site for PC [21, 22].
The glandular structure of the prostate is composed of epithelial lined acini that are encapsulated by the fibrous basal membrane and surrounded by a dense fibromuscular stroma. The epithelial bilayer constitute of three distinct cell types, luminal, basal and endocrine cells (*Figure 1*).

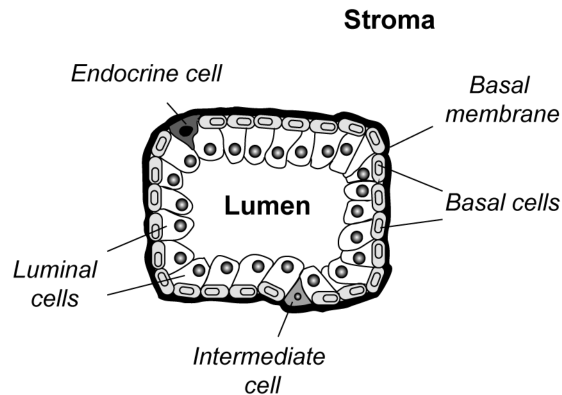

Figure 1. ***Schematic illustration of a cross section of human prostate acinus.*** *The epithelium is composed of luminal, basal, intermediate and neuroendocrine cells, separated from the surrounding stroma by the fibrous basal membrane.*

The large, columnar luminal cells express the androgen receptor (AR) transcription factor, and are strictly androgen-dependent. These cells express secretory proteins such as the prostate-specific antigen (PSA) that are secreted into the lumen. Outside the luminal layer, lining the basal membrane, are the non-secretory committed basal cells. These cells generally express insignificant levels of AR, and are not dependent on AR stimulation for survival [23]. Additionally, dispersed between the basal cells are AR-negative neuroendocrine cells. Like the luminal cells, they are secretory and secrete neuropeptides and hormones thought to regulate the development, growth and survival of the surrounding epithelial cells [24, 25]. Finally, a population of intermediate cells shares common features with luminal, basal and endocrine cells, their phenotype and expression profile suggests a hierarchal development of the epithelial bilayer [26, 27]. The basal and luminal epithelium both contains small population of stem cells, or progenitors that are thought to be able to give rise to all epithelial cells [28, 29]. During carcinogenesis, the well-organized architecture of the prostate is compromised. The basal layer and membrane are disrupted and the luminal secreted proteins, such as PSA, leaks out into the surrounding stroma and vasculature.

1.2.1.1 The epithelial origin of PC

The pursuit to identify the PC progenitor amongst the epithelial cell types has long been a central quest. The prostate carcinomas are heterogeneous, and display luminal exocrine, intermediate and neuroendocrine cell phenotypes, which has led to controversy regarding the identity of the tumor progenitor.

At the malignant stage, there is a disruption of the epithelial cell linage and subsequently skewing of the cell ratio with an dominance towards a luminal cell phenotype [30], in addition most PC are androgen-dependent and secretory, thus the luminal cell has long been postulated as the tumor progenitor. However, more recently the basal cell has come into spotlight as an alternative cell of origin [31-33]. Also a PC stem cell or intermediate cell origin has been proposed [34].

The stem cell hypothesis suggests that a genetically unstable tumor progenitor retains unlimited self-renewal ability, while a subgroup differentiates into malignant cells with features of the mature cells of the epithelium [35]. A second model proposes a clonal expansion of the tumor progenitor that by sequential accumulation of genetic and epigenetic aberrations in the progeny leads to the cell heterogeneity seen in PC tumors. These two models are not necessarily mutually exclusive, but might together contribute to the heterogeneity and late onset of PC [36, 37].

1.2.1.2 The stroma

The heterogeneous prostatic stroma is composed of smooth muscle cells, fibroblasts and infiltrating immune cells, that are imbedded in a collagenous matrix together with blood vessels, lymphatic vessels and nerves [38].

AR positive stromal cells, both smooth muscle cells and fibroblasts, have been shown to regulate differentiation, growth and survival of the epithelial, cells via the production of growth factors – andromedins - in response to AR stimulation [39-41]. The stromal cellular composition and expression profile is distinct in different prostatic zones; this has been suggested to contribute to the predisposition of cancer development in the peripheral zone [38, 42-45].

Early during tumor development, phenotypic and genotypic alterations of the stroma occur – collectively referred to as reactive stroma [46, 47]. These alterations are believed to be a response to the disrupted architecture and leakiness of the epithelial layer. The changes are similar to wound repair and includes matrix remodeling and altered expression of repair-associated growth factors and cytokines [48]. During the transition from normal to reactive stroma, the smooth muscles cells are replaced by cancer-associated fibroblasts (CAFs), myofibroblast and immune cells [47].

1.2.2 *Androgens and AR signaling*

As a part of the male reproductive system, the prostate is strictly dependent on androgens for normal development, maintenance and function. The most abundant circulating androgen is testosterone (T), which are mainly produced by the testis. The remaining fraction of T comes from precursors, dehydroepiandrosterone (DHEA) and androstenedione (AD), produced in the adrenal glands and converted to T in peripheral tissue [49]. The production of androgens is regulated via the hypothalamic-pituitary-gonadal axis (Figure 2), which starts in the brain with the endocrine secretion of gonadotropin-releasing hormone (GnRH) and corticotrophin (CRH) from the hypothalamus. GnRH diffuses into the nearby pituitary, which release luteinzing hormone (LH) and adrenocorticotropic hormone (ACTH). LH stimulates the leydig cells of the testis to produce testosterone, while ACTH stimulates the release of the adrenal androgen precursors', DHEA and AD.

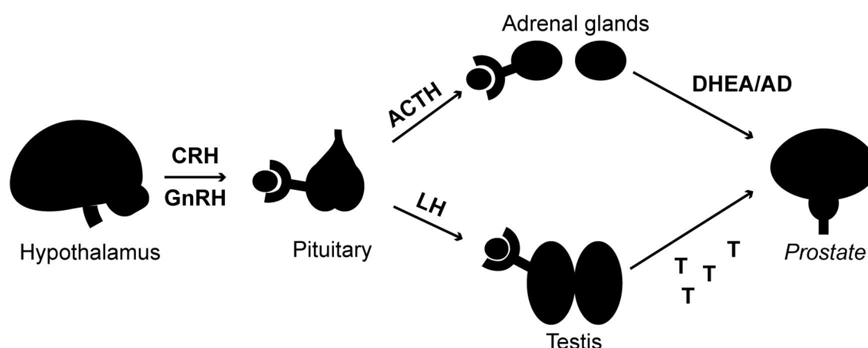

Figure 2. ***The hypothalamic-pituitary-gonadal axis*** *the forward signaling. In addition, feedback- loops at every step firmly regulate the release of signaling molecules (not drawn).*

The produced testosterone diffuses into the prostate where it is catalyzed by 5α-reductase to the more bioactive dihydrotestosterone (DHT). This conversion mainly occurs in the stroma but also in the epithelial cells. The effects of androgens are mediated by the main prostatic transcription factor, the AR. Both T and DHT has the ability to bind AR, however DHT has higher affinity for AR and a lower dissociation rate than T and are thus more potent [50, 51].

Inactivated AR is present in the cytosol where it is stabilized by heat shock proteins such as HSP70 and HSP90 [52]. Activation of the AR is a multistep-process that includes phosphorylation and protein interactions. Simplified (Figure 3), AR ligand binding induces a conformational change that results in dissociation of bound HSPs. Subsequent homodimerization and phosphorylation stabilizes and activates the ligand-receptor complex, which

thereafter translocate into the nucleus where it associates with co-regulators [53] and RNA polymerase II. The complex modulates gene expression of target genes by the binding to specific androgen response elements (AREs) [54, 55].

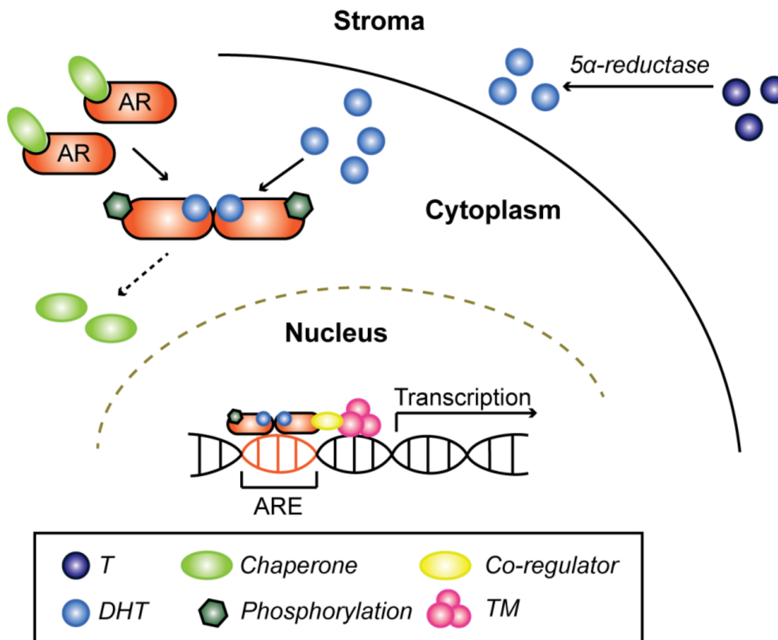

Figure 3. Schematic illustration of AR activation as described in the text. TM, Transcription machinery; RNA polymerase II and additional co-regulators.

In the normal prostate, binding of androgens to the stromal AR stimulates the growth of epithelial cells via paracrine andromedin signaling, while AR stimulation in epithelial cells has a suppressive effect by inhibition of the andromedin-stimulated proliferation [56]. However, at the malignant stage autocrine signaling in the epithelial cells instead has a stimulating effect on proliferation and survival [57, 58]. This adaptation could, at least in part, be attributed to altered expression of the oncogenic tumor transcription factor cMYC, which are down-regulated in response to AR signaling in normal epithelial cells, however induced by androgen stimulation in PC cells [59].

1.2.3 *Prostate cancer pathology and diagnosis*

Prostate cancer is diagnosed as localized, locally advanced or metastatic disease. The vast majority of identified PC are confined within the prostate, referred to as localized [60]. The natural course of PC is generally slow and asymptomatic, thus most men die with, not from the disease. In fact, it has

been estimated that as many as 80% of males over the age of 80 harbors an undiagnosed local foci of PC [61, 62]. Locally advanced PC is characterized by invasive growth in the surrounding tissue and is sometimes associated with symptoms of the lower urinary tract and hematuria. In metastatic disease PC cells has disseminated via the blood or lymphatic circulation and repopulated distant sites, mainly bone, but also commonly distant lymph nodes and liver [63]. At this stage of PC, symptoms such as bone pain and fractures are common.

Due to the asymptomatic course of local prostate cancer, it is often detected by routine PSA blood testing. Following diagnostic investigation includes rectal palpation, magnetic resonance imaging (MRI) and ultrasound together with sampling of needle biopsies that are evaluated histologically [15, 64]. The purpose of these examinations is to identify patients that would benefit from continued monitoring e.g. active surveillance [65] or patients that requires aggressive treatment.

In the adult male, PSA are essentially expressed exclusively by prostate epithelial cells, including malignant cells. In addition, the majority of prostate derived bone metastases express PSA [66]. Thus PSA is an intuitively good marker for the detection of both local and metastatic PC. However, the architecture of the prostate is compromised also in benign pathologies of the prostate, including BPH and prostatitis, therefore the PSA value is not sufficient for PC diagnosis [67], and associated with an imminent risk for over-diagnosis and over-treatment [68]. The guidelines for PSA advises that: A PSA blood level of 0-3 ng/ml is considered normal, above 10 ng/ml is indicative of local tumor growth and with a PSA level that exceeds 100 ng/ml, metastatic diseases can be suspected [69]. However, PSA does not discriminate between indolent and aggressive forms of PC [70, 71].

For assessment of tumor aggressiveness, histological evaluation is essential. The Gleason system is an important tool as it consider the differentiation of the tumor as a measurement of aggressiveness with high prognostic accuracy, especially for the identification of low risk patients [72]. The Gleason grade (GD) classifies tumors on a scale graded 2-5, where 5 is the most malignant grade [73]. An overall Gleason score (GS) is calculated from first, the most common pattern within all tissue and second, the highest grade observed.
The most clinically used staging system for prostate cancer is the TNM system. TNM summarize the stage of cancer considering the tumor size and/or extension (T), lymph node involvement (N) and the presence of distant metastasis (M). T is considered on a 1-4 scale, reflecting increasingly large and invasive tumors. T1-T2 designates localized PC, while T3-T4 is locally advanced. N and M are divided into 0 = negative, 1 = positive or X = not assessed [74]. The

nature of the metastatic spread are directly correlated to survival, M1 patients has a poor prognosis with an average survival of 2-3 years, while N1 patients has a median cancer-specific survival of 8 years [15].

One of the great difficulties when it comes to PC diagnosis is to accurately estimate and separate the aggressiveness of the tumor at diagnosis. While the majority of PC remains indolent with a slow growing course, others will progress to advanced disease. The current clinicopathological variables are lacking satisfactory prognostic accuracy, thus the search for new markers are an important and ongoing task.

1.2.4 *Treatment of Prostate cancer*

For *localized PC*, the treatment options include radiation therapy, radical prostectomy and active surveillance. The two former are considered curative, and are applied when the patient is diagnosed with high-risk tumor and/or has a long life expectancy. Radical prostectomy is the most common curative treatment used in Sweden [15]. Overall, the risk for relapse after this type of treatment is about 30% [75]. Active surveillance is implemented for patients with low risk tumors and/or in association with an overall short life expectancy. The patient is routinely monitored and curative treatment are applied in case of clinical progress [15].

For *locally advanced* and *metastatic* disease the mainstay treatment is surgical or chemical castration, the later referred to as androgen deprivation therapy (ADT). This treatment regime has been in use since Huggins and Hodges in the early 1940s, recognized that PC where androgen-dependent [76]. Today ADT is recommended to be in use in combination with other treatments to increase survival. For local advanced PC, this includes radiation therapy [77], while docetaxel and abirateron/enzalutamide has been shown to increase survival for metastatic disease [78].

The aim of ADT is to chemically, decrease the level of circulating androgens and consequently diminish AR signaling, this is achieved by the administration of GnRH agonists or antagonists that targets the hypothalamic–pituitary axis [49], thus disrupting the testosterone production in the Leydig cells of the testis. An alternative to GnRH agonists/antagonists is surgical castration. The effects of androgens can further be suppressed by AR antagonists such as bicalutamide, which are administrated in combination with ADT, then referred to as full androgen blockade [15]. However, despite initial good clinical response to ADT and survival enhancing combination therapies, the majority of patients will eventually experience a tumor relapse [78].

Generally, *Castration-resistant PC (CRPC)* remains dependent on AR signaling for growth and survival, thus ADT remains as the basis treatment also at this

stage [79]. Therapies used in combination with ADT, includes chemotherapy and radiation therapy. To bypass the resistance-mechanisms associated with the development of CRPC (*see resistance-mechanisms*), drugs like abiraterone acetate (abiraterone in short) and enzalutamide are used in clinic with good survival benefit [80]. Abiraterone targets a critical step in testosterone synthesis, by the inhibition of CYP17 [81]. Enzalutamide is a multi-mechanistic drug, that suppress AR signaling by inhibition of ligand binding, nuclear translocation and DNA interaction of activated AR [82]. Apalutamide and darolutamide are new drugs with the same mode of action as enzalutamide. A common problem with these new treatments is the increased incidence of highly aggressive PC upon development of resistance [79, 83]. For prevention and delay of cancer-related skeletal events, additional treatment is advised for metastatic CRPC, these treatments target the activation of osteoclasts [84].

1.3 **Castration-resistant PC (CRPC)**

Castration-resistant PC (CRPC) are defined by disease progression despite ADT, based on the following criteria, continuous rise in PSA (biochemical failure), progression of pre-existing disease and/or occurrence of new metastases [85]. CRPC is a highly aggressive disease that generally develops within a few years following ADT, and despite new treatment options the median survival after PC relapse is only 3-4 years [78]. ADT has been shown to induce resistance-mechanisms that trigger aggressive androgen-independent tumor growth and metastasis [86, 87]. Resistance mechanisms to ADT and AR inhibition can be divided into three categories - restored AR signaling, AR bypass and complete androgen independence [79].

1.3.1 *Mechanisms of castration resistance*

The development of the majority of CRPC is associated resistance-mechanisms that enables restored AR-signaling. This is reached by various mechanisms including AR amplification, AR mutations, ligand independent AR activation and intratumoral steroidogenesis.

1.3.1.1 AR amplification

AR amplification and subsequently elevated levels of AR, hypersensitizes the PC cells to castration levels of androgens. AR amplification is prominent in CRPC but rare in HNPC and thus thought to be treatment dependent [88], this is supported by the increased occurrence of AR amplification in response to abiraterone and enzalutamide treatment [89]. Increased AR expression is also direct associated with ADT in response to transcriptional alterations [90].

1.3.1.2 Gain-of-function AR mutations

In CRPC, the AR ligand binding domain (LBD) has been recognized as a hotspot for mutations, with a frequency of approximately 20-30% of the most abundant mutations [91, 92]. At least some of these mutations (e.g. T878A/S) have been shown to generate AR promiscuity, that is, it can be stimulated by ligands other than androgens [93-95].

1.3.1.3 Ligand independent AR activation

AR variants (ARVs) that lack the AR LBD are yet another common aberration in both advanced PC and CRPC. The absence of the LBD, results in a constitutively active AR, which are able to enter the nucleus and modulate transcription without ligand binding. This feature proposes an important role for ARVs during development of resistance, importantly in regard to second-line treatment (abiraterone and enzalutamide) [79]. ARVs can be the result of genomic alterations of the AR gene [96], or splice variants induced by the selective pressure of ADT [97, 98]. Studies of ARV7 has shown that it regulates transcription of a unique set of AR-independent genes in addition to AR-responsive genes, suggesting that, at least, this splice variant has an overlapping but distinct role compared to full length-AR in PC cells [4, 99].
Ligand independent activation of AR can also occur by cross-talk between the androgen-signaling pathway and other pathways, leading to the phosphorylation and activation of AR in response to interleukins and growth factors such as interleukin-6 (IL-6) that activate the STAT3 pathway, or insulin-like growth factor 1 (IGF-1), HER2, Keratinocyte growth factor (KGF) and epidermal growth factor (EGF), which activates the PI3K/AKT and phosphorylation of AR in the absence of ligand [79, 100-102].

1.3.1.4 Persistent androgens

ADT targets the secretion of GnRH from the hypothalamus (Figure 2); hence exclusively affect the production of androgens in testis. However, physiological significant amounts of androgens remain in the tumor despite ADT [79]. The primary source of these androgens is the adrenal androgen precursors, mainly DHEA-S, which remains high in CRPC, but my also stem from *de novo* steroidogenisis from cholesterol in the tumor [103-105]. Aberrant expression of several steroidogenesis-regulating factors has been associated with castration-resistance [106], e.g expression of Steroid 17-alpha-hydroxylase/17,20 lyase (Cyp17) is found in all PC and up-regulated in CRPC [107]. Cyp17, the target of abiraterone acetate, regulates two steps in the conversion of cholesterol to DHEA-S. Another example of altered steroidogenesis in CRPC is the up-regulation of steroid-5α-reductase

isoenzyme-1 (SRDA1) which facilitates the conversion of adrenal AD [108]. Both these alteration enables the bypass of testosterone.

1.3.1.5 Androgen receptor bypass and true independence

When the AR is bypassed, the expression of AR target genes are regulated by other hormone receptors such as glucocorticoid receptor (GR) that been shown to regulate transcription of AR target genes in PC [79]. A minor subset of PC is AR negative, and driven by alternative signaling pathways e.g. N-MYC. Like in the case of AR bypass, these tumors are unresponsive to the AR-associated drugs available. These tumors are often of neuroendocrine or small cell carcinoma subtypes [79].

1.4 **Progression of PC**

Several signaling pathways are induced during cancer development and progression. These pathways regulate biological processes that lead to increased tumor cell proliferation, survival and metastasis in response to various stimuli, such as growth factors, interleukins and oncogenic signals induced by the demand for adaption under the harsh tumor conditions.

1.4.1 *Epithelial-mesenchymal transition (EMT)*

About 20 years ago, it was first hypothesized that cancerogenesis is related to abnormal re-awakening of developmental mechanisms normally restrained to organogenesis [109]. During the embryonic development of the prostate, cells of the glandular tissue passes through several cycles of epithelial-mesenchymal and mesenchymal-epithelial transitions (EMT and MET) to form the epithelial layer [110].

During EMT epithelial cells lose epithelial characteristics and acquire a mesenchymal phenotype with increased motility and potential to evade the surrounding tissue through loss of adherence to neighboring cells [111, 112]. It is a dynamic and reversible biological process, which involves several biological pathways and crosstalk between the cells [113-118]. For instance, central pathways like wnt, the signal transducer and activator of transcription 3 (STAT3) and phosphoinositide 3-kinase (PI3K)/AKT pathways has been linked to EMT and metastasis [119-121]. In line, these pathways are induced in association to PC tumor progression and/or metastasis [120, 122-126]. Furthermore, ADT has been shown to induce EMT at an early stage during treatment [127].

