

Estrogens and the G protein-coupled estrogen receptor in prostate cancer: angels or demons?

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Tese para obtenção do Grau de Doutor em
Biomedicina
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Dedicatória

Dedico esta Tese de Doutorado a todos aqueles que lutam, já lutaram ou ainda terão de lutar contra o cancro. Uma doença sorrateira que chega de forma inesperada e altera todos os planos de uma vida.

Dedico também esta Tese aos investigadores que todos os dias acordam com o propósito de contribuir para que a luta dos primeiros seja mais leve e, quem sabe, encontrar uma cura.

"Eu tentei 99 vezes e falhei, mas na centésima tentativa eu consegui,
nunca desista de seus objetivos mesmo que esses pareçam impossíveis,
a próxima tentativa pode ser a vitoriosa."

Albert Einstein

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Lista de Publicações

Publications included in the thesis:

Figueira MI, Cardoso HJ, Carvalho TMA, Correia S, Henrique RM, Santos AO, Maia C, Socorro S. G protein-coupled estrogen receptor actions targeting the hallmarks of cancer in human prostate cells: from cell fate to metabolic reprogramming. Submitted to *European Urology Oncology*

Figueira MI, Cardoso HJ, Fonseca LRS, Silvestre S, Socorro S. Diosgenin effects in suppressing proliferation and promoting apoptosis of human prostate cancer cells in an interplay with the G protein-coupled estrogen receptor. Submitted to *Molecules*

Figueira MI, Carvalho TMA, Monteiro J, Cardoso HJ, Vaz CV, Duarte AP, Socorro S. Estrogens actions in prostate cancer: an update with a focus on phytoestrogens. Submitted to *Medicinal Research Reviews*

Figueira MI, Cardoso HJ, Correia S, Maia CJ, Socorro S. The stem cell factor (SCF)/c-KIT system in carcinogenesis of reproductive tissues: What does the hormonal regulation tell us? *Cancer letters*. 2017; 405:10-21

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Cardoso HJ, **Figueira MI**, Carvalho TMA, Serra CDM, Vaz CV, Madureira PA, Socorro S. Androgens and low density lipoprotein-cholesterol interplay in modulating prostate cancer cell fate and metabolism. *Pathology Research and Practice*. 2022 Dec; 240:154181.

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Cardoso HJ, Vaz CV, Correia S, **Figueira MI**, Marques R, Maia CJ, *et al*. Paradoxical and contradictory effects of imatinib in two cell line models of hormone-refractory prostate cancer. *The Prostate*. 2015; 75(9):923-35

Resumo

O cancro da próstata (CaP) é o segundo tipo de cancro mais comum entre os homens, com uma incidência crescente a nível mundial. É uma neoplasia altamente dependente da ação hormonal, tendo os androgénios um papel crucial no seu desenvolvimento e progressão. Contudo, também os estrogénios têm influência no cancro da próstata. De facto, os estrogénios foram usados no tratamento do CaP durante décadas, após os trabalhos pioneiros de Huggins e Hodges que demonstraram que o CaP é sensível à ação das hormonas, sendo estimulado pela ação dos androgénios e inibido pela supressão dos níveis de androgénios ou pela administração de estrogénios. No entanto, apesar da administração de estrogénios ser eficaz no atraso da progressão do CaP metastático, esta terapia foi descontinuada devido aos seus efeitos adversos colaterais. Ao longo dos anos, inúmeros estudos têm sido feitos com o objetivo de decifrar o papel dos estrogénios no CaP e de encontrar novas alternativas terapêuticas com base nos estrogénios, sem os efeitos adversos. Todavia, a contradição relativa à ação dos estrogénios continua a ser evidente na literatura até hoje. Muitos estudos apontam os estrogénios como agentes causadores de CaP, contribuindo para o seu desenvolvimento e progressão. Contudo, também há uma panóplia de estudos que defendem os estrogénios como protetores contra o CaP, suprimindo o crescimento tumoral, induzindo apoptose e inibindo a metastização, o que está de acordo com a sua eficácia como agentes terapêuticos. Esta atividade dual foi associada à diversidade de recetores ativados pelos estrogénios, estando os efeitos pro-carcinogénicos associados com a ativação do recetor de estrogénios α (ER α), enquanto que os efeitos anti-carcinogénicos têm sido atribuídos à ativação do ER β . Além disso, a descoberta do recetor de estrogénios acoplado a proteína G (GPER) aumentou a complexidade dos mecanismos que orquestram as ações dos estrogénios. O GPER é um recetor de estrogénios membranar que intervém nos efeitos rápidos e não genómicos dos estrogénios, através da mobilização de cálcio intracelular e da ativação de várias vias de sinalização. Uma quantidade substancial de evidências descreve os efeitos anti-tumorigénicos do GPER, suprimindo o crescimento e progressão tumoral em diferentes tipos de cancro. Uma área de investigação com um crescente interesse está relacionada com o estudo dos efeitos anti-carcinogénicos dos fitoestrogénios, suportada pela evidência de que a incidência do CaP é mais baixa nos países orientais, nos quais tipicamente existe o consumo de elevadas quantidades destes compostos como consequência de uma dieta rica em plantas. Adicionalmente, tem sido demonstrado que os mecanismos de ação de alguns destes compostos requerem a ativação do GPER.

A presente tese teve como objetivo responder a algumas das questões existentes sobre os estrogénios e as ações do GPER em células de CaP, permitindo estabelecer alicerces para o desenvolvimento de terapias baseadas em estrogénios novas e seguras. Primeiramente, foram investigadas as ações dos estrogénios como reguladores da apoptose e proliferação em células da próstata, e a sua interação com o sistema SCF/c-KIT, um conjunto de ligando e recetor altamente envolvido na carcinogénese e alvo de hormonas esteroides. Aqui, foi demonstrado que o 17 β -estradiol (E2) reduziu a expressão do sistema SCF/c-KIT quer em linhas celulares humanas de CaP, quer em próstata de rato *in vivo*. Além disso o E2 mostrou efeitos na supressão da proliferação e na indução da apoptose n próstata de rato. Estes resultados suportam a ideia do papel protetor dos estrogénios contra o CaP. Considerando os resultados promissores noutros tipos de cancro, foi determinado o efeito da ativação do GPER em células de CaP, abrangendo vários “hallmarks” do cancro, desde a decisão do destino celular à reprogramação metabólica. Verificou-se que o GPER é altamente expresso em casos de CaP comparativamente a casos de hiperplasia benigna da próstata (BPH). Além disso, a imunoreatividade do GPER foi inversamente correlacionada com os níveis de antigénio prostático específico (PSA). Também se verificaram níveis de GPER mais altos nas células neoplásicas LNCaP do que nas células não neoplásicas PNT1A. No entanto, células de CaP resistente à castração (CRPC) mostraram ter expressão de GPER reduzida comparativamente à linha celular responsiva aos androgénios, suportando a ideia de que os níveis de proteína GPER podem ser alterados pelos androgénios, o que foi comprovado neste estudo. Verificou-se que o GPER estava localizado na membrana celular, no retículo endoplasmático e, residualmente, no núcleo as células da próstata, e que esta localização subcelular foi alterada pelo G1, agonista específico do GPER. O tratamento com G1 mostrou uma multiplicidade de efeitos benéficos no CaP, afetando uma panóplia de “hallmarks” do cancro. Este tratamento reduziu a viabilidade e proliferação das células de CaP, bloqueando o ciclo celular e aumentando a apoptose. Também se verificou a capacidade do G1 reduzir a migração e invasão, concomitantemente com a redução da transição epitelial-mesenquimal (EMT), dependendo da linha celular. Adicionalmente, a ativação do GPER pelo G1 aumentou o metabolismo energético e o stress oxidativo das células de CaP. Por último, avaliou-se como é que compostos naturais, como a diosgenina, podem influenciar o comportamento do CaP considerando a sua interação com o GPER. A diosgenina diminuiu a viabilidade das células responsivas a androgénios e das CRPC, sem afetar a viabilidade das células não neoplásicas PNT1A. Além disso, o composto induziu apoptose e alterou o metabolismo glicolítico das células de CaP. Os efeitos da diosgenina foram incrementados aquando do tratamento combinado com G1,

suportado pela capacidade da diosgenina aumentar os níveis de expressão do GPER. Adicionalmente, o “knockdown” do gene *GPER*, através da tecnologia de siRNA suprimiu o aumento da apoptose causado pelo tratamento com diosgenina nas células e CaP, o que implica este recetor no mecanismo de atuação da diosgenina. No geral, as descobertas desta tese suportam o papel protetor dos estrogénios no CaP, revelando novos alvos moleculares e processos celulares. Além disso, estes resultados fornecem informação importante para se encontrar novas opções terapêuticas, nomeadamente, baseadas em estrogénios, para desacelerar a progressão da doença e melhorar a sobrevivência os doentes com CaP.

Palavras-chave

Cancro da próstata (PCa), estrogénios, recetor de estrogénios acoplado a proteína G (GPER), diosgenina

Resumo Alargado

O cancro da próstata (CaP) é o segundo tipo de cancro mais comum entre os homens. A sua incidência continua a aumentar a nível mundial, facto motivado em parte pelo aumento da esperança média de vida, uma vez que a idade é o principal fator de risco para o desenvolvimento deste tipo de cancro. É uma neoplasia altamente dependente da ação hormonal, tendo os androgénios um papel crucial no seu desenvolvimento e progressão, uma vez que estas hormonas estão associadas a aumentos de proliferação e resistência à apoptose na próstata. Contudo, também os estrogénios, hormonas maioritariamente associadas ao sexo feminino, têm influência no CaP. De facto, os estrogénios foram usados no tratamento do CaP durante décadas, após os trabalhos pioneiros de Huggins e Hodges que demonstraram que o CaP é sensível à ação das hormonas, sendo estimulado pela ação dos androgénios e inibido pela supressão dos níveis de androgénios ou pela administração de estrogénios. No entanto, apesar da administração de estrogénios ser eficaz no atraso da progressão do CaP metastático, esta terapia foi descontinuada devido aos seus efeitos adversos colaterais, nomeadamente a nível cardiovascular. Ao longo dos anos, inúmeros estudos têm sido feitos com o objetivo de decifrar o papel dos estrogénios no CaP e de encontrar novas alternativas terapêuticas com base nos estrogénios e sem os conhecidos efeitos adversos. Todavia, a contradição relativa à ação dos estrogénios continua a ser evidente na literatura até hoje. Muitos estudos apontam os estrogénios como agentes causadores de CaP, contribuindo para o seu desenvolvimento e progressão. Estes estudos reportam um aumento da proliferação e das capacidades de migração e invasão das células de CaP, como resposta aos estrogénios através de diversos mecanismos. Contudo, também há uma panóplia de estudos que defendem os estrogénios como protetores contra o CaP, suprimindo o crescimento tumoral, induzindo apoptose e inibindo a metastização, o que está de acordo com a sua eficácia como agentes terapêuticos. Esta atividade dual foi associada à diversidade de recetores ativados pelos estrogénios, estando os efeitos pro-carcinogénicos associados com a ativação do recetor de estrogénios α (ER α), enquanto que os efeitos anti-carcinogénicos têm sido atribuídos à ativação do ER β . Estes recetores são conhecidos como recetores clássicos nucleares que funcionam ativando a transcrição génica. Além disso, a descoberta do recetor de estrogénios acoplado a proteína G (GPER) aumentou a complexidade dos mecanismos que orquestram as ações dos estrogénios. O GPER é um recetor de estrogénios membranar que intervém nos efeitos rápidos e não genómicos dos estrogénios, através da mobilização de cálcio intracelular e da ativação de várias vias de sinalização. Uma

quantidade substancial de evidências descreve os efeitos anti-tumorigénicos do GPER, suprimindo o crescimento e progressão tumoral em diferentes tipos de cancro. Contudo, os efeitos do GPER não são muito claros, havendo também uma dualidade de efeitos pro- ou anti-tumorigénicos, dependendo do tipo de cancro e muitas, vezes no mesmo tipo de cancro. Os poucos estudos existentes sobre a ação do GPER no CaP também retratam esta divergência de resultados. Na tentativa de reintroduzir os estrogénios como terapia para o CaP pela sua eficácia, muitos estudos têm sido feitos, procurando alternativas seguras. Uma área de investigação com um crescente interesse está relacionada com o estudo dos efeitos anti-carcinogénicos dos fitoestrogénios, compostos derivados de plantas, com capacidade de interagir com recetores de estrogénios. Esta linha de investigação é fortemente suportada pelo facto de a incidência do CaP ser baixa nos países orientais, nos quais tipicamente existe o consumo de elevadas quantidades destes compostos como consequência de uma dieta rica em plantas. Assim, vários estudos investigaram os efeitos de compostos como a genisteína, o resveratrol, a quercetina, o equol, entre outros e reportaram que os mesmos são opções terapêuticas promissoras, como terapia isolada ou em combinação com outras terapias convencionais, aumentando a eficácia e reduzindo os efeitos adversos das mesmas. Adicionalmente, tem sido demonstrado que os mecanismos de ação de alguns destes compostos requerem a ativação do GPER. Considerando a problemática apresentada e as lacunas científicas existentes, a presente tese teve como objetivo responder a algumas das questões sobre as ações dos estrogénios do GPER em células de CaP, permitindo estabelecer alicerces para o desenvolvimento de novas e seguras terapias baseadas em estrogénios. Primeiramente, foram investigadas as ações dos estrogénios como reguladores da apoptose e proliferação em células da próstata, e a sua interação com o sistema SCF/c-KIT, um conjunto de ligando e recetor altamente envolvido na carcinogénese, através da ativação de várias vias de sinalização, como a fosfatidilinositol3-quinase (PI3-K), a Src, a JAK/STAT, fosfolipase-C (PLC- γ) e a MAPK. Neste trabalho, foram utilizadas linhas celulares de próstata não-neoplásicas (PNT1A) e neoplásicas responsivas a androgénios (LNCaP) ou resistentes a castração (DU145 e PC3). O tratamento destas células com 17 β -estradiol (E2) reduziu a expressão do sistema SCF/c-KIT quer nas linhas celulares humanas de cancro da próstata, quer em próstata de rato in vivo. Apesar desta redução destas proteínas, o efeito do E2 não foi uniforme em todas as linhas celulares, tendo-se verificado um aumento das proteínas c-KIT e SCF nas células PC3, que representam um estágio mais agressivo da doença, apontando para a importância de monitorização do CaP com a sua progressão. Além disso, o E2 mostrou efeitos na supressão da proliferação e na indução da apoptose nas próstatas de rato. No geral, estes resultados suportam a ideia do papel

protetor dos estrogénios contra o CaP. Considerando os resultados promissores noutros tipos de cancro, foi determinado o efeito da ativação do GPER em células de CaP, abrangendo vários “hallmarks” do cancro, desde a decisão do destino celular à reprogramação metabólica. Os “hallmarks” do cancro são um conjunto de características adquiridas pelas células ao longo da sua transformação neoplásica e incluem instabilidade e mutações genéticas, aumento da proliferação, imortalidade, escape aos supressores de crescimento, resistência à morte celular, desregulação do metabolismo celular, angiogénese, escape à ação imunológica, inflamação, ativação de capacidades de invasão e migração e ainda a plasticidade fenotípica, a reprogramação epigenética, alterações no microbioma e senescência. Antes de avaliar o efeito do GPER em algumas destas características, analisou-se o seu padrão de expressão em casos de CaP humanos e verificou-se que o GPER é altamente expresso nos tumores comparativamente a casos de hiperplasia benigna da próstata (BPH). Além disso, a imunoreatividade do GPER foi inversamente correlacionada com os níveis de PSA. Também se observaram níveis de GPER mais altos nas células neoplásicas LNCaP do que nas células não neoplásicas PNT1A. No entanto, células de CaP resistente à castração (CRPC) mostraram ter expressão de GPER reduzida comparativamente à linha celular responsiva aos androgénios, suportando a ideia de que os níveis de proteína GPER podem ser alterados pelos androgénios, o que foi comprovado neste estudo. O tratamento das células de CaP com 5 α -dihidrotestosterona (DHT) reduziu a expressão de GPER nas células PNT1A e LNCaP. A localização subcelular do GPER também foi avaliada e verificou-se que o recetor estava localizado na membrana celular, no retículo endoplasmático e, residualmente, no núcleo as células da próstata. Foi ainda demonstrado que a localização subcelular do GPER foi alterada pelo G1, agonista específico do GPER. O tratamento com G1 mostrou uma multiplicidade de efeitos benéficos no CaP, afetando uma panóplia de “hallmarks” do cancro. Este tratamento reduziu a viabilidade e proliferação das células de CaP, bloqueando o ciclo celular na fase G2/M, com alteração nos níveis de expressão das proteínas c-Myc na sua forma fosforilada e do sistema SCF e c-KIT. Adicionalmente, o GPER alterou os níveis de Akt e MAPK nas suas formas fosforiladas. O GPER mostrou ainda capacidade de aumentar a apoptose através da ativação quer da via intrínseca ou mitocondrial, quer da via extrínseca ou mediada por recetores Fas. A ativação do GPER aumentou a expressão de marcadores envolvidos nas duas vias apoptóticas como a Bax, caspase-9, caspase-8, Fas-R e Fas-L, p53, culminado na ativação da caspase-3, o “endpoint” no cruzamento de ambas as vias apoptóticas. Este aumento da apoptose nas células tratadas com G1 foi também confirmado pelo aumento da degradação de DNA, característico de células apoptóticas, analisado pelo ensaio TUNEL. Também se

verificou a capacidade do G1 reduzir a migração e invasão, concomitantemente com a redução da transição epitelial-mesenquimal (EMT), aumentando a expressão de marcadores epiteliais como a E-caderina e reduzindo marcadores mesenquimais como a N-caderina e a vimentina, ao longo o tempo, dependendo da linha celular. Adicionalmente, a ativação do GPER pelo G1 aumentou o metabolismo da glucose, dos aminoácidos e dos lípidos, bem como o stress oxidativo das células de CaP. Por último, avaliou-se como é que compostos naturais, como a diosgenina, podem influenciar o comportamento do CaP considerando a sua interação com o GPER. A diosgenina diminuiu a viabilidade das células responsivas a androgénios e das CRPC, sem afetar a viabilidade das células não neoplásicas PNT1A. Além disso, o composto induziu apoptose, principalmente, através da ativação da via extrínseca, e alterou o metabolismo glicolítico das células de CaP. Os efeitos da diosgenina foram incrementados aquando do tratamento combinado com G1, suportado pela capacidade da diosgenina aumentar os níveis de expressão do GPER. Adicionalmente, o “knockdown” do gene *GPER*, através da tecnologia de siRNA suprimiu o aumento da apoptose causado pelo tratamento com diosgenina nas células de CaP, o que indica que este recetor está envolvido no mecanismo de atuação da diosgenina no CaP. No geral, as descobertas desta tese suportam o papel protetor dos estrogénios no CaP, revelando novos alvos moleculares e processos celulares. Além disso, estes resultados fornecem informação importante para se encontrar novas opções terapêuticas, nomeadamente, baseadas em estrogénios, para desacelerar a progressão da doença e melhorar a sobrevivência os doentes com CaP.