The biological process of EMT is coordinated by transcription factors from the SNAIL [128, 129], zinc-finger E-box-binding (ZEB) [130] and TWIST families

[131], which modulates the expression of targets genes to promote dissemination and invasion [132, 133]. This includes altered expressions of cytoskeletal proteins (e.g. vimentin and α-SMA) and proteinases (mainly MMPs) that induce motility and degrade the extracellular matrix [134]. Furthermore, a major hallmark of EMT is a shift in cadherin expression, that is, suppression of epithelia cadherins such as CDH1/E-cadherin and induction of mesenchymal cadherins like N-cadherin and cadherin-11. Simplified, E-cadherin is important for cell-cell adherence, thus keeping the organization of cells in the tissue, while N-cadherin and cadherin 11 are important for cell movement by interactions with neighboring cells and the cytoskeleton [135, 136].

1.4.2 *Hypoxia and hypoxia inducible factor 1 (HIF1)*

A common feature of solid tumors is the decreased level of oxygen, *hypoxia*, in the tumor tissue [137]. Hypoxia has been shown to promote carcinogenesis and an aggressive cancer-cell phenotype [138, 139]. Furthermore, the low oxygen levels within the tumor, promote cancer-endorsing crosstalk between tumor cells and the surrounding stroma [8, 140, 141]. In line, hypoxia is an independent negative prognostic factor in solid tumors [142].

The major hypoxia associated transcription factor, hypoxia inducible factor 1 (HIF1), consists of two subunits; the β-subunit and the hypoxia stabilized α-subunit. Without stabilization, the HIF1α subunit is rapidly degraded and HIF1 activity prohibited [143]. High expression of HIF1α in prostatic intraepithelial neoplasia (PIN), the precursor of PC, but low expression in benign prostatic hyperplasia (BPH), suggests that induction of HIF1α is an early event during PC development [144].

In addition to stimulation by hypoxia, HIF1 activity can be induced by oncogenic signals, e.g. via interplay with the PI3K-Akt and IL6/STAT3 pathways [145-150]. Moreover, cells with potential to evade hypoxia induced apoptosis by induction of anti-apoptotic proteins such as B-cell lymphoma 2 (BCL-2) are enriched in the hypoxic tumour environment [151-153]. In turn, BCL-2 has been shown to stabilize HIF1α [154] and induce the transcription of vascular endothelial growth factor (VEGF) [155, 156], the primary target of HIF1.

HIF1 regulates the transcription of numerous genes involved in the metastatic process, including TWIST, VEGFA, matrix metalloproteinases (MMPs) and IL-6 (*reviewed in* [145]). However, although hypoxia has been shown to initiate epithelial-mesenchymal transition (EMT) by induction of transcription factors such as TWIST and snail, it is not necessarily a full EMT associated with increased tumor cell motility [157, 158].

Furthermore, HIF1 has been shown to contribute to castration-resistant tumor growth, in part by its ability to facilitate AR activation under androgen reduced conditions [159, 160] and further, by induction of metabolic alterations in an AR-independent manner [161].

1.4.3 *PI3K/AKT and IL-6/STAT3 pathways*

The two pathways that are addressed in the work of this thesis (paper I) are the PI3K/AKT and IL-6/STAT3 pathways. The PI3K/AKT pathway is commonly up-regulated in PC in association with cancer progression, metastasis and castration-resistant tumor growth [120, 122, 125, 126]. The pathway is initiated by the stimulation of a tyrosine kinase receptor (RTK) or G- protein coupled receptor (GPCR), the pathway includes several phosphorylation steps including the phosphorylation/activation of the effector RAC-alpha serine/threonine-protein kinase (AKT) [162]. Following activation, AKT phosphorylates and regulate down-stream oncogenic signals directly e.g. (BAD, BAX, FOXO) [163, 164] or indirectly (mTORC, via the inhibition of TSC2) [165, 166].

The rate limiting step in the PI3K/AKT pathway, is the phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP2), to (3,4,5)-trisphosphate (PIP3), which recruits AKT to the plasma membrane enabling its activation. The phosphorylation of PIP2 is catalyzed by phosphoinositide 3-kinase (PI3K) while the dephosphorylation is mediated by the tumor suppressor phosphatase tensin homolog (PTEN). Genomic alterations in the PI3K/AKT pathway is frequent in PC [126], e.g. PTEN which is often found silenced in primary PC and even more frequent in in metastatic PC [167-169], which results in a continuously active pathway.
Furthermore, the AKT pathway and the AR pathway cross-communicate in a reciprocal compensatory manner, meaning loss of one enhances the other [120, 125, 170]. Additionally, AKT mediated phosphorylation has been shown to inhibit AR degradation [171]. The association between the AR and AKT pathways has evoked interest and the clinical benefit of combination treatments of AKT and AR targeting drugs are investigated in ongoing clinical trials.

The IL-6/STAT3 pathway is initiated by stimulation of the interleukin-6 receptor, which activates Janus kinases (JAK). JAK subsequently phosphorylates/activates the signal transducer and activator of transcription 3 (STAT3) transcription factor [172]. Increased STAT3 activity has been reported in PC [122, 123] especially in bone metastases [121]. STAT3 activity has been shown to induce EMT in cancer [173, 174]. In, PC models, induction of EMT by increased STAT3 signaling has been shown to promote stem-like properties [175, 176]. However,

down-regulation of STAT3 signaling in the PTEN deficient PC cell line LNCaP has been shown to induce the cells metastatic ability [119].

1.5 Prostate cancer bone metastases

The development of bone metastases is frequently occurring complication in PC and associated with poor prognosis as PC is incurable once it has settle in the bone. Additionally, bone metastases are associated with poor quality of life associated with skeletal-related events (pathologic fracture, spinal cord compression, necessity for radiation to bone (for pain or impending fracture) or surgery to bone) [177, 178].

For the development of PC bone metastases, the first step is the dissociation of the tumor cell from the primary site. However, dissemination of primary PC cells is not enough for the development of PC metastases in bone. The metastatic process includes several additional steps including, invasion, intravasation, anti-anoikis, extravasation, homing and regrowth, each of these steps are rate limiting and critical; thus only a small fraction of the disseminating tumor cells (DTCs) actually forms distant tumors [179].

For establishment in the bone, it has been proposed that the primary tumors prepare the target tissue for the DTC, by secretion of factors, including parathyroid hormone-related protein (PTHrP), bone morphogenic proteins (BMPs) and VEGF that induces bone-turnover and angiogenesis [180, 181] Furthermore, PC tumor cells has been shown to adapt features of the residents of the bone (osteoblasts) – so called osteomimicry - in order to facilitate communication with the surrounding cells, and thus enhance their own survival [182]. This process is initiated during EMT, through the induction of the bone associated runt-related transcription factor 2 (RUNX2) [183, 184]. Furthermore, of the DTCs that reach the distant organ, a large fraction remains dormant and can reside at this stage for years [181, 185, 186]. Reawakening of dormant PC tumor cells has, for example been shown in association the suppression of the MAPK/ERK and PI3K/AKT pathways and subsequent induction of MET in response to factors produced by adjacent stroma cells [187, 188].

PC bone tumors display mixed sclerotic-lytic properties with excessive bone formation, hence considered to have a sclerotic phenotype [189, 190]. The increased bone mass are the results of exaggerating activity of the osteoblasts, which are the bone forming cells. Osteoblasts arise from mesenchymal stem cells (MSCs) [191] that undergoes a strictly regulated differentiation process to reach maturity and the ability to form the calcified matrix that constitute the bone. One of the regulators for this process is RUNX2, which regulates the

transcription of several genes associated with osteoblasts differentiation, directly or indirectly via its down-stream target, osterix [192, 193].

Bone resorption is mediated by osteoclast. The osteoclasts are derived from hematopoietic progenitors, that are activated in response to the receptor activator of nuclear factor κβ ligand (RANKL), which initiate activation mediated by the progenitor expressed receptor, RANK [194]. The differentiation is inhibited by an osteoblast secreted factor, osteoprotegerin (OPG), which binds to RANKL hence preventing it from interaction with RANK. Normally, bone remodeling is a continuous process that is regulated both locally and systemically by factors such as parathyroid hormone (PTH), glucocorticoids, and estradiol. Under normal conditions the ratio between osteoblasts and osteoclasts, and their activity is strictly balanced. However, it is well known that cancer can corrupt the bone remodeling process, to favor the development of bone metastases shifting the balance to an either sclerotic or lytic tumor phenotype [195, 196].

PC tumor cells in the bone, enters an autocatalytic cycle, by continuous production of osteoblast promoting factors including, PTHrP and BMPs which stimulate osteoblasts differentiation and activity and increase bone-turnover. Activated osteoblast respond by increased production of tumor promoting factors including transforming growth factor beta (TGFβ), IGF-1 and VEGF [197]. In addition, increased bone-turnover induces release of tumor stimulating factors such as TGFβ and IGF-1 that are incorporated in the bone matrix [181, 193].

1.5.1 *Shifting the balance*

RUNX2 is considered one of the major factors associated with the development of PC bone metastases. However, PC clinical studies have shown somewhat varied results regarding the prevalence of RUNX2 in primary tumors and bone metastases, as well as its clinical potential [198-200]. Experimental studies however, propose that RUNX expression is associated with aggressive an PC phenotype [201, 202]. RUNX2 has been shown to induce the expression of PTHrP and a lytic phenotype of breast cancer [203]. In line, induced RUNX2 expression in osteoblasts has been shown to reduce the anabolic effects of PTH [204]. In bone, PTHrP and PTH signaling via, parathyroid hormone 1 receptor (PTHR1) in osteoblasts, has catabolic effects when administrated continuously [205, 206], while anabolic effects with intermitted administration [207-211]. The paracrine anabolic effect of PTH/ PTHrP has been shown in association with induced osteoblast differentiation, reduced apoptosis [208-210] and re-activation of bone-lining cells [211]. The catabolic effects of prolonged endocrine stimulation, is associated with increases expression of RANKL, while suppressed expression of bone stimulating factors like OPG [205, 206]. The

RANKL/OPG balance has been studied in patients with breast, lung and prostate cancer bone metastases. For lung and breast cancers, which are associated with lytic bone metastases, both RANKL and OPG were increased, with an elevated RANKL/OPG ratio. However, for PC only OPG was increased, thus associated with a decreased RANKL/OPG ratio and subsequently a sclerotic phenotype [212].

1.6 Regulator of G-protein signaling 2 (RGS2)

RGS2 is located on 1q31 [213], it is a quite conserved gene with orthologs to the human RGS2 in about 200 species (NCBI). The gene is comprised of 5 exons and its promoter is associated with a CpG island (UCSC). The RGS2 protein is a small, rather uncomplicated protein. It harbors a highly preserved core-domain domain, which is characteristic for all RGS proteins, flanked by short amino and carboxyl terminal sequences. Four separate biological forms of RGS2 have been described, with supposedly distinct modes of action and cellular localization [214].

The role for RGS2 in cancer is poorly understood. However, several distinct modes of actions have been described for RGS2, by which it can affect cells in both cancer promoting as well as suppressive ways.

Aberrant RGS2 expression has been reported for several types of solid cancer including prostate cancer where RGS2 expression is generally decreased compared to normal or benign glandular tissue [215, 216]. In breast cancer there has been reports of both high [217] and low [218, 219] RGS2 levels compared to normal tissue. Considering the clinical relevance of RGS2 expression, low RGS2 level has been shown as unfavorable prognostic marker for colon cancer [220] and breast cancer [221]. The association between low RGS2 and poor prognosis in breast cancer was supported by *The human protein atlas* (https://www.proteinatlas.org/ENSG00000116741-RGS2/pathology), which further suggests that high RGS2 is an unfavorable marker for renal and stomach cancer. Additionally, in lung adenocarcinoma, high RGS2 expression was associated with poor overall survival, and identified as an independent prognostic factor [222].

RGS2, was initially identified as a putative lymphocyte G0/G1 switch gene, then annotated G0S8, isolated by its transient induction in response to cell cycle stimulation [223, 224]. During the same time, it was shown that RGS2 expression was transiently elevated during G0/G1 cell cycle transition [224, 225] and sequencing of RGS2/GS08 suggested shared similarities with genes involved in the cell cycle and in the immune system [225]. However, RGS2 was later renamed, after identification of the highly preserved core-domain

signifying of RGS proteins (the GAP domain) [226]. Since, RGS2 has mainly been considered for its inhibiting role of G-protein signaling.

1.6.1 *Short about G-protein signaling and RGS proteins*

G-protein coupled receptors (GPCRs) are important signal conveyers in all eukaryote organisms, there function are carried out by activation of the receptor and subsequent activation of G-proteins, which are categorized into four families (Gi/Go, Gs, Gq/G11 and G12/G13) annotated by the associated α-subunit. G-proteins are in its inactive form composed of three subunits that upon activation (binding of GTP) dissociates and mediates signals both via the GTP-activated α-subunit and the free βγ-complex. Hydrolyze of the GTP stops the signal and initiates the reassembling of the subunits. Hydrolyze is enhanced by specific proteins regulator of G-protein signaling (RGS) proteins, which act like α-subunit specific GTPase activating proteins (GAPs) [5, 227]. This emphasizes the importance of RGS proteins.

In addition, down-stream effects of G-protein signaling are depending on subtype of both the receptor and G-protein, as well as on regulatory proteins that conveys the signal between the receptor and the specific G-protein. Additionally, is the down-stream effect dependent on the present effector-molecule [228]. Thus can one receptor mediate different signals and activate or inhibit distinct separate pathways depending on the cellular context.

1.6.2 *RGS2, G-protein signaling and down-stream effects*

Studies of RGS2 in regards of its ability to interfere with G-protein signaling have shown its ability to inhibit most Gα families in a context dependent manner. However, RGS2 has a strong preference for Gαq and low affinity for Gαi [229, 230]. Generalizing, Gq/11 and Gi signaling has opposing effects on AKT activation. Gi signaling have a stimulating effect whilst Gq/11 has an overall attenuating effect [227], the later mediated is via direct inhibitory interaction between Gαq and PI3K [231, 232]. However, this influence of Gq/11 signaling is not straightforward, suggesting that it depends on the cellular context (receptor and effector). Furthermore, there seem to be some consensus regarding the durance of Gq signaling, constitutive Gq/11 signaling has an overall inhibitory effects on AKT activation [232, 233], while temporary signaling has a stimulating effect [234, 235]. Additionally, differing observations has been made regarding RGS2 and its role in Gq/11 associated activation of AKT, while attenuating effects has been described [236], other studies show no inhibitory effect of RGS2 [235, 237]. However, RGS2 associated inhibition of the MAPK pathways have been associated via its attenuating effect on Gaq [235]. Additionally, although RGS2 does not act as a GAP for Gs, it can interact with Gs or the down-stream target adenylyl cyclase (AC) with subsequent decreased

cAMP accumulation [238-240], thus inhibiting the MAPK pathway and ERK phosphorylation [241].
In osteoblasts, RGS2 has been shown to be important for desensitizing both Gq/11 and Gs signaling [242]; Thus, affecting central signals associated with bon-remodeling such as, Ca^{2+} oscillation and the accumulation of cAMP [243]. The expression of RGS2 has been shown to be transiently induced during osteogenic differentiation of human mesenchymal stem cells [244]. However, due to lack of an evident skeleton associated phenotype in $rgs2^{-/-}$ mice [245], it has been proposed that RGS2s effect on bone development under basal conditions is limited. The significance of RGS2 has been associated to its attenuating effect on PTH and PTHrP stimulation [246], which is mediated via the Gs and Gq signaling, type 1 PTH/PTHrP receptor (PTH1R) [247]. Temporary signaling via PTH1R by intermitted stimulation by PTH and PTHrP is essential for osteoblast differentiation, proliferation and survival [248-250]. In line with the PTH1R regulatory role for RGS2 in osteblasts, RGS2 is fast and transiently upregulated in in bone in respons to PTH, PTHrP and PGE2 stimulation. Furthermore, this induction was confined to bone although the study considered other PTH1R expressing tissues (brain, heart, kidney, liver and spleen) [251].

1.6.3 *Other roles for RGS2*

In addition, RGS2 has been shown to interact with other proteins by mechanisms distinct from its GAP activity. These interactions will be described here, in relation to its biological implications.

The expression of RGS2 has been shown to increase rapidly in response to stress, such as heat shock [252] and oxidative stress induced by H_2O_2 [253]. In addition, RGS2 has ability to both suppress and global protein translation [254] and promote translation of stress related genes [255] by direct interaction with the eukaryotic initiation factor 2B (eIF2B). RGS2 has thus been proposed to be regulatory of cellular stress response.

The expression of RGS2 is further regulated by hypoxia, in a cell type and/or context specific manner. In smooth muscle cells RGS2 expression was suppressed after 48h of hypoxic exposure [256], while induced in myeloid derived suppressor cells (MDSC) after 1h exposure, however also increases in tumor derived MDSC compared to control. The authors further showed that RGS2 had pro-angiogenic properties [257]. Moreover, in pancreatic β-cells RGS2 expression has been shown to be protective from hypoxia induced apoptosis [258]. In line with this observation, in mouse embryonic fibroblasts RGS2 has been shown to facilitate HIF1 mediated transcription with positive effects on cell-lifespan, associated with decreased stress induced apoptosis and

senescence. In this context, RGS2 was shown to assist HIF1 mediated transcription by interactions with the HIF1α subunit and the DNA [259].

Additionally, RGS2 has also been shown to suppress STAT3 regulated expression via direct interaction [260]. There are also reports of nuclear localization of RGS2 both under basal conditions and in response stimulation by stress [261, 262]. Taken together, this suggests a direct role for RGS2 in regulation of transcription.

RGS2 has furthermore been shown to be involved in yet another distinct cellular context. In a neural cell model, the PC12 cell line, RGS2 has been shown to associate with α-tubulin and contribute to microtubule polymerization [263]. In mouse oocytes, inhibition of the interaction between RGS2 and β-tubulin disrupted the meiotic spindle and chromosomal separation [264]. In addition, in HeLa cells RGS2 knockdown resulted in improper mitotic spindle organization and orientation, and significant mitotic delay [265].

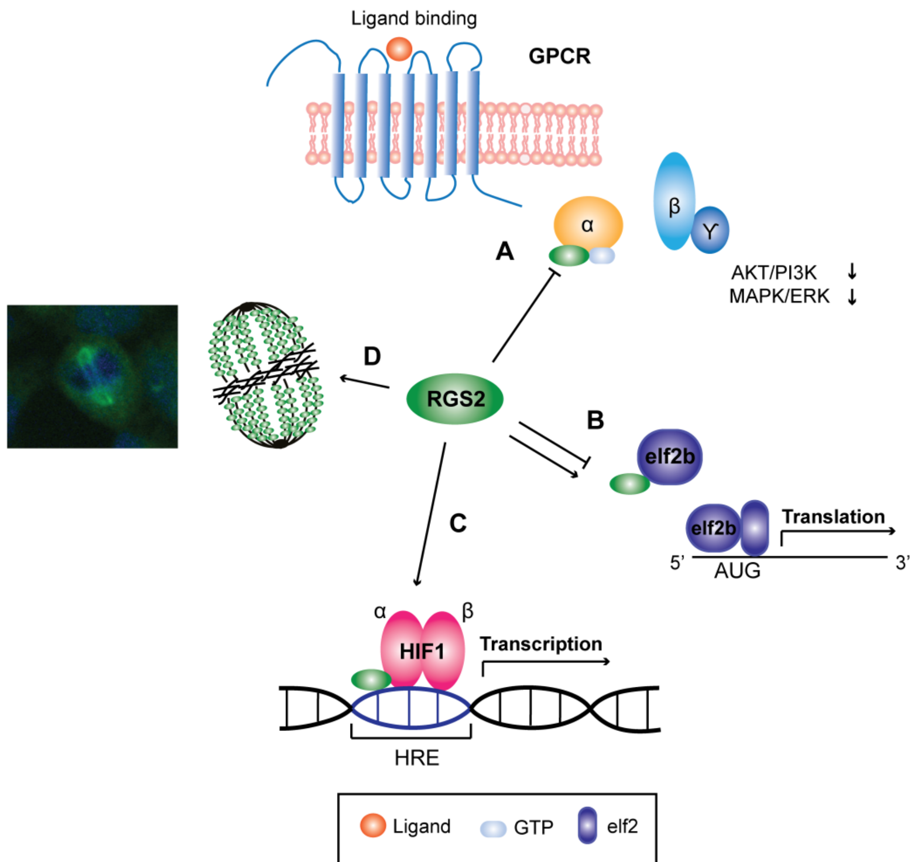

Figure 4. ***Illustration of the described modes of actions for RGS2.***

(A) Attenuation of GPCR signaling by inhibition of the α-subunit, mainly via the RGS2 GAP activity directed towards Gαq signaling. (B) Regulation of protein translation via interaction with eIF2B. (C) Regulation of transcription by co-regulatory role via interaction with transcription factors or DNA, e.g. assisting of HIF1 mediated transcription by binding with the HIF1α subunit and/or direct interaction with the DNA strand. (D) Positive effects on mitotic-spindle formation and cell division, by regulatory interaction during polymerization of microtubule. Integrated photo depictures a dividing LNCaP cell stained for RGS2. HRE, Hypoxia-response element; AUG, the protein translation initiation codon. Not depictured are the ribosomal protein translational complex and transcriptional complex.

2 AIMS

The overall aim of this thesis was to analyze RGS2 expression in prostate cancer, with main emphasis on development of advanced disease.

- To analyze the expression of RGS2 at different stages of prostate cancer in relation to cancer progression and tumor phenotype

- To assess the regulation of RGS2 expression at the different stages of prostate cancer

- To evaluate the clinical value of RGS2 as a prognostic biomarker for prostate cancer

3 MATERIAL AND METHODS

In this thesis the expression of RGS2 has been evaluated in association to the development and progression of PC. The general expression of RGS2 in benign and malignant prostatic tissue and its clinical implication has been evaluated in archival specimens. The regulation of RGS2 expression and subsequent influence on tumor phenotype at different stages and conditions of PC has been assessed *in vivo* and *in vitro*.

3.1 Clinical Specimens

RGS2 expression has been evaluated in benign prostate hyperplasia (BPH), primary tumors of hormone-naïve PC (HNPC), castration-resistant PC (CRPC), and in bone metastases (mHNPC and mCRPC).
Paraffin-embedded archival prostatic specimens from transurethral resection of the prostate (TURP) and needle biopsies were retrospectively collected. BPH and HNPC patients were a diagnosed based on findings in the TURP material, while CRPC patients received TURP as palliative treatment for symptoms associated with local progress.
PC bone metastatic tissue has been surgically removed due to SRE associated patient discomfort. For all studies, the use of anonymized material has been approved by the local ethical committees – at the Sahlgrenska University hospital in Gothenburg (paper I, II, IV) and Central Hospital in Västerås (Paper III).
(For specification of the use of clinical tissue for the culture of primary fibroblast, see Experimental *in vitro* systems)

3.1.1 *Clinical specimens Paper I*

RGS2 was assessed with histological staining of archival TURP material from BPH (n=25) and early low-risk HNPC (n=28) to evaluate early expressional differences between benign and malignant tissue. The HNPC specimens were classified as stage T1b tumors - that is, unapparent non-palpable tumors with histological tumor finding in more than 5% of the resected tissue. *For clinicophatological characteristics see paper II, table 1, cohort II.*

The homogeneity of the T1b specimens, led to the inclusion of a second archival material retrieved by needle biopsy (n=45), for assessment of RGS2 expression in correlation to PC progression and for evaluation of clinical potential. The patients had locally advanced PC with PSA >80 ng/ml or metastatic PC, thus received ADT treatment immediately following diagnosis.
Clinicopathological characteristics: T stage T1c-T4. Distribution of M stage followed: M1=26 M0=12 and MX=7. In addition, one patient had distant lymph

node metastatic status N1, the remaining patients were NX. Gleason score ranged from 6-9, with a median score of 7. PSA value ranged from 12-2900 ng/ml, with a median value of 208 ng/ml.
Clinical value of RGS2 expression was evaluated alone or in association with known clinicopathological variables. The cause of death was determined by examination of clinical records. Patients included for cancer specific survival (CSS) and hazard analysis, n=43. Two patients were excluded due to insufficient or inadequate medical journal data.

3.1.2 *Clinical specimens Paper II*

For comparison of RGS2 expression in low-risk compared to advanced PC and for assessment of alterations associated with castration-resistance, TURP specimens from HNPC and locally advanced CRPC (n=22) was included. *For clinicophatological characteristics, see Paper II, Table 1, Cohort I (CRPC) and Cohort II (HNPC).*
The CRPC patients were included based on local progress post treatment with GnRH analogues, total ablation therapy (TAB) or surgical castration. For evaluation of CSS all patients were included.

For assessment of changes in RGS2 expression associated with castration, case matched primary tumor specimens were included. The samples were retrieved by needle biopsy before and after approximately 3 months of ADT (n=28). *For clinicophatological characteristics, see Paper II, Table 1, Cohort III.* All patients were included for analysis of failure-free survival and hazard associated with RGS2 expression.

3.1.3 *Clinical specimens Paper III*

RGS2 was evaluated on tissue micro arrays, constructed from a large cohort of HNPC, retrieved by TURP and followed by watchful waiting until progress. For assessment of RGS2 staining in tumor epithelium, tumor stroma, normal epithelium, and normal stroma in relation to CCS the number of patients were 182, 185, 178 and 193 respectively. The corresponding numbers for AR staining were 278, 275, 282 and 281. F*urther characterization is available in material and methods Paper III.*

3.1.4 *Clinical specimens Paper IV*

RGS2 and RUNX2 protein expression was assessed in contemporary paraffin-embedded material with immunohistochemistry for comparison between HNPC metastases (n=5) and CRPC metastases (n=13). RGS2 gene expression was furthermore evaluated in frozen, RNA later preserved, tissue from the same patients for correlation with AR, PSA and PTHLP.

3.2 **Experimental *in vitro* studies**

Although experimental set-ups in *in vitro* do not recapitulate the heterogeneity of a tumor, the usage of a representative *in vitro* model is a time and cost efficient complement to *in vivo* studies to minimize the ethical issue regarding the usage of animal experiments. Thus, the cell cultures have been an important tool for controlled studies of RGS2 regulation and phenotypic alterations associated with changes in RGS2 expression.

3.2.1 *Cell lines and culture*

The experiments has been conducted with primarily commercial available cell lines, with addition of the in-house castration-resistant LNCaP derivate, LNCaP-19 [266]. For experiments with immortalized cell lines, the passaging were kept to a minimum and no passages above 18 from the original passage were used for the work of this thesis. The cells were routinely tested for mycoplasma contamination.

3.2.1.1 LNCaP-FGC (Lymph Node Carcinoma of the Prostate – Fast Growing Clone)

(Paper I, Paper II and Paper IV) The main cell line used in the thesis has been LNCAP (ATCC® CRL-1740™). LNCaP has been used as the backbone for the studies of RGS2 in this thesis, based on its relatively high RGS2 expression. Furthermore, LNCAP grow readily both *in vitro* and *in vivo* (subcutaneously and orthotopicaly).
The LNCaP cell line is a androgen-dependent/sensitive PC model derived from a lymph node metastasis [267]. LNCaP is one of the most common androgen-sensitive cell lines used in PC research and under basal conditions it express the full length AR (AR-FL) [79]. It is generally considered androgen-dependent; however it harbors the AR T878A gain-of-function mutation which allows promiscuous response to additional ligands [93].

3.2.1.2 LNCaP-19

(Paper I, PaperII and Paper II). LNCaP-19 is a castration-resistant derivate of LNCaP that was established by prolonged passaging in hormone-depleted media [266]. LNCaP-19 expresses low levels of RGS2 under standard culture conditions. It expresses the AR, but insignificant levels of PSA under standard conditions. However, LNCaP-19 is androgen-responsive as hormonal stimulation of the AR induces PSA expression. LNCaP-19 grows well *in vitro* and *in vivo* (subcutaneously, orthotopically and intratibially), cell growth is however inhibited by hormone stimulation [266, 268].

3.2.1.3 VCAP

(Paper II). The androgen-responsive VCaP (ATCC® CRL-2876™), where included in the study of RGS2 expression in response to anti-androgen treatment with enzalutamide. VCaP is derived from a human vertebral bone metastasis from a CRPC patient. The cell line was passaged in mouse prior to culture establishment *in vitro*. VCaP express low levels of RGS2 under standard culture conditions ([269], supplementary data).

3.2.1.4 22Rv1

(Paper II) 22Rv1 (ATCC® CRL-2505™) is a castration-resistant derivate of the androgen-dependent CWR22R cell line which are derived from a primary prostate tumor [270]. The 22RV1 cell line was established by serial passaging in castrated mice prior to establishment *in vitro* [271]. 22RV1 express high levels of RGS2 compared to LNCaP under standard culture conditions ([269], supplementary data).

3.2.1.5 WPMY-1

(Paper 1) The cell line WPMY-1 (ATCC® CRL-2854™) was established from the stromal region of a primary prostate tumor. According to morphology it is characterized as a myofibroblast. In line, it expresses high levels of smooth muscle alpha-actin and vimentin [272].

3.2.1.6 PC-3

(Paper I). PC-3 (ECCC, Wiltshire, UK) is a cell line isolated from a human vertebral castratioin-resistant PC tumor [273]. PC-3 express insignificant levels of AR and is considered a true androgen-independent cell line [274]. PC-3 express high levels of RGS2 in comparison to LNCaP ([269], supplementary data).

3.2.1.7 Primary cell culture of fibroblasts

(Paper III) The primary cultures of fibroblasts were established from radical prostatectomy or prostate adenoma enucleation tissue specimens, for PC associated or BPH associated fibroblasts respectively. No passages above 10 were used. The use of patient material was approved by the local ethical committee in Homburg. All patients had given informed consent prior to inclusion in the study.

3.2.1.8 Cell culturing standard conditions

Cells were cultured at 37 °C in humidified air with 5% CO2 under atmospheric oxygen pressure unless stated differently. LNCaP, PC-3, 22Rv1 and LNCaP-19

were cultured in RPMI-1640 (PAA Laboratories) with stable glutamine, supplemented for a final concentration of 1 mM sodium pyruvate and 4500 mg/l glucose. For complete growth media, fetal bovine serum (FBS; Gibco) was added to a final concentration of 10%, with the exception of LNCaP-19 where dextran-charcoal stripped FBS (DCC; Gibco) was used. WPMY-1 and VCaP was cultured in Dulbecco's Modified Eagle's Medium supplemented with 5% and 10% FBS respectively. Sh-clones were cultured according to protocol for LNCaP. All cells were cultured with antibiotics (Penicillin-Streptomycin; Thermo Fisher Scientific). Extra care was taken to keep the cultures subconfluent by repeated passaging.

3.2.2 *Induction of hypoxia and stabilization of HIF1α in vitro*

(Paper I) Experimental approach to evaluate the effect of reduced oxygen levels and HIF1α stabilization as a potential mechanism behind the down regulation of RGS2 documented in the clinical specimens of T1b PC tumors compared to BPH.
The cells (LNCaP and LNCaP-19) were cultured under reduced oxygen levels (1% O2) in a hypoxia chamber (Sci-tive-N hypoxia workstation, Ruskinn Technology). Control cells were cultured under atmospheric oxygen in parallel. The experiment was terminated after 48 and 72 hours, when samples were collected. The hypoxia chamber was manually calibrated, and oxygen levels were verified frequently. For preparation of cell lysates, cells were kept on ice, rinsed with cold D-PBS (PAA Laboratories) and lysed directly in the culture flask to reduce HIF1α protein degradation. Complementary experiments with CoCl2 (Sigma-Aldrich, St Louis, MO) were performed to validate the hypoxia data, with an additional set of cell lines (LNCaP, LNCaP-19, PC-3 and WPMY-1). CoCl2 treatment (150 µM) was carried out under normal atmospheric oxygen for 48 or 72 hours.

3.2.3 *Knockdown of RGS2*

For assessment of the significance for RGS2 at different stages of PC, RGS2 was stably knocked-down in the LNCaP cell line, generating the ShRGS2 sub clone. The control clone (shNT) was compared to LNCaP *in vitro* and showed concomitant behavior and expression profile in direct comparison. Evaluation of *in vivo* behavior was made retrospectively, with comparable results considering tumor take, growth rate and phenotype.

The procedure in short: SureSilencing RGS2 shRNA Plasmid and non-target control ShNT (a scrambled sequence without genomic match) was purchased (KH02231N, Qiagen). Plasmids were enriched in E-coli and purified using the Rneasy Plus Universal Mini Kit (Qiagen). Plasmids were delivered using

electroporation with Nucelofector 2b and the Nucleofector Kit R (Lonza). Post transfection, cells were selected based on antibiotic resistance (Neomycin) and single clones were picked for establishment as isolated sub-clones. RGS2 expression was verified in the separate sub-clones using qPCR. The ShRGS2 clone with the lowest RGS2 expression and the ShNT clone with an RGS2 expression similar to LNCaP were chosen for further studies. The sub-clones were not cultured under continuous antibiotic pressure, but repeatedly tested for constant RGS2 expression. For instance, stable knockdown of RGS2 was assessed prior to implantation in mice, and confirmed in recovered tumor tissue.

3.2.4 *Assessment of RGS2 knockdown*

The effects of RGS2 knockdown in PC cell line LNCaP were assessed to establish the impact of RGS2. In addition to functional analysis of RGS2, morphological alterations associated with RGS2 knockdown was evaluated using bright field microscopy and phase-contrast imaging.

3.2.4.1 Growth curve

(Paper I-II) Growth rate was determined under standard growth conditions and in the hormone-deprived setting. Cells were starved for 24 hours (h) and then counted. Media was replaced with standard culture media or hormone depleted media, following the next 72 h period the cells were counted sequentially with a 24 h interval. Cell count was performed using trypan blue and the Countess™ Automated Cell Counter (Invitrogen).

3.2.4.2 Scratch assay

(Paper I) To assess the effect on motility of RGS2 knockdown, a scratch assay was performed. Cells were plated on 6-well plates and allowed to grow until ≥ 80% confluence. A scratch was made using a sterile pipette tip, culture medium was changed and position markings were made from the bottom to ensure consistency in gap evaluation. Sequential images were captures over the next 72 h, with 24 h interval, using a Nikon TMS inverted microscope equipped with a Lumenera Infinity 1 camera. The gap area was measured using the Infinity software. Gap closure was calculated respective to the initial area.

3.2.4.3 Clonogenic assay

(Paper I) To test the ability of the clones to form single colonies, 10 000 cells were plated onto BioCoat™ Poly-D-Lysine coated 6-Well plates (Corning, NY) and cultured for 21 days. Media was carefully replaced every second or third day. Formed colonies was washed, fixed with 4% PFA and stained with crystal

violet (0.5% in 4% PFA). Single colonies of approximately ≥ 30 cells were counted using an inverted microscope.

3.2.4.4 Cell cycle profile

(Paper I) Cell cycle profile was assessed under standard conditions. Cells were detached from the culture dish and centrifuged with the collected original culture media. After wash in D-PBS, the pellet were subsequently resuspended in Vindelov's reagent (75 µmol/L propidium iodide (PI), 20 mmol/L Tris, 100 mmol/L NaCl, 0.1% Nonidet p-40, and 20 µg/ml RNase adjusted to pH 8.0) at a cell concentration of ≤ 10^6 cells per ml and incubated for 30 min at 37°C prior to analysis. PI staining was analyzed with BD Accuri C6 flow cytometer. For cell cycle profile, DNA content was analyzed using the FL3 channel on linear scale. Cells were considered apoptotic when showing less then diploid DNA content (sub-G1/-log 1) on logarithmic scale via the FL2 channel.

3.2.5 *RGS2 expression in association to AR activity*

(Paper II) RGS2 expression was assessed in association to AR activity *in vitro* to evaluate findings from the clinical specimens.

3.2.5.1 Hormone starvation

To study the effect of AR activity on RGS2 expression, LNCaP was subjected to hormone depletion, established by the exchange of FBS for DCC in standard culturing media (final concentration of 10%). For comparison, AR was stimulated in a parallel set-up by the addition of 1 or 10 nm DHT. Cells were cultured under these conditions for 35 days when RNA and protein was harvested for subsequent analysis. Equivalent amount of ethanol was used a vehicle control for the hormone starved group. The purpose of the experiment was to compare the expression of RGS2 between stimulated and non-stimulated AR. Thus, phenol-free media was used to constitute for the LNCaP promiscuous promoter.

3.2.5.2 AR inhibition by enzalutamide

LNCaP, VCaP and 22Rv1 was subjected to enzalutamide treatment for 72 hours. Cells were starved for 24 h prior to change to standard growth media supplemented with 10 µM enzalutamide or equivalent amount of DMSO as vehicle control.

3.3 **Experimental *In vivo* studies**

Animal models enable studies of mechanism associated with development and progression of cancer in contexts that reproduces the heterogeneity in the tumor environment. The animal models thus offer a complexity that cannot be achieved in the cell culture. In the present thesis male athymic BALB/c nude mice (Charles River Laboratories International) were used throughout. The mice were at least 8 weeks old at the time for experimental initiation to ensure sexual maturity. For better initiation of PC growth *in vivo*, Matrigel™ (BD Biosciences) – a protein rich matrix resembling product - was used. To certify that viable cells were used throughout the experiment, good cell viability was established with trypan blue prior to inoculation and for the remaining cells after all animals was operated. The use of animals was approved by the local ethical committee in Gothenburg.

3.3.1 *Subcutaneous implantation*

(Paper I) The advantage with subcutaneous inoculation is the possibility to easily follow the tumor growth with a calliper. Thus this was the primary choice for assessment of the effect of RGS2 knockdown on tumor development and growth.

A total of 10 mice were used in the experiment, 5 and 5 were inoculated with ShRGS2 or ShNT. Two million cells, resuspended in 200 µl of 1:1 antibiotic-free media and Matrigel™, were injected on the left flank. Animals were sacrificed 10 weeks post implantation or before, if the tumors had reached the ethically approved tumor volume of 1300 mm^3. Animals were monitored weekly when also tumors were measured using a calliper. Tumor volume (V) was calculated using the formula $V = (length \times width^2)/2$. Tumor growth was normalized by calculation of volume relative to first detected tumor volume. Harvested tumors was weight and preserved with RNAlater® Solution(Ambion, Austin, TX) for future RNA/protein extraction or fixed in 4% paraformaldehyde for paraffin embedding.

3.3.2 *Orthotopic implantation*

(Paper I-II) Orthotopic implantations of PC cells were performed for analysis of RGS2 expression in a milieu that resembles the human prostate. Implantation in the dorsolateral lobe of the mouse prostate best mimics the clinical situation, regarding corresponding expression and cell composition compared to the human peripheral zone [275]. In this environment, the expression of RGS2 was studied in association with hypoxia and development of castration-resistant tumors.

Orthotopic implantation was carried out via a T-incision in the lower abdomen. When applicable, castration was performed via the same incision*. 1 million PC

cells (LNCaP and LNCaP-19) in 7 µl of BD Matrigel were implanted using a 30 gauge needle. For anesthetiza and analgesics a mixture of xylazine/ketamine (0.55 mg/kg; 110 mg/kg) were administrated prior to operation. The animals were there after kept sedated with isoflurane during surgery. Animals were sacrificed 10 weeks after implantation, or before if signs of tumor-induced discomfort were displayed. Harvested tumors tissue was weight and preserved with RNAlater® Solution for future RNA/protein extraction or fixed in 4% paraformaldehyde for paraffin embedding.
*For assessment of castration-resistant tumor growth animals were castrated, developed tumors were compared to a non-castrated control group. RGS2 expression in association to hypoxia was analyzed in non-castrated animals.

3.3.3 *Intratibial implantation*

(Paper IV) The effects of RGS2 knockdown on development and phenotype of bone metastases were evaluated by intratibial implantation of ShRGS2 (n=10) and ShNT (n=10) in castrated mice. The castration was performed mainly due to documented poor establishment of LNCaP in non-castrated animals [276], and by reason of the observed high expression of RGS2 in human CRPC bone metastatic specimens.
One million cells in 7 µl BD MatrigelTM (BD Biosciences), was implanted with a 29 G needle in the left tibia through the cortex of the anterior tuberosity using a drilling motion to avoid cortical fracture. Animals were castrated via a scrotal incision in association with the implantation. For analgesics, 5 mg/kg rimadyl was administrated 30 minutes prior to surgery and for 5 days post-surgery. During operation, the animals were sedated using isoflurane. The experiment was ended 10 weeks post implantation, when animals where sacrificed and tibia and blood was collected. Blood was stored as serum at -80°C for later assessment. Tibia was dissected and preserved in 4% paraformaldehyde for histological evaluation. The specimens were subsequently demineralized in modified formic acid and sectioned (4 µm) (Histolab, Sweden). Tumor establishment was evaluated by hematoxyline and eosin (H&E) staining of continuous sagittal sections.
For control of castration effects, 6 male C57BL/6 mice were included. Three animals were castrated and three underwent sham operation. Animals were sacrificed after 1 week, when tibia was collected. Tibia specimens were treated in accordance with previous description.

3.4 **Assessment of gene expression**

3.4.1 *RNA extraction and cDNA preparation*

(Paper I, II, IV). Total-RNA from cultured cells or tumor tissues was isolated using the AllPrep DNA/RNA Mini Kit or RNeasy mini kit (Qiagen) according to provided protocol. For tumor tissue, RNA integrity number (RIN) was determined with the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA samples with a RIN less than 6 were excluded. RNA concentrations and purity was evaluated photospectrometically with a NanoDrop™ (Thermo Fisher Scientific). cDNA was prepared by reverse transcription with SuperScript® VILO™ cDNA Synthesis Kit, according to provided protocol.

(Paper III) Total-RNA was isolated from cell pellets with RNeasy Kits (Qiagen) and reverse transcribed to cDNA using Superscript III reverse transcriptase (Thermo Fisher) and Oligo-dT Primers (Qiagen).

3.4.2 *Quantitative real-time polymerase chain reaction (qPCR)*

(Paper I-IV) Real-time PCR was performed with ABI 7500 Fast sequence Detector (Applied Biosystems) or 96 well Step One Plus system (Thermo Fisher) with gene-specific TaqMan® Assays (Applied Biosystems). TaqMan® Assays that span exons were exclusively chosen to avoid detection of potentially contaminating genomic DNA. Expression of target genes was calculated with the $2^{-\Delta\Delta CT}$ method. Endogenous controls were chosen for its stable expression under the specific experimental condition.

3.4.3 *Gene expression profile*

For a gross overview of the effects of RGS2-knockdown the analysis of a GrandPerformance CTC Assay Panel (TATAA Biocenter) [277] was performed using the ValidPrime™ assay kit (TATAA Biocenter). qPCR was performed on BioMark (Fluidigm) using the 96.96 Dynamic Array™ IFC (Integrated Fluidic Circuit). Reverse transcription and qPCR was performed at TATAA. Biocenter (Gothenburg, Sweden). Samples were analyzed in one replicate.