Palavras-chave

Cancro da próstata (PCa), estrogénios, recetor de estrogénios acoplado a proteína G (GPER), diosgenina

Abstract

Prostate cancer (PCa) is the second most frequent type of cancer among men, with an increasing incidence worldwide. PCa is highly regulated by hormones, with androgens playing a crucial role in its development and progression. However, also estrogens are accepted to influence PCa. In fact, estrogens were used in PCa treatment for decades, after the pioneer work of Huggins and Hodges demonstrating that PCa is a hormone-sensitive cancer, stimulated by androgenic activity and inhibited by suppression of androgens levels or estrogens administration. Nevertheless, despite the effectiveness of estrogens administration in delaying the progression of metastatic PCa, this therapy was discontinued because of the adverse side effects. Over the years, a vast number of studies were performed with the aim of deciphering the role of estrogens in PCa and find new estrogen-based alternatives for PCa treatment without the adverse effects. However, a contradiction in the estrogens actions remains evident in the literature until now. Many studies have pointed out estrogens as causative agents of PCa, contributing to its development and progression. Though, also a panoply of reports defended estrogens as protective against PCa, suppressing tumor growth, inducing apoptosis and inhibiting metastization, in line with their efficacy as therapeutic agents. This dual activity was associated with the diversity of receptors activated by estrogens, with the pro-carcinogenic actions associated with the activation of the estrogen receptor α (ER α), whereas anti-carcinogenic effects are linked to ER β activation. Moreover, the discovery of G protein-coupled estrogen receptor (GPER) increased the complexity of mechanisms orchestrating estrogens actions. GPER is a membrane-bound ER that mediates the rapid, non-genomic effects of estrogens by mobilizing intracellular calcium and activating several signaling pathways. A substantial amount of evidence in different cancer types has been describing the anti-tumorigenic effects of GPER suppressing tumor growth and progression. A research area with increasing interest is the determination of the anti-carcinogenic actions of phytoestrogens, strengthened by the evidence of the lower incidence of PCa in Eastern countries, which typically have high consumption of these compounds in a consequence of plant-enriched diets. Furthermore, the mechanism of action of some of these compounds in PCa cells has been shown to require GPER activation. The present thesis aimed to answer some of the existent questions on the estrogens and GPER actions in PCa cells, which would lay the foundations for the development of new and safe estrogen-based therapies. First, it was investigated the action of estrogens as regulators of apoptosis and proliferation in prostate cells in the interplay with the SCF/c-KIT system, a set of ligand and receptor

highly involved in carcinogenesis and a target of steroid hormones. Herein, it was demonstrated that E2 down-regulated the expression of the SCF/c-KIT system both in human PCa cell lines and rat prostate in vivo, which was accompanied by the effects of E2 suppressing proliferation and inducing apoptosis in rat prostates. These results supported the protective role of estrogens against PCa. Considering the promising results in other types of cancer, it was determined the effect of GPER activation in PCa cells covering several cancer hallmarks, from cell fate to metabolic reprogramming. It was found that GPER is highly expressed in PCa compared to benign prostatic hyperplasia (BPH) cases. Furthermore, GPER immunoreactivity was inversely correlated with PSA levels. GPER protein levels also were higher in neoplastic LNCaP cells than in the non-neoplastic PNT1A cells. Nevertheless, castrate-resistant PCa (CRPC) cells displayed reduced expression of GPER compared to the androgen-responsive cell line, supporting that GPER protein levels could be modulated by androgens. GPER was found to be localized at the cell membrane, endoplasmic reticulum and, residually, in the nucleus of prostate cells and this subcellular localization was altered by the GPER-specific agonist G1. G1-treatment showed a multiplicity of beneficial effects in PCa, targeting a panoply of cancer hallmarks. It reduced PCa cells viability and proliferation, arresting cell cycle and increasing apoptosis. It was also able to reduced migration and invasion, concomitantly with epithelial-mesenchymal transition (EMT) attenuation, depending on the cell line. Moreover, GPER activation by G1 increased energy metabolism and oxidative stress in PCa cells. Finally, it was evaluated how natural compounds, such as diosgenin, can influence the behavior of PCa in the interplay with GPER. Diosgenin diminished the viability of androgen-responsive and CRPC cells, without affecting the viability of non-neoplastic PNT1A cells. Moreover, the compound induced apoptosis and modulated glycolytic metabolism of PCa cells. The effects of diosgenin were enhanced when combined with G1, supported by the ability of diosgenin to augment the expression levels of GPER. Furthermore, *GPER* knockdown by a siRNA approach abrogated diosgenin effects inducing apoptosis of PCa cells, which implicates this receptor in the diosgenin mechanism of action. Globally, the findings of this thesis support the protective role of estrogens in PCa, disclosing new molecular targets and cellular processes. Moreover, it provides valuable information to find new therapeutic options, namely, estrogen-like, to deaccelerate the progression of disease and improve the survival of PCa patients.

Keywords

Prostate cancer (PCa), estrogens, G protein-coupled estrogen receptor (GPER), diosgenin

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List de Abbreviations

2F-E₂	2-fluoroestradiol
2-ME₂	2-methoxyestradiol
2-OHE₂	2-hydroxyestradiol
3α-diol	5 α -androstane-3 α ,17 β -diol
3β-adiol	5 α -androstane-3 β ,17 β -diol
4-OHE₂	4-hydroxyestradiol
7β-OH-EpiA	7 β -hydroxy-epiandrosterone
ACC	acetyl coenzyme A carboxylase
ADT	androgen deprivation therapy
AFS	anterior fibromuscular stroma
ALT	alanine aminotransferase
Apaf-1	apoptotic protease activating factor-1
APVE₂	17 α -20Z-21-[(4-amino)phenyl]-19-norpregna-1,3,5(10),20-tetraene-3,17 β -diol
AR	androgen receptor
ASCT2	alanine, serine, cysteine transporter 2
BDE-47	2,20,4,40-tetrabromodiphenyl ether
BID	bisphenol A
BPH	benign prostatic hyperplasia
C4PY	calix[4]pyrrole derivative [meso-octamethylcalix[4]pyrrole
CAF	cancer associated fibroblast
CD36	cluster of differentiation 36
CDK	cyclin-dependent kinase
ChREBP	carbohydrate response element binding protein
CK18/8	Cytokeratin-18/8
COMT	catechol-O-methyltransferase
COX2	cyclooxygenase 2
CPT1A	carnitine palmitoyltransferase 1A
CRPC	castrate-resistant prostate cancer
CS-FBS	charcoal-stripped FBS
CTGF	connective-tissue growth factor
CXCR4	C-X-C chemokine receptor type 4
CYP11A1	cholesterol side-chain cleavage enzyme

CYP19A1	aromatase
CYR61	cysteine-rich 61
CZ	central zone
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
DES	diethylstilbestrol
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
DHT	5- α -dihydrotestosterone
DISC	death-inducing signaling complex
DPN	diarylpropionitrile
DRE	digital rectal examination
E₂	17 β -estradiol
E₂-3,4-Q	estrogen-3,4-quinones
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Egr-1	early growth response 1
EMT	epithelial-mesenchymal transition
ENL	metabolite enterolactone
EPCA	early prostate cancer antigen
EPHB2	ephrin B2
ERK	extracellular-signal-regulated kinase
ERα	estrogen receptor α
ERβ	estrogen receptor β
EZH2	enhancer of zeste homolog gene 2
F-1,6-P	fructose 1,6-biphosphate
F-6-P	fructose-6-phosphate
FA	fatty acids
FADD	Fas associated death domain
FASN	fatty acid synthase
FBS	fetal bovine serum
FDA	Food and Drug Administration
FLIP	Fas-associated death domain interleukin-1 converting enzyme like inhibitory protein
FSH	follicle-stimulating hormone
G1	GPR30-specific compound 1
G-6-P	glucose 6-phosphate

Gckr	glucokinase
GLAST	glutamate aspartate transporter
GLS	glutaminase
GLT-1	glutamate transporter-1
GLUT	glucose transporter
GNNK	Gly-Asn-Asn-Lys
GnRH	gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
GP ER	G protein-coupled estrogen receptor
GPT	glutamate pyruvate transaminase
GPx	glutathione peroxidase
Grb2	growth-factor receptor-bound protein-2
GS2	glycogen synthase
GSH	glutathione
GSTP1	glutathione S-transferase P1
Gas	Gα-subunit
HGPIN	high-grade prostatic intraepithelial neoplasia
HIF1	hypoxia inducible factor 1
HK	hexokinase
HSD17β1/2	17β-hydroxysteroid dehydrogenase
IAP	inhibitor of apoptosis family of proteins
ICAM-1	intercellular cell adhesion molecule-1
IGF-1	insulin-like growth-factor-1
IGF-1R	insulin-like growth factor-1-receptor
IL1R1	IL1 receptor 1
IL-8	interleukin-8
Ins1	proinsulin 1
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
K-1	2-[piperidinoethoxyphenyl]-3-[4-hydroxyphenyl]-2H-benzo(b)pyran
KGF	keratinocyte growth factor
LC-MS/MS	liquid chromatography tandem mass spectrometry
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone

LXR	liver X receptor
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MCT	monocarboxylate transporter
MDH	malate dehydrogenase
MEC	murine endothelial cell line
MIBE	ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyl)-1-methyl-1H-indol-3-yl] but-2-enoate
MIG	monokine induced by γ interferon
MMP	matrix metalloproteinase
MRI	magnetic resonance spectroscopic imaging
MRP1	multidrug resistance-related protein
mSCF	membrane-bound SCF
mTOR	mammalian target of rapamycin
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHERF1	Na ⁺ /H ⁺ exchanger regulatory factor
OXPHOS	oxidative phosphorylation
p70S6K	p70 S6 kinase
p-AKT	phosphorylated Akt
PAP	prostatic acid phosphatase
PARP-1	caspase-3 and poly (ADP-ribose) polymerase 1
PBS	phosphate buffer saline
PCa	prostate cancer
PCB	polychlorinated biphenyl
p-cMyc	phosphorylated c-Myc
PCNA	proliferating cell nuclear antigen
PDGFA	platelet-derived growth factor subunit A
Pdx1	duodenal homeobox 1
p-ERK	phosphorylated ERK
PET	positron emission tomography
PFA	paraformaldehyde
PFK1	phosphofructokinase-1
PI	propidium iodide
PI3-K	phosphatidylinositol 3-kinase
PIA	proliferative inflammatory atrophy
PIM1	proviral integration site for Moloney murine leukemia virus-1
PIN	prostatic intraepithelial neoplasia

PKA	protein kinase A
PKC	protein kinases C
PLC-γ	phospholipase-C
p-MAPK	phosphorylated MAPK
pNA	p-nitro-aniline
PPT	propylpyrazole triol
PR	progesterone receptor
PSA	prostate-specific antigen
PSCA	prostate stem cell antigen
PSGR	prostate-specific G-protein coupled receptor
PSMA	prostate-specific membrane antigen
PTEN	phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
PZ	peripheral zone
qPCR	quantitative polymerase chain reaction
RARβ2	retinoic acid receptor β
RASSF1A	Ras association domain family 1
Rb	retinoblastoma protein
RIA	radioimmunoassay
RIPA	radioimmunoprecipitation assay buffer
ROS	reactive oxygen species
S1P	sphingosine 1 phosphate
SCF	stem cell factor
SDG	secoisolariciresinol diglucoside
SEM	standard error of the mean
SERM	selective estrogen receptor modulator
s-KIT	soluble c-KIT
SOD	superoxide dismutase
SOX4	sex determining region Y-box 4
SphK1	sphingosine kinase 1
SREBP1c	sterol regulatory element-binding protein 1c
sSCF	soluble SCF
STAT	signal transducers and activators of transcription
SV-40 Tag	simian virus-40 T-antigen
TAP	transit amplifying population
TCA	tricarboxylic acid
TDP	4,40-thiodiphenol

TET1	ten-eleven translocation 1
TGF-β	transforming growth factor beta
TMA	tissue microarray
TMPRSS2	type II transmembrane serine protease
TNBCs	triple-negative breast cancers
tr-KIT	truncated form of c-KIT
TRUS	transrectal ultrasound
TSG-6	TNF- α -stimulated gene 6
TUNEL	terminal deoxynucleotidyl transferase dUTP nick-end labeling
TZ	transition zone
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VGSC	voltage-gated sodium channel
WB	Western Blot
XIAP	X-linked inhibitor of apoptosis protein
αE₂	17 α -estradiol

Chapter 1

General overview of prostate cancer

1. General overview of prostate cancer

1.1. Onset and progression of disease: from cellular and molecular features to physiology

The prostate is the largest accessory gland of the male reproductive tract, with a walnut shape and size, approximately, 4 cm long and 2 cm wide. It is located dorsally to the symphysis pubis, anterior to the rectum, and at the base of the urinary bladder, surrounding the first part of urethra, and the two ejaculatory ducts [1, 2]. The normal function of prostate has great importance for male reproductive physiology. Prostatic secretions constitute, approximately, 30 % of the semen [3] and are related with its gelation, coagulation, and liquefaction, and some of the secreted proteins are involved in the coating and uncoating of spermatozoa and in interactions with cervical mucus [4]. Moreover, the prostatic fluid has alkaline properties that help to protect the spermatozoa from the acidic vas deferens fluid and the acidic environment of the female reproductive system [3].

More than hundred years ago, the anatomic structure of the prostate gland was, firstly divided in lobes, including an anterior lobe, a posterior lobe, two lateral lobes and a middle lobe [5]. Currently, the accepted anatomical division of prostate comprises four zones: the central zone (CZ), the peripheral zone (PZ), the transition zone (TZ) and the anterior fibromuscular stroma (AFS) (Fig. 1.1). This zonal differentiation is based on their different embryologic origins, histology, anatomic landmarks, biological functions, and is also related with pathologic disorders [1, 6]. The PZ makes up the bulk of the prostate, comprising about 70 % of the gland mass. Around 70 % of all prostate cancer (PCa) cases arise in this region. The TZ, mainly fibromuscular, consists of about only 5 % of the glandular tissue and surrounds the proximal prostatic urethra. It represents the portion of the gland that suffers a hypertrophy development with aging originating the benign prostatic hyperplasia (BPH), a pathology of the fibromuscular stroma. The CZ correspond to 25 % of the prostate and is located at the base of the prostate between PZ and TZ, surrounding the ejaculatory ducts (Fig. 1.1). This zone has the lowest incidence of PCa. Moreover, the AFS is composed by fibromuscular tissue contiguous with the bladder, without glandular tissue [1, 6-8].

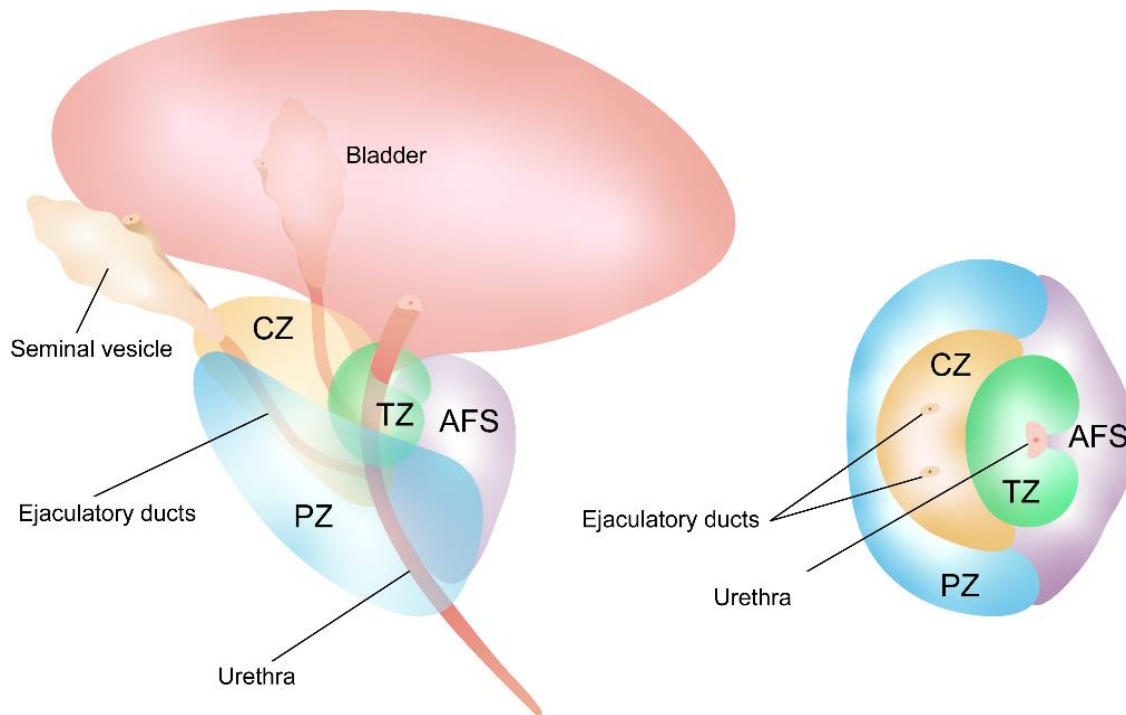


Figure 1.1. Schematic representation of the prostate anatomy divided by zones. Prostate is composed by four zones: the anterior fibromuscular stroma, the peripheral zone, the central zone, and the transition zone. The peripheral zone is the major zone of the glandular prostate. The central zone represents around 25% of the prostate mass and surrounds the ejaculatory ducts. The transition zone represents 5% of the glandular prostate mass and surrounds the proximal urethra. The anterior zone is constituted by fibromuscular stroma. It represents a third of the total volume of the prostate and does not contain glands.