3.5 **Assessment of protein expression**

3.5.1 *Immunohistochemistry (IHC)*

Immunohistochemistry (IHC) has been used as a methodology-backbone for the papers included in the thesis. IHC data from the human specimens has opened up for explorative experiments to describe the clinical observations.

IHC was used for evaluation of protein expression of RGS2 in TURP and needle biopsy specimens as well as in animal tumor xenografts. In addition, IHC was

used to evaluate expression of RUNX2 and AR in PC bone metastases specimens and TMAs, respectively.
In tumor xenografts, IHC was used to visualize hypoxic regions and blood vessels by staining for carbonic anhydrase 9 (CAIX) or HIF1A and CD31, respectively. Furthermore, proliferation was assessed by immunohistological evaluation of Ki67 expression.

All IHC protocols included the following steps: Deparaffinization, rehydration in graded ethanol, antigen retrieval, inhibition of endogenous peroxidase and blocking of non-specific epitope binding, before incubation with primary antibody. IHC staining of tumor xenografts (all markers), TURP specimens (including TMA) and bone metastases was performed using the Vectastain Elite ABC kit (Vector Laboratories), with the exception of staining for AR where the Ventana ES platform (Ventana) was used. IHC staining of the needle biopsy material was performed with EnVision FLEX system (Dako). For visualization of HRP reaction 3,3'diaminobenzidine (DAB), was used (Liquid DAB+ Substrate Chromogen System, Dako) or (iView DAB Detection kit, Ventana), the later for AR staining (paper III).

Primary antibodies used were anti-RGS2 (ab36561, AbCam), anti-CAIX (ab15086, Abcam) anti-HIF1A (ab51608, Abcam) anti-CD31 (77699, Cell signaling), anti-Ki67 (MA5-14520, Thermo Fisher Scientific), anti-RUNX2 (ab23981, Abcam) and AR (MU256-UCE, BioGenex).

3.5.1.1 Quantification of IHC staining in clinical specimens (scoring)

For IHC scoring of clinical specimens, intensity and proportion (distribution) of the staining was considered for cancer cells, stromal cells and normal epithelial cells when valid.
(Paper I, II and IV). The intensity of the epithelial staining of RGS2 was scored according to: 0 = no detectable staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining; the proportion of positive cells was scored as 0 < 5%, 1 ≤ 33%, 2 ≤ 66%, and 3 > 66% positive cells. Proportion score was multiplied by the intensity value, and these values were totaled generating a RGS2 section score.
(Paper I-II) Stromal staining was scored accordingly for stroma visible at 10X magnification with the cancer area in focus. The median of three separate hotspots rendered the stromal RGS2 section score.
(Paper III) The distribution was scored according to: “no stained cells” = 0, < 10% = 1, 10 - 50% = 2, 50 - 90% = 3 and > 90% = 4. Intensity of the dominant RGS2 staining per TMA was also performed and graded 1-4. The combined score is referred to as H – score in the paper. The main focus in paper III has been the distribution, thus are the H-score only been assessed in relation to expression of pAKT and GS.

(Paper IV) Epithelial expression of RUNX2 was scored as described above for RGS2 scoring in paper I and II. RGS2 expression in osteoblasts was scored similarly, based on number of osteoblasts with a specific intensity (0-3) out of the total number at a specific locus. Osteoblasts at 3-5 loci per tumor specimen were calculated.

3.5.1.2 Quantification of IHC in in vivo material

(Paper I) For quantification of proliferation, Ki67 positive cells were counted at 20 times (20X) magnification for two hotspots per tumor. Staining was classified according to: positive = strong staining, negative = no staining and ambiguous = weak staining. Cell count data was obtained manually with the ImageJ software.
(Paper IV) Scoring of RGS2 expression in osteoblasts was performed according to above description for scoring in human patient material.

3.5.2 *Immunocytochemistry (ICC)*

For evaluation of E-cadherin expression in ShRGS2 and ShNT, cells were cultured on 8-well glass slide culture chambers until 70% confluence. The slides were subsequently carefully washed and fixed with 4% neutral phosphate buffered formalin (4% PFA) on ice for 45 min, washed and stored in PBS at 4°C until staining (normally within two days). Anti-E-cadherin (610182; BD Transduction laboratories) was detected with Alexa FluorTM Plus 555 goat anti-mouse IgG (H+L) secondary antibody (A32727; Thermo Fisher Scientific). Slides were mounted with ProLong™ Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific).

3.5.3 *Western blot (WB)*

(Paper I-II) Western blot has been used to evaluate if transcriptional alterations are translated to protein level, to assess AKT activation and HIF1α stabilization.
Protein samples were prepared from directly lysed or pelleted cells using CelLytic M™ lysis buffer (Sigma Aldrich) and tumor tissue was lysed using RIPA buffer with 1% IGEPAL® (Sigma Aldrich). Lysis buffers were supplemented with protease and phosphate inhibitors (cOmplete mini, PhosSTOP (Roche Diagnostics)). Following sonication and centrifugation the protein concentration was determined using BCA Protein assay kit (Pierce Chemicals).

Protein samples (20-35 µg total protein) were separated according size under reducing conditions. The Invitrogen NuPAGE 4-12% Bis-Tris gradient gel (Thermo Fisher Scientific) has been used together with MOPS running buffer (Thermo Fisher Scientific) for best separation of proteins of interest. Transfer to polyvinylidene difluoride (PVDF) membranes has been performed using the i-Blot dry blot transfer system (Thermo Fisher Scientific). After transfer, the

membranes were blocked in 5% BSA, (Albumin from bovine serum; Sigma-Aldrich) or 2% Amersham™ ECL Prime Blocking Agent (GE Healthcare), the later also used as primary antibody diluent, before incubation with primary antibody. Binding of HRP conjugated secondary antibody was detected using the Amersham™ ECL select Western Blotting Detection Reagent (GE Healthcare) and the peroxidase reaction was visualized using the LAS1000 image- detection system (Fujifilm Life Science).

The following antibodies were used: anti-RGS2 (H00005997-M0; Abnova), anti-RGS2 (ab36561; AbCam), anti-TWIST (Sc-81417, Santa Cruz Biotechnology), anti-BCl-2 (ms-123-p1; NeoMarker, Fremont, CA), anti-E-Cadherin, (610182; BD Transduction laboratories), anti-Pan-AKT (4691; Cell signaling), anti-p-AKT (4058; Cell signaling), anti-STAT3 (12640; Cell signaling), anti-PSA (RB-9056-P; NeoMarkers) and anti-HIF1A (ab51608; AbCam). For control of adequate loading, anti-β actin (A5441; Sigma-Aldrich) has been used throughout.

3.5.4 *Enzyme-linked immunosorbent assay (ELISA)*

(Paper IV) Serum levels of PTHrP in animals after intratibial implantation of PC cells and tumor negative controls, was analyzed with sandwich ELISA specific for the 1-34 N-terminal region of human PTHrP (E-EL-H1478, Elabscience Biotechnology Co., Ltd.) according to protocol supplied by the manufacturer. The specificity for human PTHrP certified cancer cell-specific expression.
Base-line PTHrP expression in the cell lines were assessed with the same technique and evaluated in whole cell lysates (20 µg total protein).

3.6 **Statistics**

Statistical analyses were carried out using IBM SPSS Statistics, version 24. For all tests $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ were considered significant.

For comparisons of IHC scores, the Mann-Whitney U test was used to identify differences between groups, and Wilcoxon W paired test was used for analysis of intra-sectional differences or between TMA specimens from the same patient. For analysis of survival and FFS, Kaplan-Meier charts was created, Chi-square and Log-rank (Mantel-Cox) was used to define statistical differences. For Kaplan-Meier charts, the score was dichotomized according to median score or grouped as described. For Cox regression analysis the 95% confidence interval (CI) is specified. A log10 transformation was applied for variables with a significantly skewed distribution (p-value < 0.01 with Shapiro-Wilk test of normality). When the condition for normality was still not met the median was used as cutoff. Potential interaction between each variable and time was evaluated. Proportion hazard assumption (PH) was not violated for any of the

variables. Student's t-test was used to calculate statistical significance for differences between groups in gene expression, phenotypic properties and for osteoblast counts. The Spearman rank correlation was used throughout for evaluation of correlation of variables of interest.

4 RESULTS AND COMMENTS

4.1 (Paper I) Analysis of regulator of G-protein signalling 2 (RGS2) expression and function during prostate cancer progression

The significance of RGS2 in cancer has not been explored to any greater extent, and prognostic properties have showed varied results depending on the type of cancer investigated [220-222]. Thus, the present study was designed to evaluate RGS2 expression in PC tumors and to explore its clinical value. Additionally, RGS2 regulation and impact on tumor cell phenotype were assessed experimentally to interpret data from clinical materials.

RGS2 was initially immunohistochemically assessed in clinical specimens of BPH (n=25) and low risk T1b PC specimens (n=28), which in some cases also included normal glandular tissue (n=8). In agreement with a previous publication [215], our data confirmed that RGS2 protein levels were considerably lower in PC compared to benign or normal glandular tissue. This was also noticeable for the surrounding stroma. However, when scrutinizing the data clozely - some diversity was observed regarding the level of RGS2 expression in the PC specimens.

To assess the regulation of RGS2 expression and find a mechanism behind the heterogeneity observed in the PC specimens, RGS2 expression was immunohistochemically evaluated in orthotopic LNCaP PC xenografts in correlation to blood vessels, proliferation and hypoxia. The results showed that RGS2 expression was increased in well vascularized and proliferative areas in proximity to blood vessels. However, high levels of RGS2 were also identified in exceedingly hypoxic areas with extremely high HIF1α expression. Considering the general hypoxic environment in tumors and induction of HIF1α during development of malignancy [144], these findings were further evaluated *in vitro*. The data showed that time-limited exposure to hypoxia suppressed RGS2 expression in the prostate cancer cell lines LNCaP and LNCaP-19. Complementary experiments with stabilization of HIF1α showed corresponding results, with decreased RSG2 expression in an extended cell line panel, including an additional PC cell line (PC-3) and a myofibroblast cell line (WPMY-1). Taken together these data suggests that RGS2 is differently regulated by HIF1 in PC tumors, depending on the level and endurance of hypoxia.

The homogeneity of the low risk PC cohort in terms of clinicopathological factors, did not allow for further evaluation but suggested inclusion of a second cohort with a more complex composition for assessment of RGS2 expression in association with advanced PC (n=45). RGS2 expression did not significantly correlate with any of the clinicophatological variables available (PSA, GS, T-

stage or age). However, the data showed a strong trend for association between high RGS2 expression and positive metastatic status.
Moreover, high RGS2 expression was significantly associated with poor cancer-specific survival (CSS), with a mean survival of approximately 54 months compared to 102 months in the high and low expressing group respectively. The prognostic properties of RGS2 expression were further evaluated, showing that RGS2 and M-stage were the only prognostic factors for the present cohort. Additionally, when analyzed together with PSA, RSG2 was identified as an independent prognostic factor that outperformed PSA. Interestingly, unadjusted RGS2 showed better prognostic value than factors used for diagnosis in the clinic today (PSA, GS, T-stage). This identifies the clinical value of RGS2 expression for identification of high risk tumors.

To evaluate the impact of the RGS2 expressional differences in PC tumor specimens, RGS2 was stably knocked-down in the LNCaP PC cell line. Phenotypic alterations were evaluated in comparison to the RGS2 expressing control clone. The data showed that knockdown of RGS2 had a significant negative effect on tumor cell motility and ability to form colonies. Furthermore, the knockdown of RGS2 altered cell morphology towards a more epithelial-like cell phenotype; displayed by a more organized growth pattern, increased polygonal shape and consistent dimensions. Additionally, uni-frontal movement and observed decreased preference for three-dimensional growth suggested that the knockdown of RGS2 had a positive effect on cell polarity. These phenotypical differences were in accordance with a less metastatic tumor cell phenotype.

Additional assessment of the impact of RGS2 knockdown showed that decreased RGS2 expression was associated with reduced proliferation under standard culture conditions *in vitro* and under hypoxic pressure in subcutaneous tumor xenografts. This suggests that the high RGS2 expression documented in proliferative areas in orthotopic xenografts was not only associated but causative. Furthermore, cell cycle analysis suggested that under standard culture conditions *in vitro,* the cell population with RGS2 knockdown was less apoptotic, this was accompanied with an increased expression of BCL-2 which was also confirmed in the subcutaneous xenografts.

To evaluate the effects on tumor cell phenotype, proliferation and survival, the effect of RGS2 knockdown on pathways associated with PC development and progression was further evaluated. The data showed that while the activation of AKT was reduced by RSG2 knockdown, STAT3 protein expression was increased. Somewhat surprising, further evaluation suggested that, the knockdown of RGS2 induced an overall EMT-like expression profile, complemented by induced TWIST1, and reduced E-cadherin expression.

However, ICC evaluation of localization of E-cadherin expression did not show any obvious alterations comparing cells with RGS2 knockdown and RGS2 expressing cells.

In conclusion, these results suggest that RGS2 is generally suppressed by hypoxia in early low risk PC tumors, while induced by the increasingly hypoxic pressure in more advanced PC. Furthermore, RGS2 levels contribute to the PC tumor cell phenotype. Low RGS2 expression is associated with an indolent cancer phenotype representative of a major fraction of primary PC. In contrast, high RGS2 is associated with the proliferative metastatic phenotype that signifies progressed cancer. The data furthermore suggests that RGS2 expression has a prognostic value and the potential to distinguishing between low- and high-risk patients.
Finally, these results suggest that low RGS2 expression is associated with initiation of EMT by induction of STAT3 and TWIST1, while high RGS2 expression is associated with activation of AKT. This distinction could rationalize for the described phenotypic alterations, and suggest that RGS2 expression could be indicative of therapeutic opportunities for treatments targeting the STAT3 and PI3K/AKT signaling pathways.

4.2 (Paper II) RGS2 is prognostic for development of castration-resistance and cancer-specific survival in CRPC

The interest for RGS2 in our group was evoked by distinct expression in LNCaP-19 derived xenografts in response to growth site and availability of hormones, where RGS2 was up-regulated in orthotopic tumors compared to subcutaneous, and in intact compared to castrated animals [268]. Furthermore, a previous publication described the ability of RGS2 to inhibit androgen-independent activation of the AR in the absence of androgens, postulating a mechanism by which decreased levels of RGS2 would promote castration-resistant growth [269]. To assess these previous observations, the present study was designed to evaluate the expression of RGS2 in CRPC and in response to ADT and furthermore, assess the regulation of RGS2 experimentally in association to development of castration-resistant tumor growth.

Evaluation of RGS2 expression by IHC showed that RGS2 protein expression was significantly higher in CRPC (n=22) compared to HNPC (n=28). To verify this finding, RGS2 expression was assessed in an additional data set retrieved from the Oncomine data base (HNPC, n=10; CRPC, n=25), with concordant results.

Further evaluation of the data from the in-house patient material, showed that high RGS2 expression in the CRPC tumors was associated with significantly reduced CSS, with a mean survival of approximately 12 months compared to 24 months in the high and low expressing group respectively. For stringency, a comparison was made exclusively with patients that harbored highly dedifferentiated tumors, signified by morphology similar to GG 5. With this inclusion, the difference in CSS were even more pronounced with median survival of 8 months compared to 25 months in the high and low RGS2 expressing groups respectively. The prognostic value of RGS2 expression was further evaluated. Data showed that, in agreement with the association between high RGS2 expression and poor survival, RGS2 was confirmed as an independent prognostic factor in CRPC.

In addition, to assess the expression of RGS2 in association to ADT and acquisition of castration-resistance, RGS2 was immunohistocehmically evaluated in case matched advanced PC specimens before and after 3 months of ADT (n=28). The data showed that high levels of RGS2 expression post ADT was prognostic for decreased failure-free survival (FFS). Furthermore, when analyzed together with PSA, RGS2 was identified as an independent prognostic factor for FFS. The prognostic value for FFS was exclusive for RGS2 expression post ADT, RGS2 score before ADT was unassociated to FFS. Taken together, these data suggest that RGS2 expression could be valuable for interception of patients in need of additional treatment, at an early stage during initiation of ADT.

Further scrutinizing of the IHC score data showed that although RGS2 expression in tumor specimens before and after ADT showed similar median expression, the major proportion of specimens showed an increased RGS2 score after ADT (50%), while 21% showed an unchanged score and 29% a lower score. For evaluation of the response to ADT in respect of variation in RGS2 expression, patients were grouped according to the change in RGS2 expression. The result showed that RGS2-response was prognostic for FFS. For patients with increased or constant high RGS2 expression, the median time between initiated ADT and relapse were 11 months compared to 25 months in the group were RGS2 was decreased.
Additionally, since association between high RGS2 expression and metastasis was previously been shown in an extended form of the hormone-naive specimens (Paper I), this association was assessed in the post ADT specimens. The results showed that there was no correlation between RGS2 score post treatment and metastatic status, or between RGS2 score before and after ADT. Taken together, this suggests that regulation of RGS2 in response to ADT was associated with a distinct mechanism separate from metastatic progression.

To further assess the high RGS2 levels associated with CRPC, RGS2 expression was evaluated in association to development of castration-resistance in orthotopic LNCaP xenografts. In this model LNCaP represent a hormone-naïve tumor cell that adapt to ADT. In agreement with the patient data, RGS2 levels where higher in tumors that had developed castration-resistance compared to tumors from non-castrated hosts. Furthermore, indicative of a castration-resistance mechanism, elevated levels of AR expression and persistent AR activity, annotated by sustained PSA expression, was shown in the castration-resistant tumors.
A possible association between RGS2 level, AR level and AR activity was assessed in the Oncomine data set. Data showed a significant positive correlation between RGS2, AR and PSA in the CRPC group. Suggesting that the high levels of RGS2 expression in CRPC was associated with up-regulation of AR and sustained AR activity.

In addition, the association between RGS2 and AR was assessed in association with hormone stimulation of castration-resistant tumors. This was considered with an additional orthotopic xenograft mouse model, using the castration-resistant LNCaP-19. In contrast to LNCaP, LNCaP-19 has already acquired castration-resistance by a mechanism unassociated to induction of AR, suggested by low basal expression of both AR and PSA. The data showed that although the AR was significantly down-regulated in LNCap-19 derived tumors in response to hormone stimulation, significantly elevated levels of PSA indicated high AR activity. Notably, like PSA, RGS2 was significantly increased in tumors that developed in non-castrated animals compared to tumors from

castrated animals. This suggests that RGS2 expression was increased in response to AR stimulation.

The association between AR activity and RGS2 expression was further assessed *in vitro* by enzalutamide treatment of androgen-responsive cell lines with different AR aberrations. During the current experimental conditions the three cell lines showed different expression profiles in response to enzalutamide treatment. LNCaP exhibited substantially decreased PSA expression, indicative of adequate AR inhibition, albeit induced AR expression. RGS2 expression was significantly down regulated, in line with an AR regulated expression of RGS2. The response for the androgen-independent cell line, 22RRv1 was not as distinct, a minor decrease in PSA expression suggested insufficient inhibition of AR activity. In line, RGS2 expression showed a minor reduction in the treated group compared to the control. Interestingly, the PSA expression for VCaP cells showed no reduction of AR activity in the treated group, denoted by equivalent levels of PSA, and notably expression of AR and RGS2 was increased in the treated group compared to the control. Importantly, during standard culture conditions, 22Rv1 exhibit high levels of RGS2, while VCaP express low levels compared to LNCaP. Combined, this suggests mechanisms behind the altered expression of RGS2 in response to ADT documented in the clinical specimens. Furthermore, in light of increased RGS2 expression in association to fast relapse after initiated ADT, this suggest that RGS2 expression could be reflective of resistance-mechanisms at an early stage during anti-androgen treatments.

Wolff et al. previously suggested that RGS2 was decreased in LNCAP during hormone starvation, in association with acquisition of castration resistance [269]. In the present study, this was assessed *in vitro* by prolonged hormone-starvation of LNCaP and comparison between the expression of RGS2 in LNCaP and LNCaP-19. In agreement with the previous study, RGS2 was significantly down-regulated in LNCaP cultured under hormone-depleted conditions. Furthermore, the down-regulation of RGS2 expression corresponded with reduced AR expression and activity. These alterations were emphasized in LNCaP-19. Considering the heterogeneity of PC, this may represent a mechanism by which androgen responsive PC cells overcome ADT in the absence of hormone stimulation. However, considering data from the patient material, low levels of RGS2 in CRPC are infrequent and RGS2 expression is commonly induced during ADT. Aditionally, development of castration-resistant LNCaP tumors *in vivo* displayed increased levels of RGS2, and relatively low levels of DHT was sufficient to sustain RGS2 expression *in vitro*. Taken together, this emphasizes the ability of RGS2 to indicate persistent AR activity.
Moreover, although LNCaP-19 grows readily under hormone-depleted conditions, hormone-starved LNCaP show significant reduced growth rate. The

contribution of RGS2 was assessed by the culture of cells with RGS2 knockdown and RGS2 expressing control in the absence of presence of androgens. The data showed that cells with RGS2 knockdown displayed significantly reduced cell growth compared to the control in the hormone deprived setting, while overlapping growth curves in hormone supplemented media. This indicates that although RGS2 is down-regulated by androgen deprivation, it is still important for proliferation in the primary androgen deprived setting. Thus, suggesting that high RGS2 expressing cells have an advantage under hormone depleted conditions. This is in line with decreased FFS for patients with high levels of RGS2 after initiated ADT.

In conclusion, the present study suggests that increased RGS2 expression in association to ADT and development of CRPC are both prevalent and associated with an aggressive cancer phenotype. Furthermore, RGS2 expression is indicative of continuous AR signaling, and may identify PC patients that would benefit from additional treatment at an early stage.

4.3 (Paper III) Prognostic value of stromal expression of regulator of G-protein signaling 2 (RGS2) and androgen receptor (AR) for men with prostate cancer followed with expectancy management

In contrast to the previous studies included in this thesis, were the clinical value of RGS2 expression was evaluated in advanced forms of PC, the present patient material comprised of patients that were followed by expectancy management until signs of progress. This enables the evaluation of RGS2 expression and its clinical potential in a new setting. Furthermore, we have previously reported that RGS2 levels are significantly reduced in tumor associated stroma compared to stroma of benign tissue (Paper I), and in paper II we report that RGS2 expression is decreased in stroma of advanced CRPC compared to low grade T1b specimens. Thus, the present study was designed to assess these observations and its clinical value. The association between AR and RGS2 was further assessed.

The main aim of the present study has been to evaluate stromal expression of RGS2 and AR, with focus on cancer associated fibroblasts (CAFs) or non-CAFS (NCAFs) for fibroblasts adjacent normal tissue.
For evaluation of previous findings (paper I), the H-score (combined variable of intensity and distribution) has been used in contrast to the distribution score, referred to as IR-score (immunoreactivity score), which was the general score used in the present study.

The data showed that in agreement with previous observations, both stromal and epithelial RGS2 IR-score was decreased in PC specimens compared to normal tissue. This was also shown for AR IR-score.

Tumor cell associated RGS2-IR score was significantly increased with higher T-stage (T 1-2 vs T 3-4), but showed no significant difference considering other clinicopathological factors available (GS and M-stage).

Stromal RGS2-IR score showed significant decrease in association with increased GS (G < 7 vs GS 7 - 10), but now significant difference considering other clinicopahtological factors. While low stromal AR-IR score was significantly associated with a positive metastatic status, high GS and T-stage.
RGS2 and AR IR-score showed week but significant correlation in all cell types (CAFS, NCAFS, tumor cells and normal epithelium). Furthermore, stromal IR-score for both markers showed week but significant correlation with Ki67 index score. Taken together, this suggests that low stromal expression of both markers was associated with more advanced PC.

The expression of RGS2 and AR was further assessed *in vitro*, in primary cultures of CAFs, NCAFs and BPH derived fibroblast (BPHFs). In agreement with the IHC score data, both markers showed general decreased expression in CAFs compared to both NCAFs and BPHFs.
However, comparison of the expression of RGS2 in fibroblasts from the same prostatectomy showed that RGS2 expression was significantly decreased in CAFs compared to NCAFS, while the same comparison showed a trend for increased expression of AR. This suggests that, the expression of AR and RGS2 in tumor stroma is not necessarily linked, which could rationalize the low correlation coefficients in the patient material.

For evaluation of epithelial RGS2 expression in correlation to previously assessed clinical variables (paper I) and for evaluation of correlation to pAKT, the H-score was used. The data showed positive correlation between RGS2 expression and pAKT. This data was in agreement with our previous observation of decreased AKT activation in response to RGS2 knockdown. Taken together, this suggests that RSG2 is an important regulatory factor for the PI3K/AKT pathway in PC.
Additionally, in contrast to previous findings (paper I), RGS2 H-score showed significant correlation to GS (dichotomize GS < 8 vs GS 8 - 10). However, there was no significant correlation between RGS2 expression and M-stage, although the mean rank suggested higher expression in M1 compared to M0 patients.

Moreover, both low RGS2-IR and AR- IR-score in CAFs (less than approximately 30% positive cells) was correlated with significant reduced CSS. Loss of either RGS2 or AR was associated with poor survival compared to patients with sustained expression of both markers.
The association between low stromal IR score for either marker and poor prognosis was confirmed with cox regression analysis. However, with inclusion of Ki67 proliferation index and pAKT, only AR-IR and Ki67 were significantly prognostic for CSS. By further inclusion of GS and T-stage, only Ki-67 and GS were significantly prognostic for CSS.
Taken together, this suggests that although both stromal RGS2 and AR expression has prognostic properties for CSS survival in PC, they are not adding further prognostic value compared with strong prognostic markers such as Ki67 and GS in the current cohort.

In conclusion, the present study suggests that low stromal expression of both AR and RGS2 is associated with more aggressive PC and has prognostic properties for identification of high-risk patients. In addition, epithelial RGS2 is indicative of an activated PI3K/AKT pathway.

4.4 (Paper IV) Importance of RGS2 in prostate cancer bone metastases

The present study was design in light of the high levels of RGS2 in advanced PC and the suggested importance of RGS2 during normal bone development. Combined, this suggested that RGS2 could contribute to the sclerotic phenotype of PC bone metastases. Thus, was this ongoing study was designed to evaluate the expression of RGS2 in PC bone metastases (mPC), in general and in association with castration-resistant growth and furthermore, assess the significance of PC cell associated RGS2 expression during development of PC bone metastases and impact on tumor phenotype.

Using IHC, RGS2 was initially assessed in a patient material including hormone-naïve PC bone metastases (mHNPC, n=5) and castration-resistant PC bone metastases (mCRPC, n=13). The data showed that the general protein expression of RGS2 was high in the metastases. Additionally, RGS2 was increased in mCRPC compared to mHNPC.
Morphological evaluation of the tumor specimens showed that mHNPC displayed glandular-like structures, suggestive of sustained cell polarity. This observation was exclusive for mHNPC. This suggests that the mCRPC tumors in generally was more dedifferentiated than the mHNPC tumors.
Differential gene expression was thereafter assessed comparing the two tumor types considering gene expression for RGS2, AR and KLK3 (PSA). The data showed that RGS2 and AR expression was significantly up-regulated in mCRPC compared to mHNPC, while PSA displayed a statistically non-significant reversed expression. Moreover, evaluation of correlation between the different markers suggested that RGS2 expression was positively correlated with AR, while negatively correlated with PSA. PSA was also negatively correlated with AR. In light of previously described association between low levels of tissue PSA, despite increased nuclear staining of AR, and exceptionally aggressive PC bone metastases [278], these data suggest that high RGS2 expression is associated with a dedifferentiated aggressive mPC tumor phenotype.

Furthermore, assessment of gene expression of PTHLH (PTHrP) showed that PTHLH was increased in mCRPC compared to mHNPC. Correlation analysis showed a trend for correlation between RGS2 and PTHLH, while PTHLH showed negative correlation with PSA. It has experimentally been shown that PTHrP is down-regulated in AR-positive PC cells in a DHT-dependent manner [279]. Furthermore, PSA exhibit enzymatic ability to cleave PTHrP into an inactive form [280, 281]. Taken together with induced RGS2 expression in PTHrP stimulated osteoblasts [208, 251], these data suggest a mechanism behind the up-regulation of RGS2 seen in epithelial cells of mCRPC.

The impact of RGS2 tumor epithelial expression in bone metastases was assessed with intratibial inoculation of LNCaP cells with stable knockdown (ShRGS2) and its non-target control (ShNT). The results showed that tumor cells with RGS2 knockdown displayed hampered ability to develop tumors in the bone compared to the RGS2 expressing control, with tumor take in 4 out of 10 and 7 out of 9 operated animals, respectively.
Established tumors from both groups reduced the castration-associated loss of trabecular bone and displayed a mixed blastic-lytic phenotype. However, RGS2 expressing tumors showed augmented ability to form bone compared to tumors with RGS2 knockdown. This was associated with a significantly increased number of osteoblasts in the tumor-bone interface. The increased number of osteoblasts was local, in close association to the PC cells, suggesting that the stimulating factor is associated with paracrine cell-cell communication.

Furthermore, for ShRGS2 derived tumors, 2 of the total 4 tumors displayed an evidently lytic phenotype - where the tumors grew outside the cortical bone - while none of the control tumors displayed extra-cortical growth. In agreement, the area of cortical bone in ShRGS2 tumors was reduced, compared to both control tumor-bearing tibia and tumor-negative control tibia. In contrast to the seemingly paracrine stimulation of the osteoblasts, the induction of osteolysis was observed also distant from tumor cells, suggesting endocrine communication between the tumor cells and bone.

Expression of basic bone modulating factors was assessed in the cell lines for identification of a potential mechanism behind the decreased bone formation and lytic phenotype associated with RGS2 knockdown. The data showed that under basic culture conditions, RUNX2 gene expression was up-regulated in ShRGS2 compared to ShNT. In line, the RUNX2 regulated PTHrP protein showed a trend for increased expression. Additionally, analysis of human specific PTHrP in serum from the animal experiment confirmed that both ShRGS2 and ShNT tumors expressed the protein. However, there was a non-significant trend towards increased levels of PTHrP in ShRGS2 compared to the control. This is in line with high RUNX2 level in association with increased PTHrP expression and lytic properties of breast and PC cells [198, 203, 204]. Taken together, these data suggest an important regulatory role for tumor cell associated RGS2 expression in association to the sclerotic phenotype of PC bone metastasis.

Moreover, RGS2 expression in osteoblasts was evaluated with IHC in the patient material. Data showed that there was an increased level of RGS2 in in osteoblasts from mCRPC compared to mHNPC, in line with elevated levels of PTHLH. Together with described osteoblast stimulating effect of RGS2 in response to PTHrP signaling [246], this emphasizes the importance of

osteoblast associated RGS2 for conservation of the sclerotic phenotype of PC bone metastases under high PTHLH conditions.
The expression of RGS2 in osteoblasts was further assessed, in association to castration and different tumor cell associated osteogenic properties. This was performed by intratibial implantation of LNCaP, that grow poorly in non-castrated bone, and LNCaP-19 that has extraordinary ability to form osteoblastic tumors in both the castrated and non-castrated state [276]. The data showed RGS2 expression was increased in osteoblasts in LNCaP derived tumors from castrated mice compared to non-castrated. However, this variance was not observed in LNCaP-19 derived tumors, where osteoblasts from castrated and non-castrated animals displayed RGS2 levels corresponding to the expression seen in LNCaP tumors developed in castrated animals. This emphasizes that high RGS2 expression is associated with active osteoblasts in association with bone stimulating PC tumor cells.

In conclusion, the present study suggests that RGS2 has a dual and complex regulatory function in PC bone metastases. RGS2 is highly expressed in the epithelial cells with induced expression in the dedifferentiated tumor cells of mCRPC. Additionally, the data suggests that RGS2 is important for the tumor establishment in bone and contribute to the sclerotic phenotype of bone metastases, possibly to some extent by regulation of the expression of PTHrP and RUNX2. Furthermore, high levels of RGS2 in active, tumor-associated osteoblasts suggest the importance of RGS2 for conservation of the sclerotic phenotype of PC bone metastases.

5 GENERAL DISCUSSION

PC is often a slow-growing and symptom-free disease with good prognosis. However, there is a proportion that will progress and metastasize. Importantly, the survival rate drops significantly once the PC is no longer confined in the prostate or surrounding tissue [282]. Furthermore, considering bone metastatic PC and CRPC, they both represent incurable stages of PC associated with poor quality of life [78, 79, 83, 177, 178].
So, how can one distinguish the good from the bad? And how does one treat the incurable?

5.1 Lack of biomarkers

A large problem in the field of PC is the lack of reliable biomarkers for diagnosis, with ability to distinguish between indolent and aggressive forms.
PSA is the primary biomarker used in the clinic for monitoring PC. The declined mortality observed over the last decades could at least in part be credited to the introduction of PSA testing, hence early detection of PC [20]. However, PSA as a biomarker has its limitations regarding both sensitivity and specificity. Thus, PSA is insufficient for treatment and disease monitoring especially at late stages of PC [71]. Despite the shortcomings of PSA, both CRPC patients and patients with metastatic disease are continuously monitored with PSA testing, with the addition of assessment of performance status and bone associated factors [alkaline phosphatase (ALP), bone pain and extent of disease on bone scan] [283]. The common drawback of these additional tools is that they are generally detectable first at an already advanced stage.

Metastasized and castration-resistant PC both represents incurable stages of PC despite life-prolonging therapeutic advancements in recent years, as therapy ultimately fails and a highly aggressive PC emerge that are unresponsive to any available treatment [79, 83]. This emphasizes the need for markers that are able to indicate relapse at an early stage and suggest for additional drug targets, for early eradication and prevention of progress. Thus, there is an urgent need for complementing new biomarkers to improved diagnosis and treatment decisions.

5.2 The multifaceted role of RGS2

To understand the biological processes involved in tumor progression and acquisition of treatment-resistance is essential for the development of curative treatments and good biomarkers. The progression of PC includes oncogenic aberrations in several signaling pathways e.g. AR, HIF1, PI3K/AKT and IL-6/STA3

pathways that together induce tumor progression and adaption to treatment by intricate interplay [145-150]. In this section follows a general discussion regarding findings presented in paper I-IV in the light of what is known about RGS2, described in the introduction, and general biological processes. This should be looked at as an addition to the discussion in the papers.

5.2.1 *The impact of a hypoxic environment*

Descriptions of the association between hypoxia and EMT/metastasis is not straight forward, there are experimental studies that suggests that hypoxia induces a partial EMT, not necessarily associated with increased but rather decreased tumor cell motility [157, 158]. This is in agreement with the observation made in paper I, and rationalizes an association between hypoxia suppressed RGS2 expression, and the indolent phenotype and EMT-like expression profile acquired in response to RGS2 knockdown. Furthermore, the observed induction of BCL-2, is in line with a hypoxia induced resistance-mechanisms such as up-regulation of anti-apoptotic proteins, including BCL-2 [151, 152]. However, hypoxia is highly associated with tumor progression metastasis and poor clinical outcome [138, 139]. This is logical, considering that tumor cells at an exceptionally hypoxic environment would either, develop resistance, disseminate or capitulate [145, 284]. The *in vivo* data suggest that RGS2 expression is induced at highly hypoxic areas. Rationalizing this finding, in the light of described modes of action for RGS2 and the experimental results presented in paper I, one could suggest that this up-regulation is representative of two biological processes – apoptosis and metastasis. Interestingly, a both protective and promoting role has been described for RGS2 in association with apoptosis [255, 258]. Also, in this context is the transcription-regulatory role for RGS2 in association to HIF1 highly relevant, especially considering the described anti-apoptotic effects [259]. Taken together this may suggest that RGS2 is closely involved in HIF1 regulated determination of cell fate.

5.2.2 *PI3K/AKT*

Positive correlation between RGS2 and pAKT observed in the PC patient material (Paper III), and the attenuating effect on AKT activation in response to RGS2-knockdown (Paper I), collectively suggests that RGS2 may have a positive regulatory role for AKT phosphorylation in PC. A reasonable explanation for this observation could be via the attenuating influence of RGS2 on Gαq signaling. The reported effects on AKT phosphorylation in association with both Gq signaling [232-235] and RGS2 [235-237] is somewhat inconsistent. However, with reference to the innate preference of RGS2 for Gaq inhibition [229, 230], this could describe a mechanism by which RGS2 influence the phosphorylation of AKT. Considering that the attenuating effect of Gαq is mediated via

interaction with PI3K [232, 233], suggests that such association would have relevance especially in PC, where silencing of PTEN is frequently occurring in association to PC progress and metastasis [167-169].

5.2.3 *STAT3*

The less metastatic phenotype observed in response to RGS2 knockdown could, at least partly, be ascribed to decreased AKT activity. In addition, knockdown of STAT3 in the LNCaP cell line has been shown to induce their metastatic ability [119] which suggests that STAT3 has inhibitory effect on metastasis. Moreover, nuclear staining of active STAT3 has been shown to be inversely correlated with development of metastasis in prostate cancer [285]. This suggests that the induced STAT3 expression contributes to the less metastatic phenotype observed in response to RGS2 knockdown.
G-proteins regulatory role of transcription and translation [286, 287], suggests a general mechanism by which RGS2 could affect the level of STAT3. However, RGS2 ability to directly interact with transcription factors and regulate their activity [259, 260] could also be responsible for the increased level of STAT3 associated with RGS2 knockdown.

5.2.4 *RGS2 and proliferation*

The association between RGS2 knockdown and decreased proliferation suggest that RGS2 has a positive regulatory role for cell division. This is in line with the earliest reports of RGS2, where RGS2 expression was shown to be induced by mitogens in lymphocytes, and thought to be associated with resumed cell division [223-225]. The regulatory role for RGS2 could be mediated indirectly via the attenuation of the Gaq inhibitory effect on PI3K/AKT (as discussed above). In addition, the regulation could be direct, associated with the stimulating interaction between RGS2 and polymerizing microtubule [263]. The consequence of this direct interaction is accentuated by the significant mitotic delay associated with RGS2 knockdown in HeLa cancer cells [265].

5.2.5 *AR and RGS2*

(Paper II) Prostate cancer is generally an AR driven malignancy also at the castration-resistant state, where resumed AR signaling is achieved by numerous mechanisms [4, 79]. In addition to the clinical relevance suggested for RGS2, the data collectively suggests that RGS2 is indicative of resumed or persistent AR signaling. The significance of this is perhaps most important considering RGS2 expression in association to early ADT, and its association to failure-free survival, where RGS2 could be indicative inadequate treatment at a stage where fast intervention could have significance for patient outcome.

Association between RGS2 and AR/AR signaling has previously been assessed to some extent. Related to our observations, has fast induction of RGS2 expression been associated with AR stimulation, both via traditional ligand binding and by antagonist stimulation of a mutated AR [288]. Furthermore, down-regulation of RGS2 in response to androgen depletion *in vitro* has been suggested as a mechanism behind acquisition of castration-resistance, related to RGS2 attenuating effect on Gq associated androgen-independent activation of the AR. However, the inhibitory effect on AR activity was abolished in the presence of androgens suggesting that only androgen-independent signaling was inhibited [269]. We recapitulated the data showing that RGS2 was down-regulated in hormone-starved LNCaP, during transition into the castration-resistant LNCaP-19, and furthermore showed that castration-levels of DHT, ample to stimulate AR, was enough to antagonize the suppression of RGS2 expression that was associated with full hormone-depletion. However, the clinical relevance of this finding could be questioned, in light of the generally high expression of RGS2 after ADT and persistent high levels of RGS2 in CRPC. Especially, considering that full androgen-deprivation is hard to achieve in man, allowing for adrenal androgen contribution and intratumoral steroidogenesis [79]. Moreover, it should be noted that LNCaP-19 grows poorly under conditions were hormones are available, and it has previously been suggested that induced RGS2 level is a part of a suppressive expression profile for LNCaP-19 derived tumors under these conditions [268]. This suggests that tumor cells that acquired castration-resistance by down-regulation of RGS2 would be inhibited under typical CRPC tumor conditions.

5.2.6 *RGS2 and Stress*

With reference to RGS2 as a suggested stress-response protein [252, 253], and bearing in mind under which orthotopic experimental conditions RGS2 is highly expressed - that is under conditions with good oxygen/nutrient supply or under extreme hypoxic pressure adjacent necrotic areas - it could be speculated that high levels of RGS2 in the clinical specimens subjected to ADT, would signify cells that displayed signs of extraordinary stress compared to low expressing cells. Theoretically, this could be true and would than suggest a better treatment response. However, in light of the patient outcome in terms of significantly reduced time to relapse, high levels of RGS2 after ADT would rather be indicative of viable cells.

5.2.7 *RGS2 in bone metastases*

The high frequency of PC bone metastases together with its poor prognosis and major impact on the patients' quality of life [177, 178], emphasizes the importance of studies in this field. Although rgs2$^{-/-}$ mice lack of an apparent

skeleton associated phenotype [245], we did see general and high RGS2 staining of osteoblasts in our experimental mouse models (tumor negative and positive) as well as in the clinical patient material. High levels of RGS2 were also evident for the tumor cells. Taken together with the apparently stimulatory interaction between RGS2 expressing tumor cells and osteoblast, and the hampered ability for cells with RGS2-knockdown to populate the bone – it is tempting to speculate about osteomimicry, which has been described to enhance tumor cells ability to populate the bone niche (reviewed in [182]). On that note, high expression of RGS2 in association to metastasis would also, speculatively, imply that cells with high RGS2 expression would have preference for homing to the bone. Additionally, it should be mentioned that the osteotropic, bone stimulating LNCaP-19 cell line, that express low levels of RGS2 in comparison to LNCaP *in vitro*, significantly up-regulates RGS2 to comparable levels when implanted intratibially.

5.2.8 *RGS2 as a biomarker*

The treatment strategies for metastatic disease and CRPC are changing as new drugs are introduced in the clinic. However, for best treatment results it is essential to know when a certain treatment would be effective and/or in combination with which other drug. Therefore, reliable biomarkers are highly valuable.

In the present thesis, RGS2 was identified as a negative prognostic marker in both advanced HNPC and CRPC. The high expression of RGS2 after ADT in association with short failure-free survival, suggest that RGS2 has a significant value as a treatment-predictive biomarker, indicative of patients that would benefit from early inception of additional therapy such as anti-androgens, chemotherapy or radiation.

Regardless of the potentially promising value of RGS2 as a biomarker, there are some concerns.
For example, a problem with a tissue confined biomarker, opposing a secreted marker, is the availability. However, on the endorsing side, with a tissue-based marker the disease is assessed locally with low risk of detecting unassociated systemically demonstrated changes.
Furthermore, although promising prognostic value in paper I and II, RSG2 did not prevail as a prognostic marker for HNPC in paper III. There could be several reasons for this. This could indicate that epithelial RGS2 is in fact not a prognostic marker for HNPC. However, this discrepancy could also describe methodological differences.