Looking deeper into the histofunctional organization of prostate glandular component, it is composed of ducts and acini, housing different types of cells, the secretory or luminal cells, basal cells, stem cells and the neuroendocrine cells [9, 10]. The majority of epithelial cells are columnar luminal cells, responsible for production of prostatic secretions. These secretory cells are separated from the basement membrane and stroma by a layer of basal cells, which seems to be involved in the control of the molecular trafficking between luminal cells and the extracellular space. Moreover, this basal layer has been shown to contain a reservoir of stem cells with the capacity to differentiate into the columnar secretory cells [11-15].

The neuroendocrine cells, located among epithelial cells appear to be involved in the regulation of secretory action and prostate cell growth [11, 13, 16].

The non-glandular components of the prostate include the pre-prostatic sphincter, the capsule, supplying blood vessels and nerves and the fibromuscular stroma. The stroma houses a population of fibroblasts, smooth muscle cells that produce growth factors to support the development and function of the epithelial cells, and other minor inflammatory cells, nerve cells and endothelial cells [11, 17]. Also, the presence of

interstitial Cajal cells was detected in prostate, which are morphologically different from the smooth muscle cells [18, 19].

The prostate gland develops from epithelial invaginations of the posterior urogenital sinus, during the third month of gestation. At birth, prostate weighs a few grams and it undergoes a phase of exponential growth at puberty, corresponding to the rise in serum testosterone. Then prostate growth stabilizes until the end of the fourth decade of life, when growth is restarted [13]. At this phase, the balance between cell proliferation and apoptosis is disrupted, and BPH emerges [20]. This is a condition characterized by the benign growth of the prostate gland to a larger size that can be larger than 150 g [21].

Besides the deregulation of growth, aged prostate epithelium damaged by inflammation, infection, carcinogens, and cell mutations, among other factors, can undergo malignant transformation. The damage of the prostatic epithelium by inflammation, cause lesions with atrophic morphology, nonetheless the cells remain able to proliferate. This condition is named proliferative inflammatory atrophy (PIA) [22]. Moreover, morphological transitions can lead to histological lesions causing prostatic intraepithelial neoplasia (PIN) lesions, which are characterized by dysplasia of prostate luminal epithelial cells and loss of distinct basal and secretory layers [23]. The high-grade PIN (HGPIN) seems to be a precursor lesion of PCa [24]. It is related with tumor stage, Gleason score and PCa risk [25-28]. In fact, the majority of the alterations that lead to PCa development take place during the transition of benign epithelium to HGPIN [29]. In the establishment of PCa, the gland structures become progressively less organized with smaller ducts and ultimately the gland may lose these structures entirely. With further growth, the tumors invade surrounding tissues and metastasize to the lymph nodes, bladder, and bone [30]. Proliferation, neovascularization and extravasation at the primary site are events involved in metastasis [31].

Also, some evidence has been showing that PCa can slowly arise from proliferating stem cells or the transit amplifying population (TAP) from the basal compartment [32]. Stem cells are able to differentiate into luminal cells in response to androgens. Androgen stimulation promotes cells differentiation by inducing the expression of luminal cells marker, such as cytokeratin-18 (CK18), androgen receptor (AR), and prostate-specific antigen (PSA), whereas decreasing the expression of $\alpha 2\beta 1$ integrin, a molecule implicated in the maintenance of the immature basal cell phenotype [33]. Nevertheless, the controversy whether prostate tumors arise from basal cells or from the luminal cells persists [34-36].

In addition to cellular and structural changes, many molecular changes are involved in PCa initiation and progression. The expression profile of genes related with

proliferation, apoptosis, and stress response is altered due to a set of genetic events that include epigenetic changes, chromosomal alterations, somatic mutations and alternative splicing [37-39].

One of the most common genetic alterations in PCa is the fusion between two genes, the type II transmembrane serine protease (TMPRSS2) gene and the ETS transcription factor genes, ERG or ETV1. TMPRSS2 gene codifies a membrane-bound serine protease, regulated by androgens and involved in epithelial cell growth, which is overexpressed in PCa. This gene, frequently, suffers chromosomal rearrangement leading to a fusion with ETS transcription factor genes, which are involved in multiple processes, including cell proliferation and cancer cell invasion [40-42]. Thus, TMPRSS2-ERG and TMPRSS2-ETV are both fusion oncogenes that appear to be related to the development of PCa [40, 43].

The gain-of-function mutations and amplification of oncogenes and the loss-of-function mutations and deletion of tumor suppressor genes also are commonly found in PCa cases [44]. The proto-oncogene *MYC*, a nuclear transcription factor regulating cellular processes such as proliferation, apoptosis and metabolism, is amplified in PCa and linked with poorer prognosis [45-48]. The phosphoinositide-3-kinase (PI3K) pathway, well characterized in PCa, triggers cell proliferation through the activation of Akt and is switched off by the tumor-suppressor phosphatase and tensin homolog (PTEN) [49, 50]. In PCa, the overactivation of PI3K signaling results from PTEN deletion [51-54].

PCa is by nature a hormone-dependent cancer highly regulated at the initial phases by androgens, with these hormones and the AR playing a crucial role promoting cell proliferation and survival [55]. The mechanisms underpinning androgens actions in prostate carcinogenesis are diverse and include gene translocation [56, 57]; regulation of growth factors and their receptors expression [58-60]; cross-talk with the downstream effectors of growth factor signaling, such as PI3K/Akt pathway [61]; increased cyclin expression and promotion of active cyclin/cyclin-dependent kinase (CDK) complexes assembly [62, 63]; stimulation of reactive oxygen species production to levels that potentiate cell growth [64, 65]; induction of anti-apoptotic proteins expression [66]; and suppression of TNFR family (TNF- α /Fas)-induced apoptosis [67, 68].

In advanced phases of disease, PCa cells develop resistance to androgens and became capable of survive and proliferate even if the circulating androgens levels are maintained low. This stage corresponds to the castrate-resistant PCa (CRPC) [69-71]. The underlying mechanisms involved in the ability of prostate tumor cells to grow in the absence of androgens (or very low) are complex and include several AR gene and protein alterations, namely, somatic mutations, mainly, gain-of-function mutations; active AR splice variants, generated by gene splicing or genomic rearrangement; AR

overexpression/amplification; and posttranslational modifications of AR affecting receptor activity, structure, and stability, including phosphorylation, ubiquitylation, acetylation and methylation [72-81]. Also, other AR independent pathways have been implicated in the emergence of CRPC. Overall, the following pathways are recognized as the main routes contributing to the establishment of CRPC: i) the *hypersensitive pathway*, in which PCa cells still depend on AR and androgens actions, however, they display increased AR sensitivity, being able to respond to very low circulating levels of these hormones, in consequence of increased AR signaling by gene amplification, changes on the expression of AR-coregulators, and even by the enhanced activity of steroidogenic enzymes and local production of androgens [81, 82]; ii) the *promiscuous pathway* that represents the way by which PCa cells acquire the ability to circumvent the growth dependence on androgens, namely, through AR mutations that alter the specificity of ligand binding, with AR being activated by other steroid ligands, or that change the interaction of AR with co-regulators [74, 81, 82]; iii) the *outlaw pathway* that depends on the AR activation by ligand-independent mechanisms. In this case, AR can be activated by growth factors, like insulin-like growth-factor-1 (IGF-1), keratinocyte growth factor (KGF) and epidermal growth factor (EGF). Alternatively, it includes the connection with receptor tyrosine kinase, namely, by HER-2/neu (also known as ERBB2), a member of the EGF-receptor family of receptor tyrosine kinases that can act through the mitogen-activated protein kinase (MAPK) pathway and then activate AR [74, 81]; iv) the *bypass pathway*, based on the ability of PCa cells to survive independently of AR activation, by mechanisms such as neuroendocrine differentiation, inactivation of PTEN suppressor gene or upregulation of Bcl-2, which protects cells from apoptosis [81, 83]; v) the *lurker cell pathway* assumes the existence of a small population of PCa stem cells or progenitor cells that are androgen-independent and do not express AR, being positively selected and able to proliferate in CRPC [82, 83].

The reprogramming of cellular metabolism is also a mark in the onset and progression of PCa [84, 85]. In fact, since 2011, the reprogramming of energy metabolism was added to the list of cancer hallmarks, joining to the other classic features of tumor cells, such as, sustained proliferative activity even in the absence of growth stimuli, evading growth suppression, resistance to cell death, replicative immortality, induction of angiogenesis, migration, invasion and metastasis, and the ability to escape to immune destruction [86].

Normal prostate metabolism is specially focused on supporting sperm function and quality, which is partially achieved by the intense production and secretion of citrate [87, 88]. Prostate secretory epithelial cells use oxaloacetate and acetyl-CoA as carbon sources for the production of citrate [89] that unlike other cell types do not enter the tricarboxylic

acid (TCA) cycle for oxidation and energy production. Alternatively, citrate in the cytosol can be cleaved by citrate lyase to provide Acetyl-CoA for fatty acid synthesis. Nevertheless, prostatic secretory cells accumulate citrate that is secreted as the main component of prostatic fluid.

The lack of citrate oxidation in the TCA cycle is associated with the high levels of zinc found in prostate cells [90], which inhibit the activity of mitochondrial enzyme aconitase, responsible for catalyzing the conversion of citrate into isocitrate [91-94]. Thus, non-cancer prostate cells have what is known as a truncated TCA cycle [89]. Contrastingly, PCa cells display low levels of zinc [92, 94, 95], which allows aconitase activity, citrate oxidation and the reactivation of TCA cycle.

The accumulation of oncogenic mutations and the pressure of unfavorable tumor microenvironment with a heterogeneous supply of nutrients and oxygen [96], represent a driving force for reprogramming of PCa cells metabolism in order to maintain the energy supply. PCa cells have been shown to display alterations of glucose, amino acids, and lipids metabolism [97, 98].

In non-cancer cells, pyruvate from glycolysis is oxidized to acetyl-CoA and canalized to the TCA cycle, with mitochondrial oxidative phosphorylation (OXPHOS) being the primary and preferential pathway to generate ATP [99, 100]. However, cancer cells use pyruvate to produce lactate instead of its oxidation in mitochondria, even in aerobic conditions. This was the first evidence of reprogramming metabolism by cancer cells, presented by Otto Warburg [101, 102]. Despite OXPHOS is more efficient energetically, producing a high amount of ATP, glycolysis followed by lactate production is a rapid way to supply cancer cells with the energy substrates required to sustain their high proliferative rate [103-106]. Also, concerning glycolytic metabolism, PCa displays unique features. In contrast to other types of cancer that present a higher glycolytic profile early in the transformation process, PCa cells switch to the Warburg effect only in more advanced stages of disease [85, 107].

Glycolysis requires the entrance of glucose into the cell, which occurs through the activity of membrane glucose transporters (GLUTs) family members [108]. In the cell, glucose follows the glycolytic flux of several reactions being converted into pyruvate [109]. First glucose is converted to glucose 6-phosphate (G-6-P) by the enzyme hexokinase (HK) [109]. The phosphorylation of fructose-6-phosphate (F-6-P) into fructose 1,6-biphosphate (F-1,6-P) by the enzyme phosphofructokinase-1 (PFK1) is a main limiting step of glycolysis [109]. The glycolytic profile of advanced stages of PCa is ensured by increased levels of GLUTs, to provide cells with large amounts of glucose [110-115], as well as by augmented expression of HK and PFK1 [85, 116]. In glycolytic cancer cells, the final product of glycolysis, pyruvate, is driven to the anaerobic pathway being metabolized to lactate by the

activity of lactate dehydrogenase (LDH). The lactate produced is then exported to the extracellular space, a process mediated by the monocarboxylate transporters (MCTs) family members, mainly, by the MCT4 isoform [117, 118]. PCa cells produced and export lactate by the increased activity of LDH and enhanced expression of MCTs, namely, MCT1 and MCT4 [85, 119].

Alternatively to the metabolization through glycolysis, glucose can be converted into glycogen through the action of glycogen synthase [120]. This process is known as glycogenesis and represents a way of energy storage for the cell needs. Despite glycogen synthesis is mainly associated with liver and skeletal muscle cells [120], this characteristic can be found in several types of cancer cells, such as breast, kidney, uterus, bladder, ovary, skin and brain, particularly in cells undergoing neoplastic transformation [121]. Data regarding glycogen metabolism in PCa cells are scarce, but an interesting study found out that glycogen accumulation is associated with the androgen responsiveness [122].

Concerning amino acids, glutamine has been shown to have a role in cancer, including PCa cells. It enters the cells by the activity of alanine, serine, cysteine transporter 2 (ASCT2), and is converted into glutamate by the activity of mitochondrial enzyme glutaminase (GLS) [123]. Glutamine metabolism also originates α -ketoglutarate, an intermediary of TCA cycle [103, 124], and, in hypoxic conditions, glutamine can generate citrate for lipid synthesis [125]. The role of glutaminolysis has been implicated in the onset and progression of PCa, with increased expression and activity of intermediates and enzymes involved in that pathway being linked with enhanced proliferative ability [126]. Alanine is another amino acid that can be used by cells as an energy substrate. Alanine aminotransferase (ALT) is the enzyme responsible for the reversible conversion of alanine and α -ketoglutarate into pyruvate and glutamate, metabolites that can enter other metabolic routes [127]. Alanine levels are increased in PCa cells, suggesting the involvement of its metabolism in this type of cancer [128].

Lipid metabolism also has emerged as an important source of energy for cancer cells [129]. Exogenous fatty acids (FA) can enter the cell through membrane transporters, namely, the cluster of differentiation 36 (CD36), and then oxidized at the mitochondria [130]. FA β -oxidation requires the transport to the mitochondria, which is mediated by carnitine palmitoyltransferase (CPT1A) that converts the long chain FA into acylcarnitine derivatives [131]. In contrast to non-malignant cells, cancer cells have enhanced capacity to synthesize *de novo* FA. This process depends on the activity of acetyl coenzyme A carboxylase (ACC) that catalyzes the conversion of acetyl coenzyme A to malonyl-coenzyme A [132]. These products are substrates for FA synthesis through the activity of fatty acid synthase (FASN) [133]. Increased *de novo* lipid synthesis is an early event in PCa in consequence of up-regulated expression and increased activity of lipogenic enzymes,

namely, ACC and FASN [134-136]. Moreover, these alterations were correlate with worse prognosis and poor survival [84]. Furthermore, CD36 and CPT1A also seem to be upregulated in PCa cases [137-139].

Finally, PCa is highly influenced by the microenvironment conditions and nutrient availability, changing its metabolic activity in order to sustain cell division and tumor growth. The metabolic alterations underlying PCa also have been shown to be highly controlled by androgens [140] and regulated by several intracellular signal pathways including PI3K, MAPK and AMPK pathways, hypoxia inducible factor 1 (HIF1) and Myc and p53 signaling [141-151].

1.2. Brief epidemiological notes and risk factors

PCa is the second most frequent type of cancer and the fifth leading cause of death of cancer among men [152, 153]. Almost 1.4 million new cases and 375 000 deaths by PCa were estimated by the GLOBALCAN 2020 worldwide [152, 153]. In the United States, the American Cancer Society's estimated about 288 300 new cases and 34 700 deaths by PCa for 2023 [154, 155]. In Europe, PCa is the most frequently diagnosed cancer among men, representing 22.2% of all new cancers, with around 473 340 new cases and 108 090 deaths [156]. PCa is the most common male cancer in Portugal, with 6 760 new cases and 1 920 deaths estimated in 2020 [156]. The incidence and mortality of PCa differ geographically, and many factors contribute to these variances [157].

Among the panoply of factors that can trigger the development of the disease, the best characterized are aging, ethnicity, and genetic and familial factors [158-161]. Other factors positively associated with PCa include obesity, diet, physical inactivity, hyperglycemia, inflammation, infections, and environmental exposure to chemicals and hormones [162-169].

Aging is the main risk factor for PCa development [159]. PCa is more frequently diagnosed in older men, and these are more likely to die from the disease than younger men [170]. The risk increases, especially, after 50 years [171], and it is frequently associated with the prostate growth after the 40-50 decade of men's life [3, 20]. The slowly increase in prostate weight frequently leads to the onset of BPH that seems to be associated with increased risk and mortality of PCa [172].

Ethnicity is also an important risk factor for PCa. African-American men present a higher incidence of PCa than other groups [173-175]. These differences have been associated with biological factors, like the genetic predisposition to chromosome 8q24 variants [176-178] or the high rate of variations in tumor suppressor or apoptosis

regulatory genes [179, 180]. Black men show higher levels of PSA compared with White men [181]. Also, old evidence has been postulated that African men have higher androgenic levels, leading to a higher incidence of PCa [182].

A significant percentage of men with PCa has a family history. Thus, heredity has an important role in PCa development [183, 184]. Inherited genetic background is associated with increased risk, contributing to around 5 % of PCa cases [185]. A panoply of genes, including *RNASEL*, *ELAC2*, *MSR1*, *OGG1*, *CHEK2*, *BRCA2*, *PON1* and *GDF15* have been identified as conferring susceptibility to PCa [186]. Some of them encode proteins with important roles in DNA repair, cell cycle regulation, cell defense against inflammation and oxidative stress [187]. Also, the deletion of tumor suppressor genes *PTEN* and *TP53* and the activation of oncogene *MYC* are linked with PCa development and progression [45-48, 51-54]. Besides gene mutations, the gene fusion between *TMPRSS2* and *ETS* family members (*TMPRSS2-ERG* and *TMPRSS2-ETV*) increase the risk of PCa [40, 43], as well as the polymorphisms in 8q24, 17q12 and 17q24.3, 2p15, 10q11, 11q13 and Xp11 chromosomes [188, 189]. Recently, epigenetic changes, like the CpG islands hypermethylation in multiple genes sites have been associated with progression of PCa [190, 191].

Obesity has been linked with PCa, especially, with the aggressive stages of disease [192, 193]. Accordingly, it has been reported that dietary factors are associated with higher PCa risk, such as the consumption of red meat, fat and dairy products that contain saturated animal fat and calcium [194-199]. The excessive alcohol consumption and cigarette smoking also constitute risk factors for PCa development, as well as the physical inactivity, despite some controversy [200-202].

Hyperglycemia also was reported as a factor for higher risk of more aggressive or advanced PCa [203-206]. However, other evidence reported a protective role of this condition in PCa [207, 208]. Serum glucose is directly regulated by insulin, and higher glucose levels induce insulin secretion. Higher levels of insulin were found to be associated with a high risk of PCa [209, 210].

Inflammation also constitutes an important risk factor for PCA associated with high-grade or aggressive prostate tumors [211, 212]. Chronic inflammation leads to PIA [22] that can be on the basis of PIN lesions development [23], a well-known precursor of PCa [24].

Other risk factors that should be considered include sexually transmitted diseases [213], exposure to environmental carcinogens, such as herbicides, pesticides and bisphenol A [214-216].

The complexity of hormones action also has been unveiled to play a role in the risk of developing PCa. Androgens are well known to stimulate PCa cells growth and survival

[217]. Huggins and Hodges were the first to postulate that androgens promote prostate carcinogenesis [70, 218], and the pioneers of the basis for developing the hormonal therapies. The controversy emerges with the sex steroids estrogens. A class of female hormones also synthesized in men that are recognized by their mitogenic activity. However, the available published papers on the estrogens role in PCa development follow in one of two categories: i) carcinogens [219-225] or ii) protective factors [226-231].

1.3. Diagnosis and therapy

The early detection of PCa is quite important to prevent the progression of the disease for advanced stages. However, PCa remains asymptomatic for a long time, which conditions the early detection. When symptoms become evident, it includes urinary symptoms like hematospermia and hematuria, in localized PCa, and rectal obstruction, bone pain, and other systemic features of malignancy, in advanced disease [159].

Historically, the human prostatic acid phosphatase (PAP) was the first biomarker used for PCa screening. However, it showed very low sensitivity [232], being replaced by the PSA that, despite its limitations, remains being used as a diagnosis complement [233, 234]. PSA, also called kallikrein-3, is a glycoprotein produced by the prostate gland with functions in serum liquefaction [235]. In a normal situation, this protein does not cross the epithelial basement membrane and is not found in the bloodstream. However, in conditions of prostate damage, like chronic inflammation, BPH and cancer, PSA escapes back into the prostatic stroma and can be detected in the bloodstream [236, 237].

The measurement of serum PSA and the physical examination by digital rectal examination (DRE) are the first line methods for PCa screening [238-241]. Serum PSA levels higher than 4.0 ng/ml (traditional threshold level) were considered indicative of PCa [238]. However, PSA screening test presents low specificity, which leads to false negatives or false positive cases [238, 242-245]. There are cases of low PSA levels in PCa patients [245, 246] as well as the opposite, high levels in men without PCa, since PSA is also influenced by situations of prostatitis, urinary tract infections and BPH [247]. In fact, this led to overdiagnose of PCa (366). Total PSA includes distinct molecular forms such as the free PSA and complexed PSA, mainly bound to the protease inhibitor α_1 -antichymotrypsin [248]. Thus, the measurement of free vs total PSA ratio is a way to, partially, surpass the problem, distinguishing between PCa and BPH cases, for example [238, 249]. Regarding DRE, despite its given valuable information, some evidence suggests that the DRE may not significantly reduce mortality, and even result in a high number of false-positives leading to unnecessary invasive diagnostic tests that can

precipitate pain, erectile dysfunction, and urinary incontinence, as well as overdiagnosis and overtreatment of PCa [250-252].

Considering the existent limitations in PCa screening, the search for the perfect biomarker has been a research focus all over the world. The ideal biomarker should present high sensibility and sensitivity and be easily measured [158]. Among the biomarkers investigated in body fluids or tissue samples can be pointed the circulating tumor cells; serum proteins like filamin A, filamin B, and keratin-19; PCa antigen 3; prostate-specific G-protein coupled receptor (PSGR); prostate-specific membrane antigen (PSMA), prostate stem cell antigen (PSCA); early prostate cancer antigen (EPCA), enhancer of zeste homolog gene 2 (EZH2), the urokinase plasminogen activator (uPA); TMPRSS2-ERG gene fusions; exosomes; bone turnover markers; tumor micro-environment associated biomarkers and also metabolism-related molecules [253-255]. Some of these biomarkers are now entering into the clinical setting were approved by Food and Drug Administration (FDA), and the methods for their detection started being commercialized.

The definitive diagnosis of PCa is generally confirmed by means of imaging techniques like transrectal ultrasound (TRUS) and guided systematic biopsy with further histological examination [158, 159, 256]. Biopsy allows to determine the Gleason score, that measures the aggressiveness of cancer, predicting prognosis and guiding treatment [257]. However, the heterogeneity of biopsy samples could not be representative and lead to false results, and the sensitivity and specificity of greyscale TRUS in the detection of PCa is low [258, 259].

More recently, other techniques have been used to complement and improve PCa diagnosis like the positron emission tomography (PET) and magnetic resonance spectroscopic imaging (MRI) that are non-invasive imaging technologies based on the detection of metabolic biomarkers [260]. PET is useful by providing specificity to PCa diagnostic, mainly, in localized high-grade tumors, since the presence of abundant metabolites, like glucose, is required [260, 261]. The fact that PCa is less glycolytic than the other types of cancer hampers the possibility of using FDG-PET scan for the detection of tumor in the early stages [262, 263]. Nevertheless, the increased *de novo* lipid synthesis in the early stages of PCa allows the successful use of lipid precursors, like acetate and choline, as alternative tracers for PET imaging [264, 265]. MRI allows to identify local lesions within the prostate and guide the biopsy, due to the great anatomic information and resolution of the different prostate structures. MRI is also useful for detection of recurrent or residual disease after radiation therapy or surgery and indication of early metastases [261].

The therapeutic options for PCa depend on the state of the disease and should be adapted to local or metastatic PCa. For men with localized PCa, the available therapeutic options include radical prostatectomy, radiation (external-beam or brachytherapy), focal therapies (e.g. high intensity focused ultrasound, cryotherapy) and androgen deprivation therapy (ADT) [243]. Radical prostatectomy is the typical method, which consists of complete removal of the prostate gland [158]. Both surgery and radiation continue to be effective treatments for men with PSA levels greater than 10 ng/mL and those with significant DRE [241], and are frequently used in combination with hormone therapy or chemotherapy [266].

In the case of patients with small tumors that have not spread beyond the gland it is recommended the close monitoring without application of aggressive treatments, a strategy named active surveillance or watchful waiting [243]. The first involves several tests to monitor the progression with the intent to cure those who develop significant disease; the second of watchful waiting consists of treating symptoms with palliative intent [241].

When the PCa becomes metastatic and culminating in CRPC, the therapeutic options include chemotherapy, radiotherapy and hormone therapy [267]. The most well-known chemotherapeutic agents are docetaxel and cabazitaxel, which seem to be effective in improving the life-quality, but have poor results in survival outcomes [268, 269]. Radium 223 is an example used in radiotherapy for PCa, which shows the capacity to improve survival and reduce bone pain with relative lack of toxicity among CRPC patients [270]. Recently, other promising approaches have been gaining importance in PCa treatment, namely, the immunotherapy and the cancer stem cells targeted therapy [271, 272]. The immunotherapeutic approaches include cancer vaccines and immunomodulators [273]. In fact, the FDA approvals for the immunotherapeutic agents sipuleucel-T and ipilimumab and pembrolizumab started a new perspective in immuno-oncology [271].

ADT is the standard treatment for PCa since this hormone therapy is used both in localized and metastatic PCa [274-276]. ADT target the inhibition of testicular testosterone or its actions to suppress PCa progression, lowering androgens levels or blocking its effects [277]. The first approach of ADT reported the surgical castration, namely, the bilateral orchiectomy and chemical castration with hormone-releasing hormone (LHRH) agonist/antagonists [218, 278, 279]. In many cases, the progression of the disease to CRPC is inevitable, and the ADT therapies fail. At this aggressive stage, PCa cells develop resistance to androgens and continue to proliferate even if androgens levels are low, which is achieved through the mechanisms described above (81). In these situations, administration of antiandrogens like flutamide, bicalutamide, nilutamide,

enzalutamide, apalutamide and darolutamide is a second line of ADT, since they bind competitively the AR, blocking AR signaling [280-282]. CRPC cells have the ability to produce androgens, and for this reason, another therapeutic approach is the prevention of androgen biosynthesis. The main androgen biosynthesis inhibitor is the abiraterone acetate [283]. It selectively inhibits the actions of P450 C17 enzymes, namely, 20-lyase and 17 α -hydroxylase, which are essential for the synthesis of androgen [283]. Moreover, another important question is that even with castration, androgens can also be synthesized in the adrenal in the form of dehydroepiandrosterone (DHEA). To target these ones, adrenal androgen inhibitors, such a ketoconazole, corticosteroids and aminoglutethimide are sometimes adopted [280].

Estrogens, like diethylstilbestrol or estradiol, also were used as hormonal therapy in PCa. In fact, this was the first pillar of hormone therapy, but the adverse side effects, namely, cardiovascular toxicity, due to oral administration, lead to the discredit of their use [284]. However, in the last years, a renewed interest in this approach to achieving androgen deprivation has been gaining consistency with the adoption of alternative routes of administration [285, 286].

In summary, the peculiarities of prostate tumors require robust therapeutic options to improve the survival of PCa patients and deaccelerate the progression of disease to CRPC. Nevertheless, the existent options are not always effective, and investigation to better understand the molecular and cellular mechanisms involved in PCa continues to be a challenge in finding effective therapies.

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Chapter 2

Estrogens actions in prostate cancer: an update with a focus on phytoestrogens

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2. Estrogens actions in prostate cancer: an update with a focus on phytoestrogens

Abstract

Estrogens are classically known as female hormones. However, they also play an important role in the male reproductive system. In the middle of the past century, Huggins and Hodges findings established prostate cancer (PCa) as a hormone-sensitive cancer, which provided the basis for using estrogens in PCa therapy. However, despite the experimental evidence describing a panoply of anti-carcinogenic effects, estrogens were abolished in PCa treatment because of the adverse effects. On the other hand, many reports have been implicating estrogens in prostate carcinogenesis. The mechanisms underlying this duality of estrogens' actions are unclear but have been related to the distinct activity of estrogen receptors (ERs), namely, the classical ER α and ER β . Also, the actions of the membrane-bound G protein-coupled estrogen receptor (GPER) add increasing complexity to the scenario of the dual and contradictory estrogenic effects. The beneficial effects of estrogens against the development of PCa are also demonstrated by the lower incidence of PCa associated with higher consumption of phytoestrogens. Many studies have been investigating the phytoestrogens and their mechanism of action, which may lead to finding safe estrogen-based therapies for PCa treatment. This review describes the existing data on the anti- and pro-tumorigenic actions of estrogens, including the relationship with the ERs expression and intraprostatic and circulating estrogens. Moreover, it summarizes the anti-cancer effects of several natural compounds with estrogenic activity highlighting their promising features as possible PCa treatments.

Keywords

Prostate cancer (PCa), estrogens, estrogen receptors (ERs), phytoestrogens

2.1. Introduction

Estrogens are the steroid hormones mainly associated with female reproductive physiology. Nevertheless, this class of sex hormones are involved in a panoply of biological processes, both in men and women [1-3]. Regardless of sex, estrone (E_1), 17 β -estradiol (E_2), and estriol (E_3) are the primary naturally occurring estrogens, with E_2 being the most potent and prevalent one [4, 5]. The pioneering work of Huggins and Hodges [6] in the middle of the 20th century has provided the basis for the use of hormones in PCa therapy by establishing prostate cancer (PCa) as hormone-sensitive cancer, stimulated by androgenic activity and inhibited by suppression of androgens levels or estrogens administration. In fact, estrogens were used in PCa treatment between the 1940s and 1970s, with the synthetic estrogen diethylstilboestrol (DES) being an effective therapy for delaying the progression of metastatic PCa [7-12]. However, after decades of practice, estrogen therapy was discontinued due to the associated adverse effects, namely, at the cardiovascular level [8-10].

On the other hand, accumulating evidence has pointed out estrogens as causative agents of PCa, promoting cell survival and malignant transformation, leading to tumor growth and metastization [13-15]. An extensive amount of research has been carried out to understand this duality of estrogens' actions in PCa. Although the detailed mechanisms are far from being completely clarified, the double-edged sword of estrogens effects has been highly linked to the separate and distinct activity of estrogen receptors (ERs). Estrogens actions are mediated by the classical nuclear ERs, namely, ER α and ER β , and the membrane-bound G protein-coupled estrogen receptor (GPER) [16-18]. ER α and ER β act as ligand-activated transcription factors exerting direct effects in the regulation of gene expression by binding to specific DNA sequences in the target genes (genome signaling) [19, 20] or, alternatively, by interacting with other transcription factors or activating intracellular signaling cascades, affecting gene expression indirectly [21, 22]. GPER is involved in the rapid non-genomic estrogenic responses through the activation of signal-transduction mechanisms with subsequent production of intracellular second messengers, cAMP regulation and protein-kinase activation in signaling cascades, which ultimately also can lead to indirect gene expression regulation [16-18, 23].

It has been widely accepted that ER α and ER β drive differential responses in PCa [24, 25], with ER α associated with pro-tumorigenic effects, whereas ER β has been implicated in the anti-tumorigenic actions of estrogens [26-28]. The complexity of estrogens actions in PCa increases considering GPER, as this membrane receptor has been shown to trigger both tumor growth and tumor regression effects [29].

Despite the disclosure of the mechanism of action and the panoply of anti-cancer actions, research efforts have not found safe approaches for re-introducing estrogens in PCa therapy [30]. In line with the beneficial effects of estrogens in PCa treatment are the demonstrations of the lower incidence of PCa in Eastern countries associated with a plant-based diet and higher consumption of phytoestrogens [31-33], which have raised curiosity about the therapeutic potential of these compounds. At present, important lines of research explore the use of phytoestrogens and bioactive compounds with estrogen-like activity in PCa treatment [34]. Several compounds, alone or in combined approaches, have shown promising results in controlling PCa development and progression without adverse effects [35-41] and have been tested in preclinical studies and clinical trials as possible estrogen therapy [42-47]. The present review revisits the effects of natural and synthetic estrogens as anti-cancer molecules and, opposingly, in targeting the PCa hallmarks, such as cell survival, proliferative activity, resistance to apoptotic cell death, and invasiveness. The relationship with ERs expression, intraprostatic estrogens production and levels of circulating estrogens is discussed. Also, the actions of diverse natural compounds with estrogenic activity and promising anti-cancer effects are summarized, providing a scientific basis for the evolution of contemporary PCa treatment. Our main goal was to join the actual information fulfilling the scientific gaps in reviewing the subject, as the reviews published in the last three years focused only on one type of ER, a specific class of compounds or stage of PCa, or more generally have covered several types of cancer, without exhaustively detailing the effects of estrogens in PCa. Herein, an integrated and translational perspective about estrogens actions in PCa and its possible contribute to therapy is provided.

2.2. Estrogens in the male

2.2.1. Circulating levels

The measurement methodology is the first issue to be considered in any systematized analysis of steroid circulating levels. Among the different methods that can be used to quantify steroid levels in human blood samples, estrogens have been measured essentially by radioimmunoassay (RIA), chemiluminescence immunoassays and liquid chromatography tandem mass spectrometry (LC-MS/MS), which has the advantage of detecting small amounts of steroids reaching lower limits of quantification. Therefore, several authors [48, 49] indicate LC-MS/MS as the preferential methodology, though RIA

remains the most commonly used methodology for quantifying estrogen serum levels (Table 2.1).

Table 2.1. E₂ serum levels in healthy men and PCa patients.

E₂ concentration range (nM)		Methodology of measurement	Reference
Healthy men	PCa patients		
0,156	0,200*	RIA	[50]
0,110 – 0,160	0,120 – 0,160	RIA	[51]
0,114 – 0,125	0,110 – 0,128*	Dextran-coated charcoal method	[52]
0,110 – 0,160	0,120 – 0,160	RIA	[51]
0,200	-	RIA	[53]
0,093	-	Chemiluminescence immunoassay	[54]
0,200	-	RIA	[55]
0,066 - 0,221	0,066 - 0,233	-	[56]
0,125	0,121	RIA	[57]
0,070	-	Chemiluminescence immunoassay	[58]
0,100 – 0,150	0,050 - 0,300	RIA	[59]
0,235	0,247	-	[60]
0,089 – 0,120	0,086 - 0,106	Gas chromatography-mass spectrometry	[61]
0,062	0,066	RIA	[62]
0,028 – 0,167	-	Chemiluminescence immunoassay	[63]
0,106	0,106	RIA	[64]
0,122	0,122	Heterogeneous competitive magnetic separation assay	[65]
0,028 – 0,156	0,063 - 0,068	Chemiluminescence immunoassay	[66]
0,107	0,101 - 0,105	Chemiluminescence immunoassay	[67]
0,082 – 0,234	0,200	-	[48]
0,108	0,159*	Enzyme linked immunosorbent assay (ELISA)	[68]
0,132	-	RIA	[69]
0,103	-	RIA	[70]

* - statistically significant differences found

Another important aspect for comparative analysis of different studies refers to the units of measurement of the serum steroid concentrations, which also depends on the

methodology used. Herein, the reported E_2 measurements were converted to molar concentrations (nM) to allow immediate comparisons.

Data collection from the literature and summarized in Table 2.1 allows concluding that a broad range of serum E_2 concentrations can be found in men, from 0,028 to 0,235 nM. Overall, very low levels were obtained from healthy individuals between 30 and 90 years old. This wide age range encompasses the critical age for the development of PCa, which is 50 years old and onwards [71]. Moreover, the widespread values reported for E_2 serum concentrations could be linked to the age group, but also to the ethnicity or physiological conditions. Indeed, several endocrine and metabolic events, namely, the increase in adiposity and body mass index, with the augmented extragonadal aromatization of testosterone, which could influence the E_2 levels [52, 61, 72]. Furthermore, circulating estrogens appeared to be inversely associated with intraprostatic inflammation [73]. However, these factors were not always considered in the available studies.

The relationship between PCa and serum levels of E_2 has been largely controversial and the majority of studies have not found statistically significant associations between hormone concentrations and the risk of PCa [74-76]. Concerning the E_2 serum levels found in PCa patients, the concentrations also were highly variable ranging from 0,050 to 0,300 nM (Table 2.1). Although not consensual, some studies have reported a slightly significant increase of E_2 levels in PCa cases [77] (Table 2.1).

2.2.2. Intraprostatic production of estrogens

Estrogen's biosynthesis is the last reaction on the steroidogenic pathway, and it happens due to the activity of the heme protein aromatase/P450aro/CYP19A1 enzyme [78, 79]. Aromatase in the endoplasmic reticulum [80] or mitochondria [81] catalyzes the irreversible aromatization of the A ring of the androgens precursors generating estrogens. The naturally occurring estrogens, E_1 , E_2 and E_3 , are produced via aromatization of androstenedione and testosterone [5] (Fig. 2.1). E_1 can also be reversibly converted to E_2 by the enzyme 17 β -hydroxysteroid dehydrogenase, and both E_1 and E_2 are precursors of E_3 through the involvement of CYP3A4/5 [82].