Primarily, the methods used for IHC scoring are different. While scoring in paper I and paper II give emphasis to intensity, the scoring in paper III

emphasizes on distribution. The expression of RGS2 in tumor epithelial cell is rather heterogeneous with regular local differences –subpopulations, but also scattered differences in intensity. The scoring that was performed in paper III, exclude the influence of populations with lower distribution than the majority. Whilst the scoring carried out in paper I-II, takes in account the contribution of all populations. In the majority of cases, the score would be essentially the same - however at the extremes (extremely high or low intensity) the different scoring methods will affect the overall total score. Considering that a smaller population, observed at a certain time point, could be the growing population and have high penetrance at a later stage, one could suggest that it is important to consider not only the main intensity and/or the distribution. Furthermore, this would imply that the intensity is especially important regarding scoring of tumor epithelial cells. However, for scoring of the homogenous stroma, distribution is a fitting approach.
Moreover, there is an important difference in the clinical material assessed for RGS2 expression, the material in paper I is comprised of advanced PC, where the patient required immediate ADT, while the material included in paper III is mixed, with no information regarding subsequent treatment. This could suggest that the prognostic properties of RGS2 in HNPC, is actually related to the treatment predictive property suggested in paper II. However, since there was neither correlation between expression before and after ADT, or association between RGS2 expression after initiated ADT and cancer-specific survival (only failure-free survival), this is not likely.

5.2.9 *Future perspectives and concluding remarks*

This thesis describes a relevant tumor-promoting role for RGS2 in the development of advanced PC and bone metastases, which is worth further exploration, considering, the herein suggested discriminating role for RGS2 in association to AKT/PI3K and STAT3 signaling. This is especially relevant with reference to high prevalence of aberrations in the PI3K/AKT pathway associated with progressed disease [120, 122, 125, 126] and the correlation between RGS2 and pAKT (paper III). To consider RGS2 as a biomarker, this could suggest that RGS2 expression level would be indicative of treatment-windows for combination therapy with ADT and PI3K/AKT or IL-6/STAT3 inhibitors. Additionally, the phenotype acquired in association with RGS2 knockdown compared to RGS2 expressing cells, is in line with the identification of RGS2 as a negative prognostic marker for PC, although this was not confirmed in paper III (discussed above).
Moreover, the association between high levels of RGS2 and rapid treatment-failure is worth further consideration with reference to the highly aggressive form of PC that are promoted by ADT [86, 87]. Early detection of resistance,

before CRPC establishment, would be valuable considering potential to hinder, or at best eradicate, progressing cells. This is especially important considering the increased risk for metastatic disease in association with prolonged castration-therapy [181, 289-292].
Finally, the high prevalence of RGS2 in PC bone metastases calls for further investigation. The hampering effect of RGS2 knockdown on tumor establishment in the bone is especially relevant from a clinical perspective.

The data suggests that RGS2 has potential as prognostic and treatment-predictive biomarker in PC. However, further evaluation of the prognostic and predictive value of RGS2 is required to ascertain the significance of these findings.

6 CONCLUSIONS

The following conclusions were based on results and data included in the present thesis

- RGS2 expression is generally decreased in indolent hormone-naïve PC compared to benign prostate tissue
- Moderate hypoxia suppresses RGS2 expression while extreme hypoxia induce RGS2 expression in PC cells
- RGS2 is generally highly expressed in advanced PC in association with an aggressive tumor phenotype
- RGS2 appears to be involved in fundamental biological processes associated with PC progression
- High epithelial RGS2 expression is selected for during androgen deprivation therapy and is indicative of resumed AR activity and imminent castration-resistant relapse
- Stromal RGS2 expression is decreased in PC in association with increasingly advanced disease
- Tumor cell associated RGS2 expression contributes to the development of PC bone metastases
- Both epithelial and stromal RGS2 expression may have clinical value for detection of advanced prostate cancer
- Epithelial RGS2 expression has potential as a treatment-predictive biomarker for ADT

ACKNOWLEDGEMENT

There are so many of you that have made my time at SCC meaningful. Sitting here right now, with the almost finished product, it is not without mourning that it will soon be over.

I would like to express my deepest gratitude to everybody that has contributed to this thesis, supervisors, colleagues, friends and family – you all played a part in this. I would especially like to thank the following people:

From the beginning, my supervisors;
My main supervisor **Jan-Erik Damber,** thank you sincerely for taking on this task! I am especially grateful for your dedication as an educator and your honest curiosity regarding scientific findings and results. Thank you for your enthusiasm - it has been stimulating!
Karin Welen, my co-supervisor, thank you for letting me work in freedom to explore my own ideas!

To my group member, former and present, you are the best - I would never be where I am today if it was not for your positive influence and support!
Malin for being the best there can be! **Anna** for numerous crack-ups, in and outside the lab! **Delila**, for setting a good role-model! To all three of you for being there dark days to offer comfort and support. **Karin L,** for your incredible positive attitude and ability to always see the good in people, wooow! **Åsa**, For your wise and rational way - you've made such a difference. **Andreas J**, for your positive attitude and collaboration. **Jun** and **Daniel Å**, thank you for stimulating lunch-chats and giggles, and Daniel, an extra thanks to you for putting up with my “you should know this, you’re doctor”- questions. **Tajana**, ååå, Tajana, thank you for being a wonderful friend! **Anita,** for helping me with the animals, you were a real asset. **Karin J**, for teaching me how to do orthotopic implantation – thank you for taking the time! **Neha**, for nice lunch chats. **Lisa M**, thank you for your positive persona and creating a friendly atmosphere.

To all previous and current co-workers on 4th floor – for an overall supportive atmosphere and pleasant chit-chats! **Camilla,** what would I have done without you all those years of non-stop working, and what would I have done without you now? Thank you for being a true friend! **Som,** my dear friend always supportive and wise – “try not to stress about it” – yeah, that is a good motto. **Joy**, for incredible memories and friendships/family I will cherish for the rest of my life.
Therese C, you are one of those people one can always be frank and upfront with – your presence at SCCS has changed many day to the better. Thanks, also for letting me roam around in your freezers… **Ellen**, for your warm hugs and support in times of need and nice times in between! **Elin S**, for laughter and of course for “preggo-support”! I am glad I shared that experience with you! **Maja** and **Anna D**, for sending encouragement and thoughts, it was worth so much. **Nina L**, you made a major difference when you were at SCC! **Soheila**, I am glad I can call you my friend you are a wonderful person!

To the rest of the wonderful people at SCC that has contributed by nice interactions and small-talk, especially **Gülay**, **Anna W**, **Susanna**, **Marta**, **Mattias A** and **Toshima**! Thanks! **Ágota**, thank you for the “cleansing” weekend chats! **André**, thanks for being supportive! I am glad you are back! **Mia**, thanks for the laughs!

I would also like to send extra gratitude to the present and former colleagues in the “ice-draft room”. **Andreas S**, thank you for giving me a new perspective, for your eager to discuss and patience to listen to the fuzzy loops of intellectual (?) babble. **Daniel A**, for being a supportive friend and for sharing all those pics of your children ;). **Sandra**, thanks for always being so friendly and listening with patience! Thanks to all three of you, for the encouragement in association with the acceptance of the paper -I really appreciated that!

Special thanks also to **Lisa N**, for awaking the dead spirit by stimulating discussion about science (and life). You changed everything!
Vilborg, I am so glad for our meetings. You made such a difference! Thank you!

My wonderful friends outside of work. **Magnus**, the best friend one could ask for, always there to put me down if I dare to feel happy or contempt ;). **Jessica**, so beautiful from the inside and out - I miss you! **Katarina**, my dearest Katarina always in my heart! **Josefin**, Fia. I am so incredibly thank-full that I got to know you – you are the best of friends! **Roger**, you are a rock… **Ann** and **Dennis**, it has been too long! Ann you have such as positive influence!

My family, for always believing in me! **Inger** for love and comfort. **Per** for scientific discussion and support. Thanks to both of you for eagerly listening!
My wonderful beautiful siblings, each one of you are a jewel. I love you so much! All “new” members of the family, **Caspian**, **Tyra** och **Jasmine** – älskade gull-ungar! **Thomas** och **Fredrik**. Tack!
Shawn, you are the best support system one can ask for. Thank you, from the bottom of my heart for helping me with everything from shopping to moral support! I cannot express how much that has meant to me! TUSEN TACK! All those “you can do it”! Also thank you for being such a wonderful father to Alice, we are lucky to have you – you are the best!
Finally, my wonderful **Alice**. Thank you for giving my life a whole new meaning and fresh perspectives. There are no words strong enough to describe the love! You have waited too long. Snart mitt älskade underbara barn!

REFERENCES

1. Ferlay, J., et al., *Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018.* Eur J Cancer, 2018. **103**: p. 356-387.
2. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2018.* CA Cancer J Clin, 2018. **68**(1): p. 7-30.
3. Bray, F., et al., *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.* CA Cancer J Clin, 2018. **68**(6): p. 394-424.
4. Chandrasekar, T., et al., *Mechanisms of resistance in castration-resistant prostate cancer (CRPC).* Transl Androl Urol, 2015. **4**(3): p. 365-80.
5. Alberts, B., et al., *Molecular Biology of the Cell.* 2002. **Fourth Edition**.
6. Becker, W., L. Kleinsmith, and H. J, *The world of the cell.* 2002. **Fourth edition**.
7. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
8. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
9. McAllister, S.S. and R.A. Weinberg, *Tumor-host interactions: a far-reaching relationship.* J Clin Oncol, 2010. **28**(26): p. 4022-8.
10. Maslov, A.Y. and J. Vijg, *Genome instability, cancer and aging.* Biochim Biophys Acta, 2009. **1790**(10): p. 963-9.
11. Finkel, T., M. Serrano, and M.A. Blasco, *The common biology of cancer and ageing.* Nature, 2007. **448**(7155): p. 767-74.
12. Lange, E.M., et al., *Early onset prostate cancer has a significant genetic component.* Prostate, 2012. **72**(2): p. 147-156.
13. Nemesure, B., et al., *Family history of prostate cancer in a black population.* J Immigr Minor Health, 2013. **15**(6): p. 1107-12.
14. Bashir, M.N., M.R. Ahmad, and A. Malik, *Risk factors of prostate cancer: a case-control study in Faisalabad, Pakistan.* Asian Pac J Cancer Prev, 2014. **15**(23): p. 10237-40.
15. Damber, J.E. and G. Aus, *Prostate cancer.* Lancet, 2008. **371**(9625): p. 1710-21.
16. Whittemore, A.S., et al., *Prostate cancer in relation to diet, physical activity, and body size in blacks, whites, and Asians in the United States and Canada.* J Natl Cancer Inst, 1995. **87**(9): p. 652-61.
17. Joshi, A.D., et al., *Red meat and poultry, cooking practices, genetic susceptibility and risk of prostate cancer: results from a multiethnic case-control study.* Carcinogenesis, 2012. **33**(11): p. 2108-2118.
18. Carter, B.S., et al., *Hereditary Prostate-Cancer - Epidemiologic and Clinical-Features.* Journal of Urology, 1993. **150**(3): p. 797-802.

19. Hjelmborg, J.B., et al., *The heritability of prostate cancer in the Nordic Twin Study of Cancer.* Cancer Epidemiol Biomarkers Prev, 2014. **23**(11): p. 2303-10.
20. Torre, L.A., et al., *Global Cancer Incidence and Mortality Rates and Trends--An Update.* Cancer Epidemiol Biomarkers Prev, 2016. **25**(1): p. 16-27.
21. Isaacs, J.T., *Prostatic structure and function in relation to the etiology of prostatic cancer.* Prostate, 1983. **4**(4): p. 351-66.
22. McNeal, J.E., *The zonal anatomy of the prostate.* Prostate, 1981. **2**(1): p. 35-49.
23. Toivanen, R. and M.M. Shen, *Prostate organogenesis: tissue induction, hormonal regulation and cell type specification.* Development, 2017. **144**(8): p. 1382-1398.
24. di Sant'Agnese, P.A., *Neuroendocrine cells of the prostate and neuroendocrine differentiation in prostatic carcinoma: a review of morphologic aspects.* Urology, 1998. **51**(5A Suppl): p. 121-4.
25. Abrahamsson, P.A., *Neuroendocrine cells in tumour growth of the prostate.* Endocr Relat Cancer, 1999. **6**(4): p. 503-19.
26. Robinson, E.J., D.E. Neal, and A.T. Collins, *Basal cells are progenitors of luminal cells in primary cultures of differentiating human prostatic epithelium.* Prostate, 1998. **37**(3): p. 149-60.
27. Bonkhoff, H., U. Stein, and K. Remberger, *Multidirectional differentiation in the normal, hyperplastic, and neoplastic human prostate: simultaneous demonstration of cell-specific epithelial markers.* Hum Pathol, 1994. **25**(1): p. 42-6.
28. Isaacs, J.T. and D.S. Coffey, *Etiology and disease process of benign prostatic hyperplasia.* Prostate Suppl, 1989. **2**: p. 33-50.
29. Signoretti, S., et al., *p63 regulates commitment to the prostate cell lineage.* Proc Natl Acad Sci U S A, 2005. **102**(32): p. 11355-60.
30. Grisanzio, C. and S. Signoretti, *p63 in prostate biology and pathology.* J Cell Biochem, 2008. **103**(5): p. 1354-68.
31. Strand, D.W. and A.S. Goldstein, *The many ways to make a luminal cell and a prostate cancer cell.* Endocrine-Related Cancer, 2015. **22**(6): p. T187-T197.
32. Wang, Z.A., et al., *Luminal Cells Are Favored as the Cell of Origin for Prostate Cancer.* Cell Reports, 2014. **8**(5): p. 1339-1346.
33. Zhao, S.G., et al., *Associations of Luminal and Basal Subtyping of Prostate Cancer With Prognosis and Response to Androgen Deprivation Therapy.* JAMA Oncol, 2017. **3**(12): p. 1663-1672.
34. Xin, L., *Cells of origin for cancer: an updated view from prostate cancer.* Oncogene, 2013. **32**(32): p. 3655-63.
35. van Leenders, G.J. and J.A. Schalken, *Epithelial cell differentiation in the human prostate epithelium: implications for the pathogenesis and therapy of prostate cancer.* Crit Rev Oncol Hematol, 2003. **46 Suppl**: p. S3-10.

36. Packer, J.R. and N.J. Maitland, *The molecular and cellular origin of human prostate cancer.* Biochim Biophys Acta, 2016. **1863**(6 Pt A): p. 1238-60.
37. Shackleton, M., et al., *Heterogeneity in cancer: cancer stem cells versus clonal evolution.* Cell, 2009. **138**(5): p. 822-9.
38. McNeal, J.E., *Normal histology of the prostate.* Am J Surg Pathol, 1988. **12**(8): p. 619-33.
39. Cunha, G.R., et al., *The endocrinology and developmental biology of the prostate.* Endocrine reviews, 1987. **8**(3): p. 338-62.
40. Taylor, R.A. and G.P. Risbridger, *Prostatic Tumor Stroma: A Key Player in Cancer Progression.* Current Cancer Drug Targets, 2008. **8**(6): p. 490-497.
41. Niu, Y.N. and S.J. Xia, *Stroma-epithelium crosstalk in prostate cancer.* Asian J Androl, 2009. **11**(1): p. 28-35.
42. Hayward, S.W., M.A. Rosen, and G.R. Cunha, *Stromal-epithelial interactions in the normal and neoplastic prostate.* Br J Urol, 1997. **79 Suppl 2**: p. 18-26.
43. Shaikhibrahim, Z., et al., *Genes differentially expressed in the peripheral zone compared to the transitional zone of the normal human prostate and their potential regulation by ETS factors.* Molecular Medicine Reports, 2012. **5**(1): p. 32-36.
44. van der Heul-Nieuwenhuijsen, L., et al., *Gene expression profiling of the human prostate zones.* BJU Int, 2006. **98**(4): p. 886-97.
45. Farnsworth, W.E., *Prostate stroma: physiology.* Prostate, 1999. **38**(1): p. 60-72.
46. 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.
47. Tuxhorn, J.A., et al., *Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling.* Clin Cancer Res, 2002. **8**(9): p. 2912-23.
48. Desmouliere, A., C. Chaponnier, and G. Gabbiani, *Tissue repair, contraction, and the myofibroblast.* Wound Repair and Regeneration, 2005. **13**(1): p. 7-12.
49. Taplin, M.E. and S.M. Ho, *Clinical review 134: The endocrinology of prostate cancer.* J Clin Endocrinol Metab, 2001. **86**(8): p. 3467-77.
50. Gao, W., C.E. Bohl, and J.T. Dalton, *Chemistry and structural biology of androgen receptor.* Chem Rev, 2005. **105**(9): p. 3352-70.
51. Wilson, E.M. and F.S. French, *Binding Properties of Androgen Receptors - Evidence for Identical Receptors in Rat Testis, Epididymis, and Prostate.* Journal of Biological Chemistry, 1976. **251**(18): p. 5620-5629.
52. Smith, D.F. and D.O. Toft, *Minireview: the intersection of steroid receptors with molecular chaperones: observations and questions.* Mol Endocrinol, 2008. **22**(10): p. 2229-40.

53. Heemers, H.V. and D.J. Tindall, *Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex.* Endocr Rev, 2007. **28**(7): p. 778-808.
54. Lonergan, P.E. and D.J. Tindall, *Androgen receptor signaling in prostate cancer development and progression.* J Carcinog, 2011. **10**: p. 20.
55. Shang, Y., M. Myers, and M. Brown, *Formation of the androgen receptor transcription complex.* Mol Cell, 2002. **9**(3): p. 601-10.
56. Litvinov, I.V., A.M. De Marzo, and J.T. Isaacs, *Is the Achilles' heel for prostate cancer therapy a gain of function in androgen receptor signaling?* Journal of Clinical Endocrinology & Metabolism, 2003. **88**(7): p. 2972-2982.
57. Gao, J., J.T. Arnold, and J.T. Isaacs, *Conversion from a paracrine to an autocrine mechanism of androgen-stimulated growth during malignant transformation of prostatic epithelial cells.* Cancer Res, 2001. **61**(13): p. 5038-44.
58. Isaacs, J.T. and W.B. Isaacs, *Androgen receptor outwits prostate cancer drugs.* Nat Med, 2004. **10**(1): p. 26-7.
59. Vander Griend, D.J., I.V. Litvinov, and J.T. Isaacs, *Conversion of androgen receptor signaling from a growth suppressor in normal prostate epithelial cells to an oncogene in prostate cancer cells involves a gain of function in c-Myc regulation.* Int J Biol Sci, 2014. **10**(6): p. 627-42.
60. Sun, F., et al., in *Therapies for Clinically Localized Prostate Cancer: Update of a 2008 Systematic Review*. 2014: Rockville (MD).
61. Kabalin, J.N., et al., *Unsuspected adenocarcinoma of the prostate in patients undergoing cystoprostatectomy for other causes: incidence, histology and morphometric observations.* J Urol, 1989. **141**(5): p. 1091-4; discussion 1093-4.
62. Holund, B., *Latent prostatic cancer in a consecutive autopsy series.* Scand J Urol Nephrol, 1980. **14**(1): p. 29-35.
63. Gandaglia, G., et al., *Distribution of metastatic sites in patients with prostate cancer: A population-based analysis.* Prostate, 2014. **74**(2): p. 210-6.
64. Catala, V., et al., *Questions and answers on prostate multiparameter magnetic resonance imaging: Everything a urologist should know.* Actas Urologicas Espanolas, 2016. **40**(6): p. 339-352.
65. Carter, H.B., *Management of low (favourable)-risk prostate cancer.* BJU Int, 2011. **108**(11): p. 1684-95.
66. Roudier, M.P., et al., *Phenotypic heterogeneity of end-stage prostate carcinoma metastatic to bone.* Hum Pathol, 2003. **34**(7): p. 646-53.
67. Nadler, R.B., et al., *Effect of inflammation and benign prostatic hyperplasia on elevated serum prostate specific antigen levels.* J Urol, 1995. **154**(2 Pt 1): p. 407-13.
68. Schroder, F.H., et al., *Screening and prostate-cancer mortality in a randomized European study.* N Engl J Med, 2009. **360**(13): p. 1320-8.