Aromatization of androgens in the male occurs mainly in the somatic cells of the testis, but also in other peripheral organs, including the prostate [83-85]. Prostate cells have been shown to express a functional aromatase that uses external androgen precursors, such as dehydroepiandrosterone (DHEA) and dehydroepiandrosterone

sulphate (DHEA-S) and circulating testosterone to producing estrogenic compounds (Fig. 2.1) [78, 86].

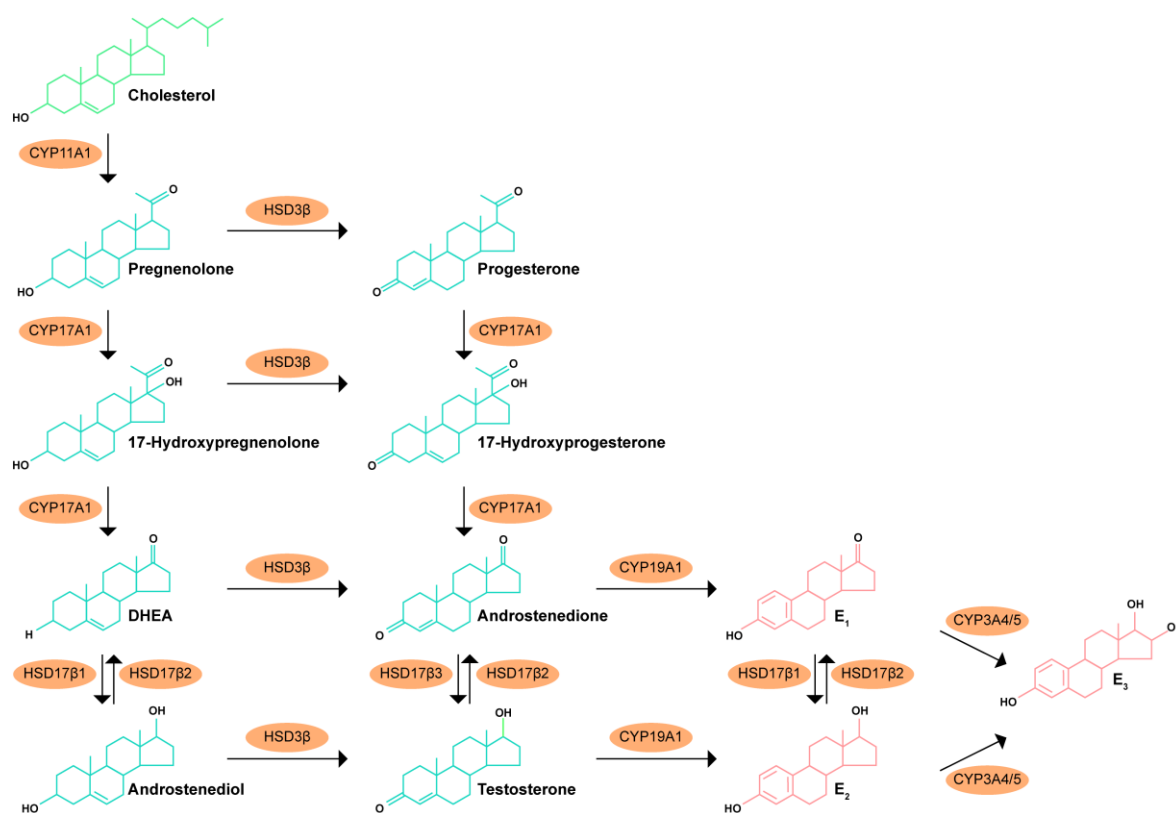


Figure 2.1. Estrogen biosynthesis pathway. Cholesterol is the precursor of steroid hormones. It originates pregnenolone through the cholesterol side-chain cleavage enzyme (CYP11A1) that is converted to progesterone by β -hydroxysteroid dehydrogenase (HSD3 β). This enzyme is also responsible for the conversion of 17-hydroxypregnenolone to 17-hydroxyprogesterone, dehydroepiandrosterone (DHEA) to androstenedione and androstenediol to testosterone. Estrogens are produced through the activity of aromatase (CYP19A1), which converts androstenedione and testosterone to estrone (E₁) and 17 β -estradiol (E₂), respectively. E₁ is reversibly converted to E₂ by the enzyme 17 β -hydroxysteroid dehydrogenase (HSD17 β 1/2), and both can originate E₃ through the cytochrome P450 3A4 /5 (CYP3A4/5).

Estrogen synthesis in the human prostate was first pointed out by Matzin and Soloway [84] based on the immunohistochemical localization of aromatase in prostatic tissues. This was followed by the characterization of aromatase expression in both the epithelial and stromal cells of human prostate [85], and later on by the demonstration that prostatic stromal cells have the ability to synthesize E₂ from testosterone [87]. Over the years, the local production of estrogens in the prostate, and its functional relevance, have been studied. Manipulating aromatase expression levels or activity have shown to directly affect the intraprostatic E₂ levels. Moreover, the localization of aromatase in the prostate cells is “conveniently” adjacent to ERs, the mediators of estrogens actions in regulating prostate physiology [88, 89]. Up-regulation of aromatase activity increased the

intracellular levels of estrogens in prostate cells concomitantly with ER α and GPER activation [83, 90]. On the contrary, upon aromatase inhibition, intraprostatic E₂ levels are significantly reduced [91].

Altered intraprostatic estrogen biosynthesis has been reported in neoplastic prostate. Results from the STERKPROSER trial suggested that human cancerous prostates have higher activity of aromatase producing more E₂ compared with the non-cancerous prostate tissues [77]. Moreover, an approximately 30-fold increase in aromatase expression was found in PCa metastatic tissues in comparison with primary tumors [92].

2.2.3. Estrogen receptors expression in the normal and pathological prostate

The classical nuclear ER α and ER β , and the membrane-bound GPER have been shown to display a distinct expression and subcellular localization in PCa tissues and cells, which underlies specific mechanisms of action and different physiological effects. Moreover, an additional complexity has been characterizing the ERs actions, driven mainly by the existence of genetic polymorphisms and splice variants. Several splice variants have been identified for both ER α and ER β . ER α displays, at least, five distinct mRNA isoforms, ER α -A-E [93], characterized by the deletions at the C-terminal ligand-binding domain [93]. ER β also presents five different mRNA isoforms (ER β 1-5), generated by the truncation or amino acids insertion in the ligand-binding domain [94]. Also, the expression of ER β variants has been shown to be accurately regulated. ER β 1 seems to be primarily regulated at the transcriptional level, whereas ER β 2 and ER β 5 are controlled by the interplay between transcriptional and post-transcriptional regulation [95].

2.2.3.1. Humans

ERs expression in human prostate goes back to foetal development, with a distinct expression pattern being reported for ER α and ER β . ER α expression was found in embryological structures, namely, in the luminal cells of the ventral urogenital epithelium, basal cells of the dorsal urogenital epithelium, prostatic utricle, distal periurethral ducts, peripheral stroma and posterior prostatic duct after 19 weeks of gestation [96]. However, Adams *et al.* [97] failed to detect the ER α protein in normal prostate epithelium or stroma at any stage of fetal development, reporting that this receptor starts to be expressed at 1 month postnatal development with a localization exclusive to the periacinar stromal nuclei. Concerning ER β , Shapiro and colleagues [96] observed its expression throughout

the urogenital epithelium, ejaculatory ducts, müllerian ducts and the entire prostate stroma between the 7 and 22 gestational weeks. Adams *et al.* [97] also showed that ER β is strongly expressed in the nuclei of epithelial and stromal prostate cells at midgestation. Moreover, this pattern is maintained in the neonatal and early prepubertal prostate [97]. Nevertheless, approximately at the age ten, the ER β seems to be predominantly expressed in the basal epithelial cells, similar to what happens in the adult prostate [97].

At least for our best knowledge, there are no reports characterizing the expression of GPER in the prostate developmental stages.

Several studies have characterized the expression of ERs in human adult prostatic tissue (Fig. 2.2). ER α is localized in stromal cells, namely, in periglandular regions [98], and sporadically in the basal epithelium [99]. In what concerns ER β , it is consistently observed in basal and luminal prostate epithelium with occasional staining of the stroma [98, 100-103]. Overall, it is accepted that in non-neoplastic prostate, ER α is confined to the stroma and ER β is the predominant receptor in epithelial cells. Recently, Rago *et al.* [104] reported the strong expression of GPER in the cytoplasm of basal epithelial cells, but not in luminal secretory epithelial cells, and with a weak staining in the cytoplasm of stromal cells.

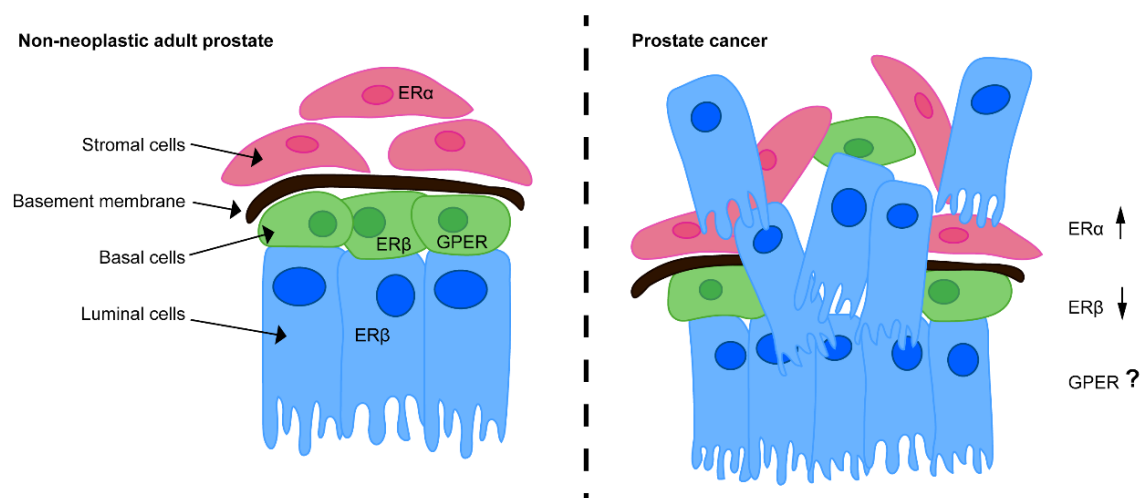


Figure 2.2. Estrogen receptors expression in non-neoplastic adult human prostate and prostate cancer. In adult non-neoplastic prostate (left panel), estrogen receptor α (ER α) is mainly associated with the stromal compartment. Opposingly, estrogen receptor β (ER β) expression occurs mainly in basal and luminal cells of prostate epithelium. G protein-coupled estrogen receptor (GPER) is present in basal epithelial cells. In prostate cancer (PCa, right panel), a reduction of ER β and an augment of ER α is observed with disease progression. Concerning GPER expression in PCa the existent data are not consensual.

Altered expression of ERs has been associated with prostate pathological conditions. In benign prostatic hyperplasia (BPH) cases, a decreased expression of ER α compared to the normal prostate was reported, whereas ER β protein levels display no

change [105]. Contrarily, Gangkak *et al.* [106] observed a significant increase in the ER α immunoreactivity, both in the epithelium and stroma of BPH specimens in comparison to controls, as well as, an augment in ER β immunoreactivity in the epithelium, but not in the stroma. Regarding the ERs cellular distribution in BPH, it follows the pattern of normal prostate. ER α is mainly expressed in stromal cells and ER β is detected in the basal layer of epithelium, and sporadically in the epithelial and stromal cells [105].

Concerning the neoplastic prostate tissues, the expression of both ER α and ER β subtypes has been reported (Fig. 2.2), with their pattern deserving some considerations. Several studies have described the decrease or even loss of ER β expression in high-grade prostatic intraepithelial neoplasia (HGPIN) [102] and PCa cases [98, 101, 102, 107-111]. An elegant study performed by Ji and colleagues [112] examined pairs of PCa tissue and surrounding morphologically normal prostate from the same individual, avoiding any variability in receptor basal expression. This study confirmed the huge decrease of ER β expression in tumors [112]. Moreover, loss of ER β expression has been shown to follow progression of disease and tumor staging. An interesting study found that ER β is expressed in 90 % of the studied BPH cases, whereas 53% of the prostatic intraepithelial neoplasia (PIN) and 82.8 % of the adenocarcinoma cases were negative for ER β [113], confirming the downregulation of ER β expression associated with the progression from hyperplastic prostate epithelium to PCa. Furthermore, ER β was detected in grade 3 prostate carcinomas, but its expression diminished greatly or was absent in the majority of grade 4/5 carcinomas [98]. ER β expression was also decreased or absent in several cases of recurrent adenocarcinoma [102] or hormone-refractory prostate tumors [107].

In what concerns the ERs variants, the information on their expression in PCa cases is very limited. However, the presence of several ER β splicing isoforms (approximately 60-, 57-, and 53 kDa) has been suggested [114]. A recent study also reported that the ER β 1 and ER β 2 variants are expressed in the cell nuclei and cytoplasm of PCa samples, whereas the ER β 5 expression was almost cytoplasm exclusive [115].

In contrast to ER β , ER α expression has been detected in HGPIN and seems to increase with tumor progression. ER α protein expression was found in 11 % of HGPIN lesions, up to 20 % of low- to intermediate-grade prostatic adenocarcinoma cases, and in 43 % and 61 % of Gleason grade 4 and 5 carcinomas, respectively [116]. Moreover, the most significant ER α expression was observed in metastatic lesions and recurrent prostate carcinoma after hormonal therapy (91 % and 94 %, respectively) [116]. However, a previous study failed to detect ER α in metastatic lesions [117]. On the other hand, a very recent study observed that the highest expression of ER α occurred in HGPIN compared to benign prostate, PCa and metastasis, suggesting that this stage is where ER α contributes

to prostatic disease [118]. The variability in the results suggests that the localization of ER α in epithelium or stroma should be taken into account [118].

Regarding GPER, it has been detected with increased expression in HGPIN [104] and neoplastic prostate cells [104, 115]. Higher GPER expression was also found in metastatic castrate resistant PCa (CRPC) compared with primary PCa cases (80 % vs. 54 %, respectively) [119]. However, GPER expression was shown to decrease from Gleason 2 to Gleason 4 [104], suggesting a decrease of GPER with tumor progression. Further studies are needed characterizing the GPER expression pattern in human PCa.

As a crucial basis for performing *in vitro* studies aiming at disclosing the specific functions of each ER, the receptors' expression in non-neoplastic and neoplastic prostate cell lines models has been characterized (Table 2.2). Several studies have described that the non-neoplastic epithelial PNT1A cells express the ER β but not the ER α [120, 121]. On the contrary, the normal human prostate stroma cell line, WPMY-1, express ER α and GPR30, but not ER β [122]. As expected, the ERs' expression found in non-neoplastic human prostate cell lines followed the pattern observed in normal prostate tissues, with ER β present in the epithelial cells and ER α confined to the stromal compartment. Furthermore, both ER α and ER β proteins were detected in the immortalized prostatic epithelial cell (PrEC) line established from a BPH specimen (BPH-1) [123].

Regarding PCa cell lines, it was reported that the androgen-responsive, LNCaP, and the CRPC, DU145, cell lines only express ER β [123]. The lack of ER α in LNCaP and DU145 cells seems to be caused by DNA hypermethylation [123]. Interestingly, very recent studies reported that LNCaP, 22Rv1, VCaP and DU145 cells express both the ER α and ER β [124-126]. LNCaP and PC3 cells also express the variants ER β 1 and the ER β 2 [127]. The CRPC PC3 cell line seems to express both the ER α and the ER β [123], as well as the ER α -36variant [129, 130]. The existing variation of ERs expression in PCa cell lines may lay down the duality of estrogenic effects from anti- to pro-tumorigenic and indicate that culture conditions or cell passage number can easily alter ER expression. Also, it highlights the importance of characterizing cell line models for ER expression in assembling the experimental designs.

GPER expression in prostate cell line models is less characterized, but the receptor seems to be expressed in the CRPC cell line models DU145 and PC3 [128, 129]. Moreover, GPER expression is higher in the PC3 cells, which mimic a more aggressive metastatic stage of disease [129].

Table 2.2. Expression profile of classical ERs and GPER in non-neoplastic and neoplastic prostate cell line models.

	Cell line / Primary cells	Cell type	ER profile	Reference
Non- neoplastic	PrEC (primary culture)	Epithelial	ER β	[123]
	PrEC (commercial)	Epithelial	ER α (including ER α variant with exon 2 deleted) ER β (including ER β 1, ER β 2 and ER β 5)	[123, 124]
	PrSC (primary culture)	Stromal	ER α GPER	[122]
	PNT1A	Epithelial	ER β	[120]
	RWPE-1	Epithelial	ER β (including ER β 1, ER β 2 and ER β 5)	[124]
	WPMY-1	Stromal	ER α GPER	[122]
	BPH-1	Epithelial	ER α (including ER α variant with exon 2 deleted) ER β (including ER β 1, ER β 2 and ER β 5)	[123, 124]
Neoplastic	C4-2	Epithelial	ER β (including ER β 1, ER β 2 and ER β 5)	[124]
	LNCaP	Epithelial	ER α ER β (including ER β 1, ER β 2 and ER β 5)	[123, 124, 127]
	DU145	Epithelial	ER α ER β (including ER β 1, ER β 2 and ER β 5) GPER	[121, 123, 124, 128]
	PC3	Epithelial	ER α (including ER α variant with exon 2 deleted and variant ER α -36) ER β (including ER β 1, ER β 2 and ER β 5) GPER	[123, 124, 129, 130]

2.2.3.2. Rodents

Similarly to that observed in humans, both the classical ERs and the GPER are expressed in the rodents' prostate [129, 131, 132]. The expression of ER α and ER β in rat and mouse prostate was first characterized in the 90s by the Jan-Åke Gustafsson group

after the discovery of the ER β subtype [131, 132]. In adult rats, both the ER α and ER β transcripts are expressed in all the prostate lobes, dorsal, lateral and ventral [132]. The ER α is expressed at low levels, being more abundant in the lateral lobe and scarce in the ventral one [132]. It is expressed in the stroma cells, namely, in the fibromuscular tissue surrounding the prostatic collecting ducts in the posterior periurethral region, and in a thin layer of connective tissue surrounding the ejaculatory ducts, as well as in some basal epithelial cells [133-137]. On the other hand, ER β is highly expressed in the three lobes [132]. It is highly expressed in the nuclei and cytoplasm of luminal and basal epithelial cells of the ventral, dorsal and anterior prostate, and is also detected in the cytoplasm [133-137]. In mice prostate, the scenario is a little bit different, as ER α seems to be expressed in both the epithelial and stroma cells, and ER β is mainly localized in the epithelial cells [135]. Studies characterizing GPER expression in rodents' prostate are almost non-existent.

Prins *et al.* [138, 139] have determined the ontogeny of ER α and ER β expression in the rat prostate lobes, confirming that ERs expression accompanies prostate development. At birth, ER α is confined to the nuclei of mesenchymal cells in the urogenital sinus, and the proximal region of the budding prostate lobes [138]. ER α is not present in the central or distal mesenchymal cells of the prostate nor within the epithelial cells of the budding glands [138]. Between postnatal days 1 and 15, when the prostate lobes underwent branching morphogenesis, ER α is expressed in some mesenchymal cells in the proximal region of the gland [138]. Moreover, ER α is the main receptor expressed in rat prostate at 15 days after birth, and its expression starts decreasing afterwards and with morphogenesis [140]. Between days 15 and 30, at early puberty, ER α expression seems to be entirely lost within the proximal stromal cells of all prostate lobes and, on day 45, it is not detected in any of the prostate lobes [138]. Regarding ER β , which is mainly expressed in the epithelial cells, its expression increases with prostate development and growth. This is visible in the epithelial cells of luminized ducts at postnatal days 6 and 10 in the ventral prostate, and between days 10 and 15 in the dorsal and lateral lobes [139, 140]. A further marked increase in ER β expression is noted at day 30 in all prostate lobes [139]. At day 90, ER β is strongly expressed in the epithelial cells, which corresponds to the adult expression pattern [139]. Although detected in the stromal cells of distinct prostate lobes at birth, ER β expression significantly declines from neonatal day one onwards [139].

In mice, neither ER α nor ER β were detectable in the prostate epithelium in the first week of life, but both receptors are expressed after 2 and 3 weeks of development [141]. Thereafter, ER α levels decline, and no ER α -positive epithelial cells can be detected by week 4. In contrast, ER α is highly expressed in almost all stromal cells at one week of age, though its expression declines after week 1, and by week 2, very few stromal cells are

ER α positive [141]. Regarding ER β , it is expressed in the epithelial cells displaying a continuous increase towards adult expression levels, which were achieved by six weeks [141].

Investigation in the prostate of aged rats reported histopathological alterations, including hyperplasia, intraluminal concretions, nuclear atypia and PIN, associated with changes in ERs expression [136]. ER α -positive cells seem to be mainly present in areas of intraepithelial proliferation, hyperplasia, and squamous metaplasia and proliferative inflammatory atrophy (PIA), with their number being consistently increased in focal areas showing increased epithelial proliferation and the presence of inflammatory cells [90]. Moreover, ER α expression is increased in basal cells of prostate epithelial lesions of male aging rats [90]. Contrastingly, ER β expression is reduced in specific areas related to PIN, atrophic abnormalities, squamous epithelium and cellular atypia in the prostate epithelium of senile rats [136]. The pattern and intensity of ER β immunoreactivity in rat prostate did not change with age when considering the epithelium of acini with normal morphology or those with hyperplasia [136].

2.3. Estrogens as prostate carcinogens

2.3.1. Cell survival and growth, and neoplastic transformation

Estrogens are widely recognized as anti-cancer agents and were used in therapy for years, being discontinued because of their important side effects. However, several studies have defended the pro-tumorigenic effect of estrogens, reporting that these steroid hormones can contribute to the malignant transformation of prostate cells.

Estrogens have been implicated as a cause of BPH, which is predominantly characterized by the overgrowth of stromal compartment. For example, E₂ was shown to increase the proliferation of human BPH-derived stromal cells in an ER-dependent mechanism but have no effect on epithelial cells [142]. However, E₂ (1 μ M) was shown to increase the proliferation of immortalized nontumorigenic human prostate epithelial BPH-1 cells in culture for 3 days [143], though cell proliferation started being reduced after 6 days of exposure to hormone [143]. Nevertheless, DNA damage was significantly higher in BPH-1 cells treated with E₂ for 6 weeks, which was accompanied by an increased percentage of c-Myc- and cyclin D1-immunopositive cells [143]. In recombinant prostate tissue, composed of urogenital mesenchyme plus BPH-1 cells, growing under the kidney capsule of male athymic nude mice, testosterone and E₂ treatment increased the Ki-67 proliferation index compared with untreated animals [144].

Actually, different types of studies, with diverse experimental approaches and using distinct estrogenic compounds, have demonstrated that estrogens can deregulate prostate cells survival and growth (Table 2.3), which was linked to hyperplasia, dysplasia, metaplasia, and neoplasia.

The effects of estrogens inducing proliferation of human prostate cells were reported in different cell types, from non-neoplastic to androgen-sensitive and CRPC cells. E₂ concentrations from 0.01 to 5 µM (Table 2.3) increased the proliferation of human prostate epithelial cells, and androgen-sensitive LNCaP and VCaP cells [145-147]. In CRPC cells, namely, DU145 and PC3 cell lines, E₂ (0.01 and 1 µM) increased the anchorage-independent growth and inhibited apoptosis [14]. E₂ also increased the growth and anchorage-independent growth of cells isolated from the lymph node metastasis of a PCa patient in an ERα-dependent mechanism [15].

The molecular orchestrators responsible for the proliferative actions of estrogens also have been disclosed. Besides the dependency of ERα alone, the interaction between ERα, androgen receptor (AR), and Src was shown to be required for the rapid activation of the MAPK proliferation pathway in non-neoplastic cells [145]. Moreover, the augmented proliferation of LNCaP cells was associated with the increased expression of PSA, IGF-1R, IGF-1, and IGFBP-2 and the decreased expression of AR, ERβ, IGF-II, and IGFBP-3 [146].

The increased proliferation of prostate cell lines in response to estrogens is translated to *in vivo* experiments. E₂ administration (Table 2.3) was shown to decrease the population of rat prostate epithelial cells in phase G₀/G₁ whereas increasing the S and G₂/M phase cell populations [13]. In addition, E₂ caused DNA damage and induced apoptosis of prostate tissue cells, at least for short-time exposure [13].

For example, in human prostate xenografts, an early exposure to β-estradiol 3-benzoate (7 to 90 days) combined with a secondary exposure (until 200 days) increased the proliferation of prostate epithelial cells leading to the development of hyperplastic glands [148]. This was underpinned by the suppression of PTEN expression, and likely augmented activity of the PI3K-Akt signaling pathway, resulting in the inhibition apoptosis and cell cycle progression [148]. In the same line, diethylstilbestrol (DES) was reported as causative of prostate hyperplasia, since the prenatal exposure to this compound resulted in enlarged prostates in adults [149]. Beyond the induction of cell proliferation, estrogens' effects have been related with the development of other histological alterations in prostate. Several studies reported that E₂ in combination with testosterone, induced dysplasia in rat dorsolateral prostate [150-152], as well as in the lateral, ventral, dorsal and anterior regions of mice prostate [153].

Also, a variety of estrogenic compounds seems to cause squamous metaplasia in the prostate of several species [154-156]. DES induced the metaplastic transformation of

mouse prostate epithelium, mainly, in the anterior region [157]. This effect was associated with the onset of cytokeratin-10 expression, up-regulation of progesterone receptor expression, and loss of expression of the cell cycle inhibitor p27^{Kip1} [157].

Yu and colleagues observed that E₂ induced the neoplastic transformation of rat prostate epithelial cells [13]. These transformed cells displayed increased expression of several putative PCa stem cell markers, as well as changes in the hormone receptors expression, namely, increased levels of ER α and decreased expression of ER β and AR [13, 143], which indicates a change in the hormone-responsiveness accompanying tumors development. Moreover, these findings support the driven role of ER α in prostate carcinogenesis. Low doses of E₂ also were reported to increase tumor growth in DU145 and PC3 cells mouse xenograft models [14]. However, the carcinogenic effects of estrogens have been mainly demonstrated by their association with testosterone. Rat treatment with E₂ in addition to testosterone promoted tumor development, causing the formation of DNA adducts, oxidative DNA damage and lipid peroxidation [152]. In mice, the pathologic areas induced by the testosterone plus E₂ treatment showed increased number of PCNA-positive proliferating cells [153]. Moreover, addition of E₂ was shown to shift the incidence of prostate tumors to 100 % compared only with 35-40 % of incidence when testosterone was given alone [151]. These findings implicated estrogens in prostate carcinogenesis and indicated that the known effects of androgens driving cancer may depend on testosterone aromatization to E₂, which was demonstrated in animal models. Aromatase knockout mice, unable to synthesize E₂, displayed reduced incidence of PCa compared with wild-type animals in response to testosterone administration [153]. This study also showed that the presence of a functional ER α is determinant for tumor development. E₂ plus testosterone treatment was ineffective inducing PCa in *ER α* knockout mice, whereas *ER β* knockout animals displayed a biochemical and histological pattern of carcinogenesis similar to wild-type [153].

Humans are exposed to estrogens since embryological life, and prenatal exposure to estrogens was shown to have observable effects in the adult male reproductive tract, including the prostate gland [158]. Studies performed in animal models showed that exposure to low doses of estrogens during fetal development causes the enlargement of the prostate, whereas high doses implicate a reduction in the adult prostate weight [149]. Moreover, the developmental exposure to β -estradiol 3-benzoate seems to change the differentiation and epigenetic programming in a human fetal prostate xenograft model [148], which can be a mechanism contributing to carcinogenesis later in life. Research efforts are needed to ascertain the impact of fetal estrogen exposure and the development of PCa in adulthood.

Catechol estrogens are potent compounds originated from the metabolism of estrogens. This class of compounds has been identified in the prostate tissue, and also has been linked with prostate malignancy [159]. Polymorphisms in the estrogen metabolism enzymes that detoxify catechol estrogens, namely, catechol-O-methyltransferase (COMT), glutathione (GSH) and quinone reductase were associated with PCa risk [160-164]. Furthermore, prostate areas susceptible to development of carcinoma have been shown to have less protection by these enzymes. This susceptibility and pro-tumorigenic effects were related with the reaction of catechol estrogen-3,4-quinones (E_2 -3,4-Q) with DNA, which is favored by the reduced activity of COMT, GSH and quinone reductase [159, 165]. *In vitro* studies showed that exposure to the catechol estrogens 2-hydroxyestradiol (2-OHE₂) and 4-hydroxyestradiol (4-OHE₂) increased the proliferation of BPH-1 cells [143]. This was achieved by slightly diminishing the non-proliferating cell fraction (G₀/G₁-phase) while increasing the cell population in S-phase [143]. Moreover, both catechol estrogens increased the abundance of the cell cycle inducer cyclin D1 and the proto-oncogene c-Myc, which explains the shift towards the proliferative phase [143, 166]. Increased proliferative ability driven by 4-OHE₂ and 2-OHE₂ was accompanied by altered expression of molecular targets in the estrogen signaling pathways. These catechol estrogens increased the abundance of ER α and its downstream target IGF-1R, whereas reducing the levels of ER β and its downstream tumor suppressor FOXO-1, though the 4-OHE₂ effects were observed in a greater extent than that of 2-OHE₂ [143]. Furthermore, genotoxic effects linked with the neoplastic transformation of BPH-1 cells were observed in response to administration of 4-OHE₂ and 2-OHE₂ [143].

Catechol estrogens also have been shown to disturb tissue homeostasis and prostate histology architecture. The catechol estrogen 4-OH-E₂, in combination with testosterone, induced prostatic dysplasia, and the frequency of appearance of this histological phenotype was doubled by the addition of 2-fluoroestradiol (2F-E₂) to testosterone and 4-OH-E₂ [152].

In sum, several reports defended that exposure to exogenous estrogens can be tumorigenic, especially when low doses are used. The majority of *in vitro* studies reported that E₂, at least for concentrations below 1 μ M (Table 2.3), increased the proliferation of prostate cells. Noteworthy, is also the fact that the altered intraprostatic estrogen biosynthesis is associated with neoplastic transformation of prostate, by disrupting downstream signaling pathways and influencing the development and proliferation of prostate epithelial cells and stroma [167].

Table 2.3. Effect of estrogens in regulating survival and growth of prostate cells *in vitro* and *in vivo*.

Type of Study	Cell line /Animal model	Estrogenic compound	Concentration /Dose	Assay model / Administration method	Time of treatment	Method of evaluation	Effect	Activated pathways	Ref.
<i>in vitro</i>	BPH-1	E ₂	1 µM	6-well plates	Up to 6 days / 6 weeks	Sulforhodamine-B assay Comet assay	↑ Proliferation Neoplastic transformation	Genotoxic mechanism	[143]
	BPH-1	2-OHE ₂	1 µM	6-well plates	Up to 9 days / 6 weeks	Sulforhodamine-B assay Comet assay	↑ Proliferation Neoplastic transformation	Genotoxic mechanism	[143]
	BPH-1	2-OHE ₂	10 µM	6-well plates	2, 4, 7 and 10 days	Sulforhodamine-B assay	↓ Cell viability	Cytotoxicity	[143]
	BPH-1	4-OHE ₂	1 µM	6-well plates	Up to 9 days / 6 weeks	Sulforhodamine-B assay Comet assay	↑ Proliferation Neoplastic transformation	Genotoxic mechanism	[143]
	LNCaP	E ₂	0.01 µM	Multi-well plates	24h	BrdU incorporation assay	↑ Proliferation	MAPK (ERK1/2) and association of AR, ERβ and Src	[168]
	LNCaP	E ₂	0.01 - 5 µM	96 well plate	3 days	MTS PSA measurements	↑ PSA expression	ER mediated	[169]

							↑ Cell growth		
	LNCaP	αE_2	0.01 – 5 μM	96 well plate	3 days	MTS PSA measurements	↑ PSA expression ↑ Cell growth	ER mediated	[169]
	LNCaP	E_2	0.01 μM		24h / 48h	WST-1 based assay	↑ Proliferation ↑ PSA expression	↑ c-Myc	[125]
	DU145	E_2	0.01 μM	12-well plates	24h	Soft-agar colony formation assay TUNEL assay	↑ Anchorage-independent growth ↓ Apoptosis	↓ FOXO1	[14]
	DU145	E_2	1 μM	12-well plates	24h	Soft-agar colony formation assay TUNEL assay	↑ Anchorage-independent growth ↓ Apoptosis	↓ FOXO1	[14]
	PC3	E_2	0.01 μM	12-well plates	24h	Soft-agar colony formation assay TUNEL assay	↑ Anchorage-independent growth ↓ Apoptosis	↓ FOXO1	[14]
	PC3	E_2	1 μM	12-well plates	24h	Soft-agar colony formation assay	↑ Anchorage-independent	↓ FOXO1	[14]

						TUNEL assay	growth ↓ Apoptosis		
	EPN	E ₂	0.01 µM	60 mm dishes	27h / 5 min	BrdU incorporation assay	↑ Proliferation	MAPK (ERK1/2) and association of AR, ERα and Src	[145]
	NRP-152	E ₂	1 or 3 µM	96-well plates / 60 mm dishes	2–6 weeks and 3–48h	Soft agar colony formation assay Comet assay Flow cytometry	Neoplastic transformation	Genotoxic mechanism	[13]
	MDA-Pca 2b	βE ₂	0.01 - 5 µM	96 well plate	3 days	MTS PSA measurements	↑ Cell growth	ER mediated	[169]
in vivo	BALB/c mice	DES *	2 mg DES (and 18mg cholesterol)	Pellet s.c. implant	Up to 3 weeks	Immunohistochemistry for PCNA	↑ Proliferation	ERα	[157]
	Nude mice	E ₂	1 or 3 µM	E ₂ -NRP-152 cells s.c. injection in the flanks	4–8 weeks	Tumor sizes measure	↑ Neoplastic transformation	Genotoxic mechanism	[13]
	Athymic nude male mice	β-oestradiol 3-benzoate *	250 µg/kg (early exposure) + 2.5 mg pellet	S.c injection	90 days (early exposure) +	Ki-67 quantification	↑ Proliferation	PI3K-Akt pathway	[148]

			(secondary exposure)		110 days (secondary exposure)				
Xenograft BALB/cA-nu castrated male mice using DU145 and PC3 cells	E ₂	0.18 mg	Pellet s.c. implant	25 to 35 days	Tumor volume and weight measure TUNEL assay	↑ Tumor growth	↓ FOXO1 ERβ and KLF5 pathway	[14]	
Xenograft BALB/cA-nu castrated male mice using DU145 and PC3 cells	E ₂	3.4 mg	Pellet s.c. implant	25 to 35 days	Tumor volume and weight measure TUNEL assay	↓ Tumor growth	↓ FOXO1 ERβ and KLF5 pathway	[14]	
Male athymic nude mice Tissue recombinants composed of mouse urogenital mesenchyme plus an immortalized nontumorigenic human prostatic	E ₂	2.5 or 10 mg (plus Testosterone)	Silastic implants	1-4 months	Immunohistochemistry Growth indices Determination of cancer incidence	↑ Proliferation ↑ Apoptosis	Akt pathway	[144]	

epithelial cell line (BPH-1) grown under the kidney capsule								
Male NBL/Cr rats	4-OHE ₂	5 µg/day	Silastic implants	13 weeks	Measurement of DNA Adducts Measurement of 8- Hydroxyguanosine Measurement of Lipid Hydroperoxides	↑ Inflammation ↑ Dysplasia	-	[152]
Male NBL/Cr rats	2F-E ₂ *	5 µg/day	Silastic implants	13 weeks	Measurement of DNA Adducts Measurement of 8- Hydroxyguanosine Measurement of Lipid hydroperoxides	↑ Inflammation ↑ Dysplasia	-	[152]
Male NBL/Cr rats	E ₂ (+Testosterone)		s.c. silastic implants	91 weeks	Stain with hematoxylin and eosin	↑ Prostate adenocarcinoma s development	-	[151]
Male NBL/Cr rats	DES * (+Testosterone)		s.c. silastic implants	91 weeks	Stain with hematoxylin and eosin	↑ Prostate adenocarcinoma s development	-	[151]
Sprague-	E ₂		s.c. silastic	75 weeks	Stain with	↑ Prostate	-	[151]

	Dawley (Hsd:SD) rats	(+Testosterone)		implants		hematoxylin and eosin	adenocarcinoma s development		
	Sprague- Dawley (Hsd:SD) rats	DES * (+Testosterone)		s.c. silastic implants	75 weeks	Stain with hematoxylin and eosin	↑ Prostate adenocarcinoma s development	-	[151]
	CD-1 mice	E ₂ (+Testosterone)		Silastic implants	4 months	Histopathological grading Immunohistochemis try Quantification and imaging of prostates	↑ Prostate size	-	[153]
	C57BL/6 mice	E ₂ (+Testosterone)		Silastic implants	4 months	Histopathological grading Immunohistochemis try Quantification and imaging of prostates	↑ Prostate size	↓ α-actin ↓ E-cadherin	[153]
	C57BL/ 6 X J129 mice	E ₂ (+Testosterone)		Silastic implants	4 months	Histopathological grading Immunohistochemis try Quantification and	↑ Prostate size	-	[153]

						imaging of prostates			

* - synthetic

2.3.2. Progression of disease and metastization

Besides the reported early pro-tumorigenic effects of estrogens, several studies also have implicated this class of hormones in the progression of PCa and development of metastasis.