69. Damber, J.E. and R. Peeker, *Utdrag ur Urologi.* Student Literature, 2012.
70. Friedrich, M.J., *Debate continues on use of PSA testing for early detection of prostate cancer.* Jama, 2011. **305**(22): p. 2273-5.
71. Terada, N., et al., *Prognostic and predictive biomarkers in prostate cancer: latest evidence and clinical implications.* Therapeutic Advances in Medical Oncology, 2017. **9**(8): p. 565-573.
72. Sehn, J.K., *Prostate Cancer Pathology: Recent Updates and Controversies.* Missouri medicine, 2018. **115**(2): p. 151-155.
73. Gleason, D.F., *Histologic grading of prostate cancer: a perspective.* Hum Pathol, 1992. **23**(3): p. 273-9.
74. in *Prostate Cancer: Diagnosis and Treatment*. 2014: Cardiff (UK).
75. Morgan, T.M., P.H. Lange, and R.L. Vessella, *Detection and characterization of circulating and disseminated prostate cancer cells.* Front Biosci, 2007. **12**: p. 3000-9.
76. Huggins, C. and C.V. Hodges, *Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate.* CA Cancer J Clin, 1972. **22**(4): p. 232-40.
77. Fossa, S.D., et al., *Ten- and 15-yr Prostate Cancer-specific Mortality in Patients with Nonmetastatic Locally Advanced or Aggressive Intermediate Prostate Cancer, Randomized to Lifelong Endocrine Treatment Alone or Combined with Radiotherapy: Final Results of The Scandinavian Prostate Cancer Group-7.* Eur Urol, 2016. **70**(4): p. 684-691.
78. Miller, R.E. and C.J. Sweeney, *Chemotherapy for metastatic castrate-sensitive prostate cancer.* Prostate Cancer Prostatic Dis, 2016. **19**(2): p. 139-44.
79. Watson, P.A., V.K. Arora, and C.L. Sawyers, *Emerging mechanisms of resistance to androgen receptor inhibitors in prostate cancer.* Nat Rev Cancer, 2015. **15**(12): p. 701-11.
80. Roviello, G., et al., *Role of the novel generation of androgen receptor pathway targeted agents in the management of castration-resistant prostate cancer: A literature based meta-analysis of randomized trials.* European Journal of Cancer, 2016. **61**: p. 111-121.
81. Scott, L.J., *Abiraterone Acetate: A Review in Metastatic Castration-Resistant Prostrate Cancer.* Drugs, 2017. **77**(14): p. 1565-1576.
82. Scott, L.J., *Enzalutamide: A Review in Castration-Resistant Prostate Cancer.* Drugs, 2018. **78**(18): p. 1913-1924.
83. Vlachostergios, P.J., L. Puca, and H. Beltran, *Emerging Variants of Castration-Resistant Prostate Cancer.* Curr Oncol Rep, 2017. **19**(5): p. 32.
84. Cassinello, J., et al., *SEOM clinical guidelines for the treatment of metastatic prostate cancer (2017).* Clin Transl Oncol, 2018. **20**(1): p. 57-68.

85. Saad, F. and S.J. Hotte, *Guidelines for the management of castrate-resistant prostate cancer.* Can Urol Assoc J, 2010. **4**(6): p. 380-4.
86. Johnson, M.A., et al., *Castration triggers growth of previously static androgen-independent lesions in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model.* Prostate, 2005. **62**(4): p. 322-38.
87. Huss, W.J., et al., *Origin of androgen-insensitive poorly differentiated tumors in the transgenic adenocarcinoma of mouse prostate model.* Neoplasia, 2007. **9**(11): p. 938-50.
88. Visakorpi, T., et al., *In vivo amplification of the androgen receptor gene and progression of human prostate cancer.* Nat Genet, 1995. **9**(4): p. 401-6.
89. Azad, A.A., et al., *Androgen Receptor Gene Aberrations in Circulating Cell-Free DNA: Biomarkers of Therapeutic Resistance in Castration-Resistant Prostate Cancer.* Clin Cancer Res, 2015. **21**(10): p. 2315-24.
90. Cai, C.M., et al., *Androgen Receptor Gene Expression in Prostate Cancer Is Directly Suppressed by the Androgen Receptor Through Recruitment of Lysine-Specific Demethylase 1.* Cancer Cell, 2011. **20**(4): p. 457-471.
91. Robinson, D., et al., *Integrative Clinical Genomics of Advanced Prostate Cancer.* Cell, 2015. **162**(2): p. 454.
92. Beltran, H., et al., *Targeted next-generation sequencing of advanced prostate cancer identifies potential therapeutic targets and disease heterogeneity.* Eur Urol, 2013. **63**(5): p. 920-6.
93. Veldscholte, J., et al., *The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens.* The Journal of steroid biochemistry and molecular biology, 1992. **41**(3-8): p. 665-9.
94. Veldscholte, J., et al., *A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens.* Biochem Biophys Res Commun, 1990. **173**(2): p. 534-40.
95. Steketee, K., et al., *Broadened ligand responsiveness of androgen receptor mutants obtained by random amino acid substitution of H874 and mutation hot spot T877 in prostate cancer.* International Journal of Cancer, 2002. **100**(3): p. 309-317.
96. Nyquist, M.D., et al., *TALEN-engineered AR gene rearrangements reveal endocrine uncoupling of androgen receptor in prostate cancer.* Proc Natl Acad Sci U S A, 2013. **110**(43): p. 17492-7.
97. Yu, Z., et al., *Rapid induction of androgen receptor splice variants by androgen deprivation in prostate cancer.* Clin Cancer Res, 2014. **20**(6): p. 1590-600.
98. Watson, P.A., et al., *Constitutively active androgen receptor splice variants expressed in castration-resistant prostate cancer require full-length androgen receptor.* Proc Natl Acad Sci U S A, 2010. **107**(39): p. 16759-65.

99. Guo, Z.Y., et al., *A Novel Androgen Receptor Splice Variant Is Up-regulated during Prostate Cancer Progression and Promotes Androgen Depletion-Resistant Growth.* Cancer Research, 2009. **69**(6): p. 2305-2313.
100. Yang, L., et al., *Interleukin-6 differentially regulates androgen receptor transactivation via PI3K-Akt, STAT3, and MAPK, three distinct signal pathways in prostate cancer cells.* Biochem Biophys Res Commun, 2003. **305**(3): p. 462-9.
101. Ceder, Y., et al., *The Molecular Evolution of Castration-resistant Prostate Cancer.* Eur Urol Focus, 2016. **2**(5): p. 506-513.
102. 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 Res, 1994. **54**(20): p. 5474-8.
103. Locke, J.A., et al., *Androgen levels increase by intratumoral de novo steroidogenesis during progression of castration-resistant prostate cancer.* Cancer Res, 2008. **68**(15): p. 6407-15.
104. Tamae, D., et al., *The DHEA-sulfate depot following P450c17 inhibition supports the case for AKR1C3 inhibition in high risk localized and advanced castration resistant prostate cancer.* Chem Biol Interact, 2015. **234**: p. 332-8.
105. Dillard, P.R., M.F. Lin, and S.A. Khan, *Androgen-independent prostate cancer cells acquire the complete steroidogenic potential of synthesizing testosterone from cholesterol.* Mol Cell Endocrinol, 2008. **295**(1-2): p. 115-20.
106. Armandari, I., et al., *Intratumoral steroidogenesis in castration-resistant prostate cancer: a target for therapy.* Prostate Int, 2014. **2**(3): p. 105-13.
107. Montgomery, R.B., et al., *Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth.* Cancer Res, 2008. **68**(11): p. 4447-54.
108. Chang, K.H., et al., *Dihydrotestosterone synthesis bypasses testosterone to drive castration-resistant prostate cancer.* Proc Natl Acad Sci U S A, 2011. **108**(33): p. 13728-33.
109. Pierce, G.B. and W.C. Speers, *Tumors as Caricatures of the Process of Tissue Renewal - Prospects for Therapy by Directing Differentiation.* Cancer Research, 1988. **48**(8): p. 1996-2004.
110. Odero-Marah, V., et al., *Epithelial-Mesenchymal Transition (EMT) and Prostate Cancer.* Adv Exp Med Biol, 2018. **1095**: p. 101-110.
111. Kalluri, R. and R.A. Weinberg, *The basics of epithelial-mesenchymal transition.* J Clin Invest, 2009. **119**(6): p. 1420-8.
112. Das, V., et al., *The basics of epithelial-mesenchymal transition (EMT): A study from a structure, dynamics, and functional perspective.* J Cell Physiol, 2019.

113. Christiansen, J.J. and A.K. Rajasekaran, *Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis.* Cancer Research, 2006. **66**(17): p. 8319-8326.
114. Tam, W.L. and R.A. Weinberg, *The epigenetics of epithelial-mesenchymal plasticity in cancer.* Nat Med, 2013. **19**(11): p. 1438-49.
115. Huang, R.Y.J., et al., *An EMT spectrum defines an anoikis-resistant and spheroidogenic intermediate mesenchymal state that is sensitive to e-cadherin restoration by a src-kinase inhibitor, saracatinib (AZD0530).* Cell Death & Disease, 2013. **4**.
116. Kowalski, P.J., M.A. Rubin, and C.G. Kleer, *E-cadherin expression in primary carcinomas of the breast and its distant metastases.* Breast Cancer Res, 2003. **5**(6): p. R217-22.
117. Putzke, A.P., et al., *Metastatic progression of prostate cancer and e-cadherin regulation by zeb1 and SRC family kinases.* Am J Pathol, 2011. **179**(1): p. 400-10.
118. Brabletz, T., et al., *EMT in cancer.* Nat Rev Cancer, 2018. **18**(2): p. 128-134.
119. Pencik, J., et al., *STAT3 regulated ARF expression suppresses prostate cancer metastasis.* Nat Commun, 2015. **6**: p. 7736.
120. Carver, B.S., et al., *Reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient prostate cancer.* Cancer Cell, 2011. **19**(5): p. 575-86.
121. Don-Doncow, N., et al., *Expression of STAT3 in Prostate Cancer Metastases.* Eur Urol, 2017. **71**(3): p. 313-316.
122. Cancer Genome Atlas Research, N., *The Molecular Taxonomy of Primary Prostate Cancer.* Cell, 2015. **163**(4): p. 1011-25.
123. Mora, L.B., et al., *Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells.* Cancer Res, 2002. **62**(22): p. 6659-66.
124. Liao, Y., et al., *Increase of AKT/PKB expression correlates with gleason pattern in human prostate cancer.* Int J Cancer, 2003. **107**(4): p. 676-80.
125. Bitting, R.L. and A.J. Armstrong, *Targeting the PI3K/Akt/mTOR pathway in castration-resistant prostate cancer.* Endocr Relat Cancer, 2013. **20**(3): p. R83-99.
126. Taylor, B.S., et al., *Integrative genomic profiling of human prostate cancer.* Cancer Cell, 2010. **18**(1): p. 11-22.
127. Sun, Y., et al., *Androgen deprivation causes epithelial-mesenchymal transition in the prostate: implications for androgen-deprivation therapy.* Cancer Res, 2012. **72**(2): p. 527-36.
128. Barrallo-Gimeno, A. and M.A. Nieto, *The Snail genes as inducers of cell movement and survival: implications in development and cancer.* Development, 2005. **132**(14): p. 3151-3161.
129. Hajra, K.M., D.Y. Chen, and E.R. Fearon, *The SLUG zinc-finger protein represses E-cadherin in breast cancer.* Cancer Res, 2002. **62**(6): p. 1613-8.

130. Comijn, J., et al., *The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion.* Molecular Cell, 2001. **7**(6): p. 1267-1278.
131. Vesuna, F., et al., *Twist is a transcriptional repressor of E-cadherin gene expression in breast cancer.* Biochemical and Biophysical Research Communications, 2008. **367**(2): p. 235-241.
132. Nistico, P., M.J. Bissell, and D.C. Radisky, *Epithelial-mesenchymal transition: general principles and pathological relevance with special emphasis on the role of matrix metalloproteinases.* Cold Spring Harb Perspect Biol, 2012. **4**(2).
133. Thiery, J.P., et al., *Epithelial-mesenchymal transitions in development and disease.* Cell, 2009. **139**(5): p. 871-90.
134. Lamouille, S., J. Xu, and R. Derynck, *Molecular mechanisms of epithelial-mesenchymal transition.* Nature Reviews Molecular Cell Biology, 2014. **15**(3): p. 178-196.
135. Alimperti, S. and S.T. Andreadis, *CDH2 and CDH11 act as regulators of stem cell fate decisions.* Stem Cell Research, 2015. **14**(3): p. 270-282.
136. Bussemakers, M.J., et al., *Complex cadherin expression in human prostate cancer cells.* Int J Cancer, 2000. **85**(3): p. 446-50.
137. Brahimi-Horn, M.C., J. Chiche, and J. Pouyssegur, *Hypoxia and cancer.* J Mol Med (Berl), 2007. **85**(12): p. 1301-7.
138. Bristow, R.G. and R.P. Hill, *Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability.* Nat Rev Cancer, 2008. **8**(3): p. 180-92.
139. Semenza, G.L., *Regulation of cancer cell metabolism by hypoxia-inducible factor 1.* Semin Cancer Biol, 2009. **19**(1): p. 12-6.
140. Noman, M.Z., et al., *Microenvironmental hypoxia orchestrating the cell stroma cross talk, tumor progression and antitumor response.* Crit Rev Immunol, 2011. **31**(5): p. 357-77.
141. Barron, D.A. and D.R. Rowley, *The reactive stroma microenvironment and prostate cancer progression.* Endocr Relat Cancer, 2012. **19**(6): p. R187-204.
142. Carreau, A., et al., *Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia.* Journal of Cellular and Molecular Medicine, 2011. **15**(6): p. 1239-1253.
143. Wang, G.L. and G.L. Semenza, *Purification and characterization of hypoxia-inducible factor 1.* J Biol Chem, 1995. **270**(3): p. 1230-7.
144. Zhong, H., et al., *Up-regulation of hypoxia-inducible factor 1alpha is an early event in prostate carcinogenesis.* Cancer Detect Prev, 2004. **28**(2): p. 88-93.
145. Lu, X. and Y. Kang, *Hypoxia and hypoxia-inducible factors: master regulators of metastasis.* Clin Cancer Res, 2010. **16**(24): p. 5928-35.
146. Xu, Q., et al., *Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways.* Oncogene, 2005. **24**(36): p. 5552-5560.

147. Jung, J.E., et al., *STAT3 is a potential modulator of HIF-1-mediated VEGF expression in human renal carcinoma cells.* Faseb Journal, 2005. **19**(7): p. 1296-+.
148. Dodd, K.M., et al., *mTORC1 drives HIF-1 alpha and VEGF-A signalling via multiple mechanisms involving 4E-BP1, S6K1 and STAT3.* Oncogene, 2015. **34**(17): p. 2239-2250.
149. Deep, G. and G.K. Panigrahi, *Hypoxia-Induced Signaling Promotes Prostate Cancer Progression: Exosomes Role as Messenger of Hypoxic Response in Tumor Microenvironment.* Critical reviews in oncogenesis, 2015. **20**(5-6): p. 419-34.
150. Figueroa, Y.G., et al., *NF-kappaB plays a key role in hypoxia-inducible factor-1-regulated erythropoietin gene expression.* Exp Hematol, 2002. **30**(12): p. 1419-27.
151. Shimizu, S., et al., *Prevention of hypoxia-induced cell death by Bcl-2 and Bcl-xL.* Nature, 1995. **374**(6525): p. 811-3.
152. Butterworth, K.T., et al., *Hypoxia selects for androgen independent LNCaP cells with a more malignant geno- and phenotype.* Int J Cancer, 2008. **123**(4): p. 760-8.
153. Alas, S. and B. Bonavida, *Rituximab inactivates signal transducer and activation of transcription 3 (STAT3) activity in B-non-Hodgkin's lymphoma through inhibition of the interleukin 10 autocrine/paracrine loop and results in down-regulation of Bcl-2 and sensitization to cytotoxic drugs.* Cancer Research, 2001. **61**(13): p. 5137-5144.
154. Trisciuoglio, D., et al., *Bcl-2 regulates HIF-1a protein stabilization in hypoxic melanoma cells via the molecular chaperone HSP90 beta.* Ejc Supplements, 2010. **8**(7): p. 153-153.
155. Iervolino, A., et al., *Bcl-2 overexpression in human melanoma cells increases angiogenesis through VEGF mRNA stabilization and HIF-1-mediated transcriptional activity.* Faseb Journal, 2002. **16**(9): p. 1453-+.
156. Biroccio, A., et al., *Bcl-2 overexpression and hypoxia synergistically act to modulate vascular endothelial growth factor expression and in vivo angiogenesis in a breast carcinoma line.* FASEB J, 2000. **14**(5): p. 652-60.
157. Renaud, S., et al., *Role of hypoxia in epithelial-to-mesenchymal transition (EMT) in non-small cell lung cancer (NSCLC).* European Respiratory Journal, 2014. **44**.
158. Lundgren, K., B. Nordenskjold, and G. Landberg, *Hypoxia, Snail and incomplete epithelial-mesenchymal transition in breast cancer.* Br J Cancer, 2009. **101**(10): p. 1769-81.
159. Mitani, T., et al., *Coordinated action of hypoxia-inducible factor-1alpha and beta-catenin in androgen receptor signaling.* J Biol Chem, 2012. **287**(40): p. 33594-606.
160. Mitani, T., et al., *Hypoxia enhances transcriptional activity of androgen receptor through hypoxia-inducible factor-1alpha in a low androgen environment.* J Steroid Biochem Mol Biol, 2011. **123**(1-2): p. 58-64.

161. Geng, H., et al., *Interplay between hypoxia and androgen controls a metabolic switch conferring resistance to androgen/AR-targeted therapy.* Nature Communications, 2018. **9**.
162. Sarker, D., et al., *Targeting the PI3K/AKT pathway for the treatment of prostate cancer.* Clin Cancer Res, 2009. **15**(15): p. 4799-805.
163. Datta, S.R., et al., *Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery.* Cell, 1997. **91**(2): p. 231-241.
164. Zhang, X., et al., *Akt, FoxO and regulation of apoptosis.* Biochim Biophys Acta, 2011. **1813**(11): p. 1978-86.
165. Lau, M.T. and P.C. Leung, *The PI3K/Akt/mTOR signaling pathway mediates insulin-like growth factor 1-induced E-cadherin down-regulation and cell proliferation in ovarian cancer cells.* Cancer Lett, 2012. **326**(2): p. 191-8.
166. Gottlieb, T.M., et al., *Cross-talk between Akt, p53 and Mdm2: possible implications for the regulation of apoptosis.* Oncogene, 2002. **21**(8): p. 1299-1303.
167. Trotman, L.C., et al., *Pten dose dictates cancer progression in the prostate.* PLoS Biol, 2003. **1**(3): p. E59.
168. Suzuki, H., et al., *Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues.* Cancer Res, 1998. **58**(2): p. 204-9.
169. Cairns, P., et al., *Frequent inactivation of PTEN/MMAC1 in primary prostate cancer.* Cancer Res, 1997. **57**(22): p. 4997-5000.
170. Crumbaker, M., L. Khoja, and A.M. Joshua, *AR Signaling and the PI3K Pathway in Prostate Cancer.* Cancers (Basel), 2017. **9**(4).
171. Zhong, J., et al., *p300 acetyltransferase regulates androgen receptor degradation and PTEN-deficient prostate tumorigenesis.* Cancer Res, 2014. **74**(6): p. 1870-1880.
172. Johnson, D.E., R.A. O'Keefe, and J.R. Grandis, *Targeting the IL-6/JAK/STAT3 signalling axis in cancer.* Nat Rev Clin Oncol, 2018. **15**(4): p. 234-248.
173. Cui, Y., et al., *STAT3 regulates hypoxia-induced epithelial mesenchymal transition in oesophageal squamous cell cancer.* Oncology Reports, 2016. **36**(1): p. 108-116.
174. Gyamfi, J., et al., *Interleukin-6/STAT3 signalling regulates adipocyte induced epithelial-mesenchymal transition in breast cancer cells.* Sci Rep, 2018. **8**(1): p. 8859.
175. Huang, S., et al., *Interleukin-6/signal transducer and activator of transcription 3 promotes prostate cancer resistance to androgen deprivation therapy via regulating pituitary tumor transforming gene 1 expression.* Cancer Sci, 2018. **109**(3): p. 678-687.
176. Schroeder, A., et al., *Loss of Androgen Receptor Expression Promotes a Stem-like Cell Phenotype in Prostate Cancer through STAT3 Signaling.* Cancer Research, 2014. **74**(4): p. 1227-1237.

177. Fragkoulis, C., et al., *Current therapeutic options targeting bone metastasis in metastatic castration resistant prostate cancer.* J BUON, 2016. **21**(4): p. 787-791.
178. Coleman, R.E., *Clinical features of metastatic bone disease and risk of skeletal morbidity.* Clin Cancer Res, 2006. **12**(20 Pt 2): p. 6243s-6249s.
179. Sahai, E., *Illuminating the metastatic process.* Nat Rev Cancer, 2007. **7**(10): p. 737-49.
180. Bentley, H., et al., *Expression of bone morphogenetic proteins in human prostatic adenocarcinoma and benign prostatic hyperplasia.* Br J Cancer, 1992. **66**(6): p. 1159-63.
181. Weilbaecher, K.N., T.A. Guise, and L.K. McCauley, *Cancer to bone: a fatal attraction.* Nat Rev Cancer, 2011. **11**(6): p. 411-25.
182. Rucci, N. and A. Teti, *Osteomimicry: How the Seed Grows in the Soil.* Calcified Tissue International, 2018. **102**(2): p. 131-140.
183. Yuen, H.F., et al., *TWIST modulates prostate cancer cell-mediated bone cell activity and is upregulated by osteogenic induction.* Carcinogenesis, 2008. **29**(8): p. 1509-18.
184. Koeneman, K.S., F. Yeung, and L.W. Chung, *Osteomimetic properties of prostate cancer cells: a hypothesis supporting the predilection of prostate cancer metastasis and growth in the bone environment.* Prostate, 1999. **39**(4): p. 246-61.
185. Aguirre-Ghiso, J.A., *Models, mechanisms and clinical evidence for cancer dormancy.* Nat Rev Cancer, 2007. **7**(11): p. 834-46.
186. Townson, J.L. and A.F. Chambers, *Dormancy of solitary metastatic cells.* Cell Cycle, 2006. **5**(16): p. 1744-50.
187. Weidenfeld, K. and D. Barkan, *EMT and Stemness in Tumor Dormancy and Outgrowth: Are They Intertwined Processes?* Frontiers in Oncology, 2018. **8**.
188. Sosa, M.S., P. Bragado, and J.A. Aguirre-Ghiso, *Mechanisms of disseminated cancer cell dormancy: an awakening field.* Nat Rev Cancer, 2014. **14**(9): p. 611-22.
189. Roudier, M.P., et al., *Histopathological assessment of prostate cancer bone osteoblastic metastases.* J Urol, 2008. **180**(3): p. 1154-60.
190. Morrissey, C., et al., *Effects of androgen deprivation therapy and bisphosphonate treatment on bone in patients with metastatic castration-resistant prostate cancer: results from the University of Washington Rapid Autopsy Series.* J Bone Miner Res, 2013. **28**(2): p. 333-40.
191. Aubin, J.E., *Bone stem cells.* J Cell Biochem, 1998. **72 Suppl 30-31**(S30-31): p. 73-82.
192. Rutkovskiy, A., K.O. Stenslokken, and I.J. Vaage, *Osteoblast Differentiation at a Glance.* Medical Science Monitor Basic Research, 2016. **22**: p. 95-106.
193. Buijs, J.T. and G. van der Pluijm, *Osteotropic cancers: from primary tumor to bone.* Cancer Lett, 2009. **273**(2): p. 177-93.