Metastization is the complex process of dissemination of cancer to distant organs and the main cause of cancer-related deaths [170]. The occurrence of metastasis mainly depends on the migration and invasion capabilities of cancer cells, which is a consequence of the epithelial-mesenchymal transition (EMT) [171]. EMT is characterized by a switch in the expression of epithelial to mesenchymal markers, namely, the reduction of E-cadherin expression and the augment of N-cadherin and vimentin proteins [172, 173], which in the case of prostate cells has been linked with estrogenic actions, as well as, with the effect of catechol estrogens. Some of these compounds, namely, 2-OHE₂ and 4-OHE₂, have been shown to increase the invasion of prostatic cells [143], which can disrupt prostate histology architecture and promote tumor progression.

Estrogens could alter the phenotype of BPH-1 cells, down-regulating E-cadherin and up-regulating vimentin and Snail expression, thus, stimulating EMT [174]. Altered expression of EMT markers and enhanced migration and invasion properties have been described in PCa cells in response to estrogens. E₂ was shown to promote migration and invasion of distinct PCa cell line models and patient derived cells (Table 2.4). 0.01 μ M E₂ increased the migration of PacMetUT1 (isolated from the lymph node metastasis of a PCa patient) and 22Rv1 cells [15]. Accordingly, treatment with E₂ caused a change in the morphology of PacMetUT1 cells similar to the EMT process, which was confirmed by a down-regulation of E-cadherin expression and up-regulation of vimentin and Snail [15].

In vivo approaches support the outcomes of cell-based research indicating the role of estrogens in PCa progression and metastization. Mice receiving E₂ in combination with testosterone showed a dramatic reduction of α -actin and E-cadherin expression accompanying the prostate malignant transformation [153]. Similar findings were found when applying this treatment in mice harboring recombinant prostate tissue composed of urogenital mesenchyme and immortalized BPH-1 cells [144]. Metastatic carcinoma cells were identified in renal lymph nodes, lung and liver, accompanied by a drastic reduction of E-cadherin expression [144].

ER α has been indicated as the “guilty” ER in mediating the pro-migration and -invasion effects of estrogens. Indeed, the enhanced migratory capabilities observed in PCa cell lines and patient’s metastasis-derived cells were reported to be dependent on ER α signaling [15]. However, ER α might promote the metastatic process by favoring the “seed” of PCa cells in other organs. The “seed and soil” hypothesis proposed by Stephen Paget to

explain the emergence of metastasis [175] compares the dissemination of tumor cells to the distribution of seeds, arguing that only the seeds that find the proper soil will survive. The rationale underneath this model supports those cells from a primary tumor will establish metastasis if they find a well-matched organ. PCa preferentially metastasizes to bone [176], and interestingly it was shown that estrogens signaling through ER α stimulates the osteoblast-like properties of PCa cells, demonstrating its important role in the formation of osteoblastic lesions [15]. This suggests that estrogens signaling can be a relevant mechanism driving the progression of the disease for more aggressive stages and the emergence of bone metastasis. Therefore, characterizing the ERs' expression, mainly ER α , in PCa cases could be a useful prognosis tool in evaluating the progression of disease. Research is needed to deeply clarify this usefulness, though the presence of ER α , or the combination of ER α -positivity with low ER β expression, correlated with worse biochemical recurrence, disease progression and survival outcomes [177].

The stroma component also has been shown to play a triggering role in mediating the metastatic effects of estrogens. An interesting work revealed that conditioned medium from E₂-stimulated prostate stromal cells can promote migration of DU145 and PC3 PCa cells [178]. This increased migration seems to be mediated by enolase 1 [178], a key enzyme in glycolytic metabolism, also known as pyruvate dehydrogenase phosphatase, which catalyzes the transformation of 2-phosphate-D-glycerate to phosphoric acid-pyruvate, as well as the reverse conversion of phosphoric acid-pyruvate to 2-phosphate-D-glycerate for glycogen synthesis [179]. Enolase 1 expression is frequently increased in tumors [180], and due to its role in anaerobic glycolysis, it is thought to promote tumor development and progression [179]. E₂ 0.01 μ M enhanced the stability of enolase 1 in prostate stromal cells and promoted its secretion to the extracellular matrix, in an ER α -dependent mechanism [178]. Studies with recombinant enolase 1 showed that this enzyme bounds to the membrane of PCa cells promoting cell migration in a paracrine manner, via their plasminogen receptor activity [178]. The secretion of stromal cell-derived enolase 1 and its association with the surface of PCa cells, recruits and activates plasminogen, and thereby promotes the remodeling of extracellular matrix and migration of PCa cells [178, 181, 182].

Another mechanism related with the effect of E₂ promoting migration and invasion was the up-regulation of the sex determining region Y-box 4 (SOX4) [147], a developmental transcription factor overexpressed in many types of human tumors [183]. SOX4 was shown to regulate the expression of metastasis-related genes promoting EMT and PCa progression [184].

Also, the augmented intraprostatic production of estrogens seems to promote progression of CRPC and favor tumor metastasis. Aromatase knockdown and the

aromatase inhibitor letrozole were shown to increase overall survival and inhibit metastasis in castrated PC3 xenograft mice [185], which contributed implying estrogens in PCa metastization. The estrogenic effects in the PC3 xenograft mice were proposed to occur through the activity of ER α and its interaction with the estrogen response elements in the *MMP12* gene, regulating the expression of matrix metalloproteinase 12 (MMP-12) [185]. Also, *MMP-12* knockdown suppressed the invasion of PC3 cells [185]. The MMP-12 is one of the MMPs involved in metastasis [186, 187] and in the case of prostate it was shown to mediate the interactions between PCa cells and bone marrow stromal cells during bone metastasis [188], which further supports the role of ER α as a driver of PCa metastization to the bone.

In summary, in agreement with the pro-proliferative effects, low concentration/doses of estrogens support increased migration and invasion of PCa cells, leading to tumor progression and mestastization.

Table 2.4. Effect of estrogens in regulating prostate cells migration and invasion *in vitro* and *in vivo*.

Type of Study	Cell line /Animal model	Estrogenic compound	Concentration /Dose	Assay model / Administration method	Time of treatment	Method of evaluation	Effect	Activated pathway	Ref.
<i>in vitro</i>	LNCaP	E ₂	0.01 µM		24h / 72h	Immunoblotting Wound-healing assay Invasion assay	↑ Migration ↑ Invasion EMT	↑ N-cadherin ↑ Vimentin ↓ E-cadherin	[125]
	LNCaP	E ₂	0.01 µM	12-well plates	5 days	Alkaline phosphatase activity assay	↑ Osteoblast-like properties	ERα signaling	[15]
	LNCaP	E ₂	0.01 µM	96-well plates	72h	Wound healing Invasion assays	↑ Migration ↑ Invasion	SOX4 up-regulation	[147]
	22Rv1	E ₂	0.01 µM	24-well Boyden chambers	18h	Migration assay	↑ Migration	ERα signaling	[15]
	RWPE-1	E ₂	0.01 µM	96-well plates	48h / 72h 8 days	RT-PCR analysis WB	EMT	ERα signaling	[174]
	C4-2	E ₂	0.01 µM	24-well Boyden chambers	18h	Migration assay	↑ Migration	ERα signaling	[15]
	PacMetUT1 isolated from the lymph node metastasis	E ₂	0.01 µM	24-well Boyden chambers	18h	Migration assay	↑ Migration EMT	ERα signaling	[15]
	DU145	Conditioned medium	0.01 µM	15-cm dishes	48h	Transwell migration assay	↑ Migration	ERα signaling	[178]

		PrSC from BPH patients or WPMY-1 cells treated with E ₂				Wound healing assay		ENO1	
DU145	E ₂	0.01 μM		24h	Wound healing assay Cell invasion assay	↑ Migration ↑ Invasion	ERα signaling ERβ signaling Galectin-3 signaling	[27]	
DU145	E ₂	0.01 μM		48h	Cell Invasion Analysis Colony Formation Analysis (Soft Agar)	↑ Invasion	SRC	[189]	
PC3	E ₂	0.01 μM			Cell Invasion Analysis Colony Formation Analysis (Soft Agar)	↑ Invasion	SRC	[189]	
PC3	Conditioned medium PrSC from BPH patients or WPMY-1 cells treated with E ₂	0.01 μM	15-cm dishes	48h	Transwell migration assay Wound healing assay	↑ Migration	ERα signaling ENO1	[178]	
PC3	E ₂	0.01 μM	Incubation of cells	Overnight	Matrigel invasion assay	↑ Invasion	ERα/MMP-12	[185]	

				24-well, 8.0-μm pore size				activation	
	PC3	E ₂	0.0001 and 0.01 μM		24h	Wound healing assay Cell invasion assay Colony Formation Analysis (Soft Agar)	↑ Migration ↑ Invasion	ERα and ERβ signaling β-Catenin signaling	[190]
<i>in vivo</i>	CD-1, C57BL/6, and C57BL/6 x J129 mice	E ₂	2.5 or 10 mg (plus Testosterone)	Silastic implants	4 months	Quantification and imaging of prostates Immunohistochemistry	Carcinogenesis	ERα signaling	[153]
	Male NBL/Cr rats	E ₂		Silastic implants s.c.	16 weeks	Measurement of DNA Adducts Measurement of 8-Hydroxyguanosine Measurement of Lipid Hydroperoxides	↑ Tumor incidence	DNA adduct Oxidative DNA damage Lipid peroxidation	[152]
	Male NBL/Cr rats	4-OHE ₂	5 μg/day	Silastic implants	13 weeks	Measurement of DNA Adducts Measurement of 8-Hydroxyguanosine Measurement of Lipid Hydroperoxides	Carcinogenesis	-	[152]

	Male NBL/Cr rats	2F-E ₂ *	5 µg/day	Silastic implants	13 weeks	Measurement of DNA Adducts Measurement of 8-Hydroxyguanosine Measurement of Lipid Hydroperoxides	Carcinogenesis	-	[152]
	Male athymic nude mice Tissue recombinants composed of mouse urogenital mesenchyme plus BPH-1	E ₂	2.5 or 10 mg (plus Testosterone)	Silastic implants	1-4 months	Immunohistochemistry Growth indices Determination of cancer incidence	↑ Progression ↑ Metastization	Akt pathway	[144]

* - synthetic

2.4. Evidence of estrogens as protective agents in prostate cancer

2.4.1. Anti-proliferative and pro-apoptotic effects

2.4.1.1. Endogenous estrogens

In line with the use of estrogens as therapeutic agents in PCa, a panoply of studies has described their protective and anti-tumorigenic properties over the years.

In vitro and *in vivo* evidence have shown that E₂, the most potent endogenous estrogen, is capable of reducing PCa cell viability and proliferation (Table 2.5). This effect was reported in PCa cell lines, either in androgen-sensitive, LAPC-4, LNCaP and 22Rv1 cells, as well as in AR-negative CRPC models, namely, PC3 cells [169, 191-194]. Treatment with E₂ concentrations up to 10 µM, showed to reduce the DHT-induced cell growth of LNCaP and LAPC-4 cells [169, 192], whereas lower concentrations, ranging from 1 nM to 1 µM, were able to diminish the proliferation of 22Rv1 and PC3 cell lines [191, 193]. The same effect was shared by the E₂ stereo-isomer 17α-estradiol (αE₂), with 0.1 - 10 µM concentrations suppressing the proliferation of the androgen-sensitive PCa cell lines [169, 192]. Moreover, the E₂ effects counteracting proliferation and viability of PCa cells were followed by an increase of apoptosis. E₂ concentrations between 1 nM and 1 µM increased apoptosis in LAPC-4 cells and also in CRPC cells, namely, 22Rv1 and PC3 [191, 193, 194]. In addition, stimulation of PCa cells with both E₂ and αE₂ had other interesting effects, such as the reversion of the DHT- or cortisol-induced PSA expression, and the reduction of the DHT-induced DNA biosynthesis [169, 192].

The results obtained *in vitro* were translated into the *in vivo* setting. Wistar rats treated with a supraphysiological dose of E₂ showed a marked reduction of prostate weight underpinned by decreased cell proliferation and increased apoptosis compared to rats receiving placebo [195]. Several studies in animal tumor models (Table 2.5) also reported the effects of estrogens counteracting growth of malignant prostate tissue. In rodents, E₂, αE₂ or estradiol benzoate treatment reduced prostate tumor growth (size and volume) by suppressing proliferation of tumors cells and inducing apoptosis, both in androgen-sensitive and CRPC tumor models [169, 192, 196-201]. In addition, improvement of survival was described in the mice LuCaP 35 xenograft tumor model treated with E₂ [196]. Noteworthy, other reported effects of E₂ in mice included the reduction of testosterone and DHT serum concentrations and PSA levels [192, 196], which further corroborates the anti-tumorigenic effects of this hormone.

Also, the anti-tumor role of the endogenous metabolite of E_2 , 2-Methoxyestradiol (2-ME₂), has been widely studied in PCa. Kumar *et al.* [202] were the first in demonstrating that 2-ME₂ inhibits the growth of non-neoplastic prostate epithelial cells. This growth inhibitory effect and the reduction of cell proliferation by 2-ME₂ was also observed in a variety of PCa cell lines models, both in androgens-sensitive and CRPC cell lines (Table 2.5) [202-207]. Not surprisingly, 2-ME₂ has been highly associated with apoptosis induction in PCa (Table 2.5) [202-206, 208-213].

In vivo findings, both in rats and mice further supported the enormous amount of the *in vitro* evidence of the 2-ME₂ anti-tumorigenic role. Copenhagen X Fisher F1 male rats transplanted with Dunning R3327-PAP prostate tumors treated with 12.5 mg/kg/day of 2-ME₂ showed reduced tumor growth accompanied by increased apoptosis [208]. Accordingly, in both androgen-sensitive or CRPC xenograft mice models, 2-ME₂ treatment was able to reduce tumor growth, and augment apoptosis (Table 2.5) [203, 205, 206, 213-216]. Also in the TRAMP mice model, in which tumor development resemble the progression of human PCa (hyperplasia, 8-12 weeks; neoplasia, 15-18 weeks; metastasis, 24 weeks), the effect of 2-ME₂ treatment reducing tumor growth was confirmed. Administration of 2-ME₂ had an important role during the various stages of PCa, reducing prostate weight, malignant transformation, neoplastic progression, as well as, promoting tumor regression [205, 213, 216]. Moreover, these effects were accompanied by a reduction of testosterone [216] and PSA levels [205]. However, Ganapathy and colleagues [213] observed a reduction of apoptosis in TRAMP mice treated with 2-ME₂, which was suggested to be related with restoring of normal tissue architecture.

Some years ago, it was demonstrated that the DHT metabolites, 5 α -androstane-3 α ,17 β -diol (3 α -diol) and 5 α -androstane-3 β ,17 β -diol (3 β -Adiol) have estrogenic activities via activation of ER β [217-219]. Also, these estrogenic metabolites of DHT were shown to have anti-cancer properties. 0.1 μ M (and concentrations below) 3 β -Adiol diminished the proliferation and increased apoptosis of both androgen-sensitive and CRPC cells [220-222]. Accordingly, the proliferation of established tumors in PC3 xenografts mice tumor models was substantially reduced by a 3-weeks treatment with 3 β -Adiol [221].

ERs and GPER, and their downstream effectors in signaling pathways, are the molecular players involved in the control of prostate cell growth by estrogens [169, 192, 194]. However, the protective role of estrogens in PCa has been mainly attributed to ER β , which has been shown to have anti-proliferative and pro-apoptotic effects in PCa models [223, 224].

Over the years, the downstream effectors of the ERs actions have been disclosed. The stem cell factor (SCF) and its tyrosine kinase receptor c-KIT, with a recognized role in the control of cell proliferation and apoptosis, are important drivers of carcinogenesis in several tissues [225-227]. The SCF/c-KIT system was shown to be involved in the control of non-neoplastic prostate cell growth and a target of estrogens regulation. Treatment with supraphysiological doses of E₂ strongly diminished rat prostate weight, suppressed cell proliferation and induced apoptosis, underpinned by the diminished expression of SCF and c-KIT [195].

In PCa cells and tissues, estrogens actions in controlling cell proliferation strictly depended on the control of several cell cycle regulators, namely, cyclins, cyclin-dependent kinases (CDK), and CDK inhibitors. The anti-proliferative effects of estrogenic compounds such as αE₂, E₂ and 2-ME₂ have been related with cell cycle arrest at S, G₁ and G₂/M phases. αE₂ and E₂ seem to reduce cyclin A and cyclin D1 expression [192], and are involved in cyclin D1 phosphorylation through p38 kinase activation [194]. Cyclin D1 works in G₁ and cyclin A in S and G₂ phases [228]. The reduction of these cyclins disrupts cell cycle progression, reducing PCa proliferation. 2-ME₂ was shown to induce G₂/M arrest, increasing the G₂/M population, with a concomitant decrease in the G₁ population [202]. This G₂ block can be explained by the inhibition of CDK-1 activity, since 2-ME₂ leads to the accumulation of phosphorylated-CDK-1, by inducing the expression of the inhibitory Wee 1 kinase, which mediates the phosphorylation of CDK-1 at tyrosine 15 residue making it inactive [202, 229, 230]. 2-ME₂ administration also was shown to increase the expression levels of p53 and CDK inhibitor p21, which further detailed the molecular targets underlying the effect of this estrogenic compound disrupting cell cycle [206, 231].

Concerning the mechanistic involved in the pro-apoptotic effects of estrogens, both the extrinsic and intrinsic pathway of apoptosis have been shown to be activated by E₂ and estrogenic metabolites. E₂- and 2-ME₂-induced PCa cell apoptosis was shown to be mediated by the membrane death receptor Fas and its ligand (Fas L), with the subsequent activation of caspase-8 [195, 210, 213]. In fact, E₂ and its metabolite 2-ME₂ increased the expression of Fas and Fas L, as well as caspase-8 [195]. Activation of the extrinsic pathway of apoptosis upon Fas/Fas L binding causes the formation of the death inducing signaling complex (DISC) by the recruitment of several molecules, including the Fas associated death domain (FADD) and procaspase-8, which becomes activated [232-234]. 2-ME₂ was shown to augment DISC formation through inhibition of Akt, sensitizing PCa cells to Fas-mediated apoptosis [210]. The anti-apoptotic protein Fas-associated death domain interleukin-1 converting enzyme like inhibitory protein (FLIP) is a key inhibitor of Fas-induced apoptosis by competing with

procaspase-8 for FADD binding, thus preventing the recruitment of caspase-8 to FADD, or by inhibiting caspase-8 activation through DISC [235, 236]. Several studies reported that PCa cells treated with 2-ME₂ display diminished FLIP expression [209, 210, 213].

2-ME₂ pro-apoptotic effects through the activation of the intrinsic pathway of apoptosis encompass the regulation of several members of the Bcl-2 family, altering the ratio of pro- and anti-apoptotic proteins. This seems to occur by an indirect effect of 2-ME₂ reducing the levels of phosphorylated Akt, as well as by other mechanisms [208, 212, 215, 237-242]. Exposure to 2-ME₂ increased the levels of the pro-apoptotic proteins Bax and Bim and reduced the expression of Bcl-2, an anti-apoptotic member of the family [208, 213, 215, 243]. The pro-apoptotic Bid that promotes leakage of cytochrome C from mitochondria connects the intrinsic and extrinsic apoptotic pathways, as it is activated by caspase-8 [244, 245]. A study showed that 2-ME₂ could induce Bid cleavage with consequent reduction of Bcl-2 and caspase-8 activation [213].

2-ME₂ induction of apoptosis in PCa cells also seems to be correlated with the reduced levels of X-linked inhibitor of apoptosis protein (XIAP), a member of the inhibitor of apoptosis family of proteins (IAP) that inhibits caspase activity [231].

The pro-apoptotic actions of the 3 β -Adiol metabolite were shown to induce apoptosis of PCa cells by means of the intrinsic pathway through the reduction of Bcl-2, increase in Bax expression, and involvement of caspase-9, which culminates in the augmented activity of caspases-3/7 [220]. Furthermore, the 3 β -Adiol activation of apoptosis by the mitochondrial pathway seems to be triggered by the increased transcription of the transcription factor FOXO3a that causes an increase in the expression of p53 upregulated modulator of apoptosis (PUMA) [220].

2.4.1.2. Synthetic estrogens

Several synthetic compounds with estrogenic activity have been developed all over the years with the perspective of being used as anti-cancer molecules, suppressing PCa cell growth. With this rationale behind, the synthesized compounds were generally developed as ER β agonists, as this receptor has been assigned to have anti-proliferative and pro-apoptotic properties. Examples include the 8 β -VE₂ and 17 α -20Z-21-[(4-amino)phenyl]-19-norpregna-1,3,5(10),20-tetraene-3,17 β -diolthe (APVE₂) compounds (Table 2.5).

High doses of 8 β -VE₂ induced apoptosis in PCa cell lines and the prostate of rodent tumor models (Table 2.5), which was related to the interference in androgens/AR signaling. Androgens are the main players promoting PCa cells survival,

and androgens deprivation is well known to block prostate cell growth [246, 247]. For this reason, androgen deprivation therapy (ADT) is a gold standard in PCa treatment. A recent study demonstrated that stimulating ER β activity with the specific agonist 8 β -VE₂ diminished survival and increased apoptosis of VCaP cells [248]. This human PCa cell line overexpresses the AR and its splice variants linked with the resistance to ADT. Interestingly, 8 β -VE₂ treatment reduced the overexpression of AR and AR variants in VCaP cells under androgen deprivation [248], which renders this compound interesting in overcoming resistance to treatments. Results in mice models of BPH and PCa treated with 8 β -VE₂, demonstrated that ER β causes apoptosis through the activation of the extrinsic pathway and mediated by the TNF α , in an androgen independent manner [249, 250]. Other studies reported that 8 β -VE₂ induces apoptosis of PCa cells by activating both the extrinsic and intrinsic pathways [220, 249]. 8 β -VE₂ showed to increase the expression of caspase-8, with the subsequent activation of caspase-3, in a TNF α mediated process [249]. Concerning the intrinsic pathway, 8 β -VE₂ reduced Bcl-2 expression and increased the cleavage of caspase-9 [220]. Furthermore, 8 β -VE₂-induced apoptosis by the intrinsic pathway seems to be mediated by the increased levels of FOXO3a [220]. The APVE₂ compound showed to reduce PCa cells growth by arresting cell cycle at G2/M phase and inducing apoptosis, with effects perceived at nanomolar concentrations (Table 2.5) [251]. Studies concerning the anti-proliferative and pro-apoptotic actions of APVE₂ are scarce, and the mechanistic is highly unknown.

Other well-known synthetic estrogen is DES. It has been widely used for treatment of CRPC [252]. However, the molecular mechanisms underlying DES in PCa cells still need further clarification. This compound diminishes PCa cells viability [253], reduces proliferation and augments apoptosis [194, 254, 255]. DES treatment depleted the population of CRPC cells in G1 and S-phase, with an accumulation of cells in G2/M phase [254, 256]. DES was shown to decrease the phospho-ERK levels and JNK activation, increase the phosphorylation of the cell-cycle inhibitor protein p16^{INK4A} and activate p38 kinase, which then phosphorylates cyclin D1, tagging it for degradation and arresting cell cycle. This process was shown to be mediated by ER β , as well as by GPER [194].

Furthermore, DES actions suppressing cell division included the inhibition of telomerase activity, that is highly up-regulated in dividing cells, such as malignant cells [255], and the disruption of microtubule organization [256]. PCa cells stimulated with DES showed decreased density of microtubules [256], with suppression of β -tubulin isotypes I, II and IV, and α -tubulin isotypes I and IV, as well as, diminished expression of glyceraldehyde-3-phosphate dehydrogenase [256], which is a microtubule associated protein playing a role in bundling of microtubules [257].

In addition, DES showed to inhibit AR transactivation activity in PC3 and DU145 PCa cell lines, which indicates its anti-androgenic effects by attenuating the levels of AR-mediated transcription [258]. Other mechanism associated with DES includes the inhibition of HIF1 α activation and changes in a panoply of metabolites, including, lactate, phosphocreatine and reduced glutathione [259]. This suggests that DES can have a putative role in the metabolic rewiring of PCa cells.

Not surprisingly, the review of the existing knowledge concerning the role estrogens in controlling PCa growth showed the consistent and powerful effects of these hormones counteracting PCa cells proliferation. Indeed, these beneficial effects have been known for decades with estrogens being used in PCa therapy, and only disregarded because of the important adverse effects reported. Thus, the evidence of the anti-tumorigenic role of estrogens prompted the appearance of studies evaluating the effect of these hormones in combination with both chemotherapeutic drugs and natural compounds [193, 206, 207, 215, 260], as a strategy that could potentiate the action of each compound alone, allowing reducing the doses used and overcoming the problems related with collateral effects.

Table 2.5. Anti-proliferative, pro-apoptotic and growth/migration/invasion suppressing effects of estrogens in PCa cells.

Type of Study	Cell line/ Animal model	Estrogenic compound	Concentration / Dose	Assay model/ Administration method	Time of treatment	Method of evaluation	Effect	Activated pathway	Ref.
<i>in vitro</i>	PrECs	2-ME ₂	0.5, 1, 2, 3 and 5 μ M	96 well plate 6 cm dish	24 and 48 h	Trypan blue exclusion assay MTS Flow cytometry	↓ Cell growth ↑ Cells in G2/M phase ↓ Cells in G1 phase	G2/M arrest	[202]
	BPH-1	8 β -VE ₂ *	6 μ M	multichambered slides	12 h	Immunohistochemistry	↑ Apoptosis	↑ Casp-3 ↑ Casp-8	[249]
	RWPE-1	8 β -VE ₂ *	6 μ M	multichambered slides	12 h	Immunohistochemistry	↑ Apoptosis	↑ Casp-3 ↑ Casp-8	[249]
	ALVA-31	2-ME ₂	5 μ M	6 well plate 6 cm dish	1 - 6 days	Cell count in hemocytometer Analysis of cell-cycle distribution by FACS Annexin V-FITC and PI <i>in situ</i>	↓ Cell growth ↑ Cytotoxicity	-	[203]

						Caspase inhibition			
LAPC-4	αE_2	0.1 - 10 μM	96 well plate	72 h Up to 20 days	MTS assay Caspase 3/7 activity assays	$\downarrow$ DHT-induced PSA expression $\downarrow$ DHT-induced cell growth $\downarrow$ DHT-induced DNA biosynthesis	$\downarrow$ DHT-induced Cyclin A and Cyclin D1 expression	[192]	
LAPC-4	βE_2	0.1 - 10 μM	96 well plate	72 h Up to 20 days	MTS assay Caspase 3/7 activity assays	$\downarrow$ DHT-induced PSA expression $\downarrow$ DHT-induced cell growth $\downarrow$ DHT-induced DNA biosynthesis	$\downarrow$ DHT-induced Cyclin A and Cyclin D1 expression	[192]	
LAPC-4	E_2	10^{-8} - 1 μM	96 well plate	72 h	MTT assay Caspase 3 assay ROS assays	$\downarrow$ Cell viability $\uparrow$ Apoptosis	$\uparrow$ p-ERK levels $\uparrow$ ROS $\downarrow$ JNK $\uparrow$ Casp-3	[194]	

								activation ↑ Phospho-p16 ^{INK4A} ↑ Phospho-p38 ↑ Phospho-cyclin D1 ERα and ERβ mediated	
	LAPC-4	DES *	10 ⁻⁸ - 1 μM	96 well plate	72 h	MTT assay Caspase 3 assay ROS assays	↓ Cell viability ↑ Apoptosis	↓ p-ERK levels ↓ JNK ↑ Casp-3 activation ↑ Phospho-p16 ^{INK4A} ↑ Phospho-p38	[194]

								↑ Phospho-cyclin D1	
								ERβ and GPER mediated	
	LNCaP	2-ME ₂	5 μM	6 well plate 6 cm dish	1 - 6 days	Cell count in hemocytometer FACS Annexin V-FITC and PI <i>in situ</i> Caspase inhibition	↓ Cell growth ↑ Cytotoxicity	-	[203]
	LNCaP	2-ME ₂	10 μM	10 cm dish	0 - 48 h	DNA fragmentation assay TUNEL FACS	↑ Apoptosis	SAPK/JNK activation Bcl-2 phosphorylation	[208]
	LNCaP	APVE ₂ *	0.0001 - 1 μM	75 cm ² flasks 24 and 96 well plate	4 / 5 days	MTS BrdUrd uptake assay Flow cytometry	↓ Cell number	-	[251]

						Caspase assay			
						MTS			
	LNCaP-FGC	2-ME ₂	0.5 - 10 µM	96 well plate	4 - 72 h	Flow cytometry Cyclin B1 and A-dependent kinase assay Caspase-3 assay	↓ Cell growth	-	[231]
	LNCaP-FGC	2-ME ₂	2 µM	-	24 h	Flow cytometry Cyclin B1 and A-dependent kinase assay Caspase-3 assay	↑ Cells in G1 phase ↓ Cells in S phase	↓ Cyclin A-dependent kinase Activity ↑ p53 ↑ p21	[231]
	LNCaP-FGC	2-ME ₂	≥ 5 µM	-	24 h	Flow cytometry Cyclin B1 and A-dependent kinase assay Caspase-3 assay	↑ Cells in G2/M phase ↓ Cells in G1 phase ↑ Apoptosis	G2/M cell-cycle arrest ↑ Cyclin B1 and its associated kinase activity ↑ p53	[231]

								↓ XIAP	
	LNCaP	2-ME ₂	5 and 10 µM	-	48 h	ELISA Flow cytometry	↓ Cell number in G ₁ phase ↑ Cell number in S-phase ↑ Cell number in S and G ₂ /M phases ↑ Apoptosis	G ₂ /M arrest	[204]
	LNCaP	E ₂ + Cisplatin	0.1 and 0.2 µM + 10, 50 and 100 µM	96 well plate 3.6 cm dish	2 h	MTT	↓ Cell Viability	-	[260]
	LNCaP	2-ME ₂	1, 5 and 10 µM	75 cm ² flasks	48 h	Western Blot		↑ β-catenin	[243]
	LNCaP	2-ME ₂	0.5, 1, 3 and 5 µM	96 well plate 6 cm dish	24 and 72 h	MTS Apoptosis detection Colony formation assay		-	[207]
	LNCaP	2-ME ₂ +	0.5 µM +	6 cm dish	24 h	Apoptosis detection	↓ Cell growth	-	[207]

		Eugenol	41 µg/mL			Colony formation assay			
	LNCaP	2-ME ₂	0.01, 0.05, 0.1, 0.25, 0.5, 0.75 1 and 3 µM	96 well plate	72 h	Apoptosis detection	↑ Apoptosis	↑ cdc25c ↑ p34cdc2	[205]
	LNCaP	HE3235 * (in medium supplemented with AED)	0.05 µM	96 well plate 6 well plate	4 days	Cell counting in hemocytometer Annexin V-FITC	↓ Proliferation ↑ Apoptosis	-	[261]
	LNCaP	αE ₂	0.01 - 5 µM	96 well plate	3 days	MTS PSA measurements	↓ DHT- induced PSA ↓ DHT- induced cell growth	ER mediated	[169]
	LNCaP	βE ₂	0.01 - 5 µM	96 well plate	3 days	MTS PSA measurements	↓ DHT- induced PSA ↓ DHT- induced cell growth	ER mediated	[169]
	LNCaP	3β-Adiol	0.01 µM	6 well plate 6 cm dish	12, 24 and 48 h	TUNEL Annexin V assay Survival assay Immunocytochemistry	↑ Apoptosis ↓ Colony formation	↓ Bcl-2 ↑ Casp-3/7 activity ↑ PUMA ↓ p53	[220]

								↑ FOXO3a	
	LNCaP	2-ME ₂	3 μM	-	24 h	Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP)	↓ c-FLIP promoter activity	-	[209]
	LNCaP	2-ME ₂	0.5, 1, 2, 3 and 5 μM	96 well plate 6 cm dish	24 and 48 h	Trypan blue exclusion assay MTS Flow cytometry Mitotic index measurements Light microscopy TUNEL FITC-PARP assay Caspase activity assay	↓ Cell growth ↑ Cells in G2/M phase ↓ Cells in G1 phase ↓ Number of mitotic cells ↑ Apoptosis	G2/M arrest ↑ p21 ↑ p34cdc2 ↑ Wee 1 kinase ↑ phospho cdc2	[202]
	LNCaP	2-ME ₂	3 μM		2 - 24 h	Real-Time PCR analysis	↓ FLIP	-	[213]

	LNCaP	2-ME ₂	1 µM		12 - 72 h	Annexin V-fluorescein isothiocyanate (FITC)/7-amino-actinomycin D Caspase activity assay Assessment of mitochondrial membrane potential	↑ Apoptosis	↑ Bcl-2 homology 3 (BH3)-only protein harakiri (Hrk) ↑ PUMA ↑ NOXA ↑ p-JNK ↑ c-Jun expression and phosphorylation ↑ Cytochrome C release ↑ Caspase-3 activity	[242]
	LN-AI	2-ME ₂	0.5 - 1 µM	96 well plate 6 well plate	24 - 72 h	MTS Flow cytometry	↓ Cell growth ↑ Apoptosis	G2/M cell cycle arrest ↑ cleaved	[206]

						DAPI staining apoptosis assay		PARP	
						Clonogenic assay		↑ p53	
						Cyclin B1 and A- dependent kinase assay		↑ p21	
						Immunohistochemistry			
	LN-AI	2-ME ₂ + Docetaxel	0.5 - 1 μM + 0.05 - 0.1 μM	96 well plate 6 well plate	24 - 72 h	MTS		G2/M cell cycle arrest	
						Flow cytometry	↓ Cell growth	↑ cleaved PARP	
						DAPI staining apoptosis assay	↑ Apoptosis	↑ Cyclin B1- dependent kinase activity	[206]
						Clonogenic assay	↓ Colonies formation		
						Cyclin B1 and A- dependent kinase assay		↓ Cyclin A- dependent kinase activity	
						Immunohistochemistry		↑ p53	

								↑ p21	
	LN-AI/CSS	2-ME ₂	0.5 - 1 μM	96 well plate 6 well plate	24 - 72 h	MTS Flow cytometry DAPI staining apoptosis assay Clonogenic assay Cyclin B1 and A- dependent kinase assay Immunohistochemistry	↓ Cell growth ↑ Apoptosis	G2/M cell cycle arrest ↑ cleaved PARP	[206]
	LN-AI/CSS	2-ME ₂ + Docetaxel	0.5 - 1 μM + 0.05 - 0.1 μM	96 well plate 6 well plate	24 - 72 h	MTS Flow cytometry DAPI staining apoptosis assay Clonogenic assay Cyclin B1 and A- dependent kinase assay Immunohistochemistry	↓ Cell growth ↑ Apoptosis	G2/M cell cycle arrest ↑ cleaved PARP	[206]

	22Rv1	E ₂	1 µM	96 well plate	48 and 72 h	MTT Flow cytometry	↓ Proliferation ↑ Apoptosis	↑ ERβ ↑ p75NTR ↓ NF-κB translocation to the nucleus	[193]
	22Rv1	E ₂ + 5-AzaC	1 µM + 2 µg/ml	96 well plate	48 and 72 h	MTT Flow cytometry	↓ Proliferation ↑ Apoptosis	↑ ERβ ↑ p75NTR ↓