194. Luo, G., et al., *TNFalpha and RANKL promote osteoclastogenesis by upregulating RANK via the NFkappaB pathway.* Mol Med Rep, 2018. **17**(5): p. 6605-6611.
195. Vessella, R.L. and E. Corey, *Targeting factors involved in bone remodeling as treatment strategies in prostate cancer bone metastasis.* Clinical Cancer Research, 2006. **12**(20): p. 6285s-6290s.
196. Clines, G.A. and T.A. Guise, *Hypercalcaemia of malignancy and basic research on mechanisms responsible for osteolytic and osteoblastic metastasis to bone.* Endocr Relat Cancer, 2005. **12**(3): p. 549-83.
197. Shupp, A.B., et al., *Cancer Metastases to Bone: Concepts, Mechanisms, and Interactions with Bone Osteoblasts.* Cancers (Basel), 2018. **10**(6).
198. Akech, J., et al., *Runx2 association with progression of prostate cancer in patients: mechanisms mediating bone osteolysis and osteoblastic metastatic lesions.* Oncogene, 2010. **29**(6): p. 811-21.
199. Ge, C., et al., *Role of Runx2 phosphorylation in prostate cancer and association with metastatic disease.* Oncogene, 2016. **35**(3): p. 366-76.
200. Brubaker, K.D., et al., *Prostate cancer expression of runt-domain transcription factor Runx2, a key regulator of osteoblast differentiation and function.* Prostate, 2003. **56**(1): p. 13-22.
201. Yun, S.J., et al., *Transcriptional repression of RUNX2 is associated with aggressive clinicopathological outcomes, whereas nuclear location of the protein is related to metastasis in prostate cancer.* Prostate cancer and prostatic diseases, 2012. **15**(4): p. 369-73.
202. Chua, C.-W., et al., *Suppression of androgen-independent prostate cancer cell aggressiveness by FTY720: validating Runx2 as a potential antimetastatic drug screening platform.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2009. **15**(13): p. 4322-35.
203. Pratap, J., et al., *Runx2 transcriptional activation of Indian Hedgehog and a downstream bone metastatic pathway in breast cancer cells.* Cancer Res, 2008. **68**(19): p. 7795-802.
204. Merciris, D., et al., *Overexpression of the transcriptional factor Runx2 in osteoblasts abolishes the anabolic effect of parathyroid hormone in vivo.* Am J Pathol, 2007. **170**(5): p. 1676-85.
205. Frolik, C.A., et al., *Anabolic and catabolic bone effects of human parathyroid hormone (1-34) are predicted by duration of hormone exposure.* Bone, 2003. **33**(3): p. 372-9.
206. Ma, Y.L., et al., *Catabolic effects of continuous human PTH (1--38) in vivo is associated with sustained stimulation of RANKL and inhibition of osteoprotegerin and gene-associated bone formation.* Endocrinology, 2001. **142**(9): p. 4047-54.
207. Schiller, P.C., et al., *Anabolic or catabolic responses of MC3T3-E1 osteoblastic cells to parathyroid hormone depend on time and duration of treatment.* J Bone Miner Res, 1999. **14**(9): p. 1504-12.

208. Thirunavukkarasu, K., et al., *Analysis of regulator of G-protein signaling-2 (RGS-2) expression and function in osteoblastic cells.* J Cell Biochem, 2002. **85**(4): p. 837-50.
209. Jilka, R.L., *Molecular and cellular mechanisms of the anabolic effect of intermittent PTH.* Bone, 2007. **40**(6): p. 1434-46.
210. Miao, D., et al., *Osteoblast-derived PTHrP is a potent endogenous bone anabolic agent that modifies the therapeutic efficacy of administered PTH 1-34.* The Journal of clinical investigation, 2005. **115**(9): p. 2402-11.
211. Dobnig, H. and R.T. Turner, *Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells.* Endocrinology, 1995. **136**(8): p. 3632-8.
212. Mountzios, G., et al., *Abnormal bone remodeling process is due to an imbalance in the receptor activator of nuclear factor-kappa B ligand (RANKL)/osteoprotegerin (OPG) axis in patients with solid tumors metastatic to the skeleton.* Acta Oncologica, 2007. **46**(2): p. 221-229.
213. Wu, H.K., et al., *Differential expression of a basic helix-loop-helix phosphoprotein gene, G0S8, in acute leukemia and localization to human chromosome 1q31.* Leukemia, 1995. **9**(8): p. 1291-8.
214. Gu, S., et al., *Alternative translation initiation of human regulators of G-protein signaling-2 yields a set of functionally distinct proteins.* Mol Pharmacol, 2008. **73**(1): p. 1-11.
215. Wolff, D.W., et al., *Epigenetic repression of regulator of G-protein signaling 2 promotes androgen-independent prostate cancer cell growth.* Int J Cancer, 2012. **130**(7): p. 1521-31.
216. Linder, A., et al., *Analysis of regulator of G-protein signalling 2 (RGS2) expression and function during prostate cancer progression.* Sci Rep, 2018. **8**(1): p. 17259.
217. Smalley, M.J., et al., *Regulator of G-protein signalling 2 mRNA is differentially expressed in mammary epithelial subpopulations and over-expressed in the majority of breast cancers.* Breast Cancer Research, 2007. **9**(6).
218. Lyu, J.H., et al., *RGS2 suppresses breast cancer cell growth via a MCPIP1-dependent pathway.* J Cell Biochem, 2015. **116**(2): p. 260-7.
219. Ma, X.J., et al., *Gene expression profiling of the tumor microenvironment during breast cancer progression.* Breast Cancer Res, 2009. **11**(1): p. R7.
220. Jiang, Z., et al., *Analysis of RGS2 expression and prognostic significance in stage II and III colorectal cancer.* Biosci Rep, 2010. **30**(6): p. 383-90.
221. Wang, C.L., et al., *Downregulation of regulator of G protein signaling 2 expression in breast invasive carcinoma of no special type: Clinicopathological associations and prognostic relevance.* Oncology Letters, 2018. **15**(1): p. 213-220.
222. Yin, H.L., et al., *Drug-resistant CXCR4-positive cells have the molecular characteristics of EMT in NSCLC.* Gene, 2016. **594**(1): p. 23-29.

223. Siderovski, D.P., et al., *A set of human putative lymphocyte G0/G1 switch genes includes genes homologous to rodent cytokine and zinc finger protein-encoding genes.* DNA Cell Biol, 1990. **9**(8): p. 579-87.
224. Heximer, S.P., A.D. Cristillo, and D.R. Forsdyke, *Comparison of mRNA expression of two regulators of G-protein signaling, RGS1/BL34/1R20 and RGS2/G0S8, in cultured human blood mononuclear cells.* DNA Cell Biol, 1997. **16**(5): p. 589-98.
225. Siderovski, D.P., S.P. Heximer, and D.R. Forsdyke, *A human gene encoding a putative basic helix-loop-helix phosphoprotein whose mRNA increases rapidly in cycloheximide-treated blood mononuclear cells.* DNA Cell Biol, 1994. **13**(2): p. 125-47.
226. Koelle, M.R. and H.R. Horvitz, *EGL-10 regulates G protein signaling in the C. elegans nervous system and shares a conserved domain with many mammalian proteins.* Cell, 1996. **84**(1): p. 115-25.
227. Dorsam, R.T. and J.S. Gutkind, *G-protein-coupled receptors and cancer.* Nat Rev Cancer, 2007. **7**(2): p. 79-94.
228. Sato, M., et al., *Factors determining specificity of signal transduction by G-protein-coupled receptors. Regulation of signal transfer from receptor to G-protein.* J Biol Chem, 1995. **270**(25): p. 15269-76.
229. Heximer, S.P., et al., *RGS2/G0S8 is a selective inhibitor of Gqalpha function.* Proc Natl Acad Sci U S A, 1997. **94**(26): p. 14389-93.
230. Heximer, S.P., et al., *G protein selectivity is a determinant of RGS2 function.* Journal of Biological Chemistry, 1999. **274**(48): p. 34253-34259.
231. Ballou, L.M., et al., *Activated G alpha q inhibits p110 alpha phosphatidylinositol 3-kinase and Akt.* Journal of Biological Chemistry, 2003. **278**(26): p. 23472-23479.
232. Bommakanti, R.K., S. Vinayak, and W.F. Simonds, *Dual regulation of Akt/protein kinase B by heterotrimeric G protein subunits.* J Biol Chem, 2000. **275**(49): p. 38870-6.
233. Wu, E.H., B.H. Tam, and Y.H. Wong, *Constitutively active alpha subunits of G(q/11) and G(12/13) families inhibit activation of the pro-survival Akt signaling cascade.* FEBS J, 2006. **273**(11): p. 2388-98.
234. Xie, P., et al., *Activation of NF-kappa B by bradykinin through a G alpha(q)-and G beta gamma-dependent pathway that involves phosphoinositide 3-kinase and Akt.* Journal of Biological Chemistry, 2000. **275**(32): p. 24907-24914.
235. Anger, T., et al., *RGS protein specificity towards Gq- and Gi/o-mediated ERK 1/2 and Akt activation, in vitro.* J Biochem Mol Biol, 2007. **40**(6): p. 899-910.
236. Nunn, C., et al., *RGS2 inhibits beta-adrenergic receptor-induced cardiomyocyte hypertrophy.* Cell Signal, 2010. **22**(8): p. 1231-9.
237. Seredenina, T., O. Gokce, and R. Luthi-Carter, *Decreased Striatal RGS2 Expression Is Neuroprotective in Huntington's Disease (HD) and*

Exemplifies a Compensatory Aspect of HD-Induced Gene Regulation. Plos One, 2011. **6**(7).
238. Kehrl, J.H. and S. Sinnarajah, *RGS2: a multifunctional regulator of G-protein signaling.* Int J Biochem Cell Biol, 2002. **34**(5): p. 432-8.
239. Roy, A.A., et al., *RGS2 interacts with Gs and adenylyl cyclase in living cells.* Cell Signal, 2006. **18**(3): p. 336-48.
240. Salim, S., et al., *Identification of RGS2 and type V adenylyl cyclase interaction sites.* J Biol Chem, 2003. **278**(18): p. 15842-9.
241. Neves, S.R., P.T. Ram, and R. Iyengar, *G protein pathways.* Science, 2002. **296**(5573): p. 1636-9.
242. Homme, M., et al., *Vitamin D and dexamethasone inversely regulate parathyroid hormone-induced regulator of G protein signaling-2 expression in osteoblast-like cells.* Endocrinology, 2003. **144**(6): p. 2496-504.
243. Keinan, D., et al., *Role of regulator of G protein signaling proteins in bone.* Front Biosci (Landmark Ed), 2014. **19**: p. 634-48.
244. Madrigal, A., L. Tan, and Y. Zhao, *Expression regulation and functional analysis of RGS2 and RGS4 in adipogenic and osteogenic differentiation of human mesenchymal stem cells.* Biol Res, 2017. **50**(1): p. 43.
245. Oliveira-Dos-Santos, A.J., et al., *Regulation of T cell activation, anxiety, and male aggression by RGS2.* Proc Natl Acad Sci U S A, 2000. **97**(22): p. 12272-7.
246. Roy, A.A., et al., *Up-regulation of endogenous RGS2 mediates cross-desensitization between Gs and Gq signaling in osteoblasts.* J Biol Chem, 2006. **281**(43): p. 32684-93.
247. Mannstadt, M., H. Juppner, and T.J. Gardella, *Receptors for PTH and PTHrP: their biological importance and functional properties.* American Journal of Physiology-Renal Physiology, 1999. **277**(5): p. F665-F675.
248. Juppner, H., et al., *A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide.* Science, 1991. **254**(5034): p. 1024-6.
249. Abou-Samra, A.B., et al., *Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium.* Proceedings of the National Academy of Sciences of the United States of America, 1992. **89**(7): p. 2732-6.
250. Datta, N.S. and A.B. Abou-Samra, *PTH and PTHrP signaling in osteoblasts.* Cell Signal, 2009. **21**(8): p. 1245-54.
251. Miles, R.R., et al., *Dynamic regulation of RGS2 in bone: Potential new insights into parathyroid hormone signaling mechanisms.* Endocrinology, 2000. **141**(1): p. 28-36.
252. Nguyen, C.H., et al., *RGS2 is a component of the cellular stress response.* Biochemical and Biophysical Research Communications, 2012. **426**(1): p. 129-134.

253. Zmijewski, J.W., et al., *Oxidative stress and heat shock stimulate RGS2 expression in 1321N1 astrocytoma cells.* Arch Biochem Biophys, 2001. **392**(2): p. 192-6.
254. Nguyen, C.H., et al., *Translational control by RGS2.* J Cell Biol, 2009. **186**(5): p. 755-65.
255. Wang, C.J. and P. Chidiac, *RGS2 promotes the translation of stress-associated proteins ATF4 and CHOP via its eIF2B-inhibitory domain.* Cell Signal, 2019. **59**: p. 163-170.
256. Jain, N., Y.P. Tu, and P.W. Abel, *Effects of hypoxia on regulators of G-protein signaling 2 (RGS2) regulation of pulmonary arterial constriction.* Faseb Journal, 2013. **27**.
257. Boelte, K.C., et al., *Rgs2 mediates pro-angiogenic function of myeloid derived suppressor cells in the tumor microenvironment via upregulation of MCP-1.* PLoS One, 2011. **6**(4): p. e18534.
258. Dong, H., et al., *Regulator of G protein signaling 2 is a key regulator of pancreatic beta-cell mass and function.* Cell Death & Disease, 2017. **8**.
259. Du, W.W., et al., *miR-17 extends mouse lifespan by inhibiting senescence signaling mediated by MKP7.* Cell Death Dis, 2014. **5**: p. e1355.
260. Lee, H.K., et al., *RGS2 is a negative regulator of STAT3-mediated Nox1 expression.* Cell Signal, 2012. **24**(3): p. 803-9.
261. Chatterjee, T.K. and R.A. Fisher, *Cytoplasmic, nuclear, and golgi localization of RGS proteins. Evidence for N-terminal and RGS domain sequences as intracellular targeting motifs.* J Biol Chem, 2000. **275**(31): p. 24013-21.
262. Song, L., J.W. Zmijewski, and R.S. Jope, *RGS2: regulation of expression and nuclear localization.* Biochem Biophys Res Commun, 2001. **283**(1): p. 102-6.
263. Heo, K., et al., *RGS2 promotes formation of neurites by stimulating microtubule polymerization.* Cell Signal, 2006. **18**(12): p. 2182-92.
264. Jiang, M.X., et al., *Inhibition of the Binding between RGS2 and beta-Tubulin Interferes with Spindle Formation and Chromosome Segregation during Mouse Oocyte Maturation In Vitro.* Plos One, 2016. **11**(7).
265. de Souza, E.E., et al., *Human Nek7-interactor RGS2 is required for mitotic spindle organization.* Cell Cycle, 2015. **14**(4): p. 656-667.
266. Gustavsson, H., K. Welen, and J.E. Damber, *Transition of an androgen-dependent human prostate cancer cell line into an androgen-independent subline is associated with increased angiogenesis.* Prostate, 2005. **62**(4): p. 364-73.
267. Horoszewicz, J.S., et al., *The LNCaP cell line--a new model for studies on human prostatic carcinoma.* Prog Clin Biol Res, 1980. **37**: p. 115-32.
268. Jennbacken, K., et al., *The Prostatic Environment Suppresses Growth of Androgen-Independent Prostate Cancer Xenografts: An Effect Influenced by Testosterone.* Prostate, 2009. **69**(11): p. 1164-1175.

269. Cao, X., et al., *Regulator of G-protein signaling 2 (RGS2) inhibits androgen-independent activation of androgen receptor in prostate cancer cells.* Oncogene, 2006. **25**(26): p. 3719-34.
270. Wainstein, M.A., et al., *CWR22: androgen-dependent xenograft model derived from a primary human prostatic carcinoma.* Cancer Res, 1994. **54**(23): p. 6049-52.
271. Sramkoski, R.M., et al., *A new human prostate carcinoma cell line, 22R upsilon 1.* In Vitro Cellular & Developmental Biology-Animal, 1999. **35**(7): p. 403-409.
272. Webber, M.M., et al., *A human prostatic stromal myofibroblast cell line WPMY-1: a model for stromal-epithelial interactions in prostatic neoplasia.* Carcinogenesis, 1999. **20**(7): p. 1185-92.
273. Kaighn, M.E., et al., *Establishment and characterization of a human prostatic carcinoma cell line (PC-3).* Invest Urol, 1979. **17**(1): p. 16-23.
274. Dai, J., et al., *Mouse models for studying prostate cancer bone metastasis.* Bonekey Rep, 2016. **5**: p. 777.
275. Berquin, I.M., et al., *Expression signature of the mouse prostate.* J Biol Chem, 2005. **280**(43): p. 36442-51.
276. Hagberg Thulin, M., et al., *Osteoblasts stimulate the osteogenic and metastatic progression of castration-resistant prostate cancer in a novel model for in vitro and in vivo studies.* Clin Exp Metastasis, 2014. **31**(3): p. 269-83.
277. Josefsson, A., et al., *Circulating tumor cells mirror bone metastatic phenotype in prostate cancer.* Oncotarget, 2018. **9**(50): p. 29403-29413.
278. Crnalic, S., et al., *Nuclear androgen receptor staining in bone metastases is related to a poor outcome in prostate cancer patients.* Endocrine-related cancer, 2010. **17**(4): p. 885-95.
279. Pizzi, H., et al., *Androgen regulation of parathyroid hormone-related peptide production in human prostate cancer cells.* Endocrinology, 2003. **144**(3): p. 858-67.
280. Cramer, S.D., Z. Chen, and D.M. Peehl, *Prostate specific antigen cleaves parathyroid hormone-related protein in the PTH-like domain: inactivation of PTHrP-stimulated cAMP accumulation in mouse osteoblasts.* J Urol, 1996. **156**(2 Pt 1): p. 526-31.
281. Iwamura, M., et al., *Alteration of the hormonal bioactivity of parathyroid hormone-related protein (PTHrP) as a result of limited proteolysis by prostate-specific antigen.* Urology, 1996. **48**(2): p. 317-25.
282. Wu, J.N., et al., *No Improvement Noted in Overall or Cause-Specific Survival for Men Presenting With Metastatic Prostate Cancer Over a 20-Year Period.* Cancer, 2014. **120**(6): p. 818-823.
283. Albala, D.M., *Imaging and treatment recommendations in patients with castrate-resistant prostate cancer.* Rev Urol, 2017. **19**(3): p. 200-202.
284. Walsh, J.C., et al., *The clinical importance of assessing tumor hypoxia: relationship of tumor hypoxia to prognosis and therapeutic opportunities.* Antioxid Redox Signal, 2014. **21**(10): p. 1516-54.

285. Torres-Roca, J.F., et al., *Activated STAT3 as a correlate of distant metastasis in prostate cancer: A secondary analysis of radiation therapy oncology group 86-10.* Urology, 2007. **69**(3): p. 505-509.
286. Ho, M.K., et al., *Regulation of transcription factors by heterotrimeric G proteins.* Curr Mol Pharmacol, 2009. **2**(1): p. 19-31.
287. Trefier, A., et al., *G Protein-Coupled Receptors As Regulators of Localized Translation: The Forgotten Pathway?* Frontiers in Endocrinology, 2018. **9**.
288. Marques, R.B., et al., *Modulation of androgen receptor signaling in hormonal therapy-resistant prostate cancer cell lines.* PLoS One, 2011. **6**(8): p. e23144.
289. Thalmann, G.N., et al., *Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer.* Cancer research, 1994. **54**(10): p. 2577-81.
290. van der Pluijm, G., et al., *Interference with the microenvironmental support impairs the de novo formation of bone metastases in vivo.* Cancer Research, 2005. **65**(17): p. 7682-7690.
291. Reim, N.S., et al., *Cortical bone loss in androgen-deficient aged male rats is mainly caused by increased endocortical bone remodeling.* Journal of Bone and Mineral Research, 2008. **23**(5): p. 694-704.
292. Ottewell, P.D., et al., *Castration-induced bone loss triggers growth of disseminated prostate cancer cells in bone.* Endocr Relat Cancer, 2014. **21**(5): p. 769-81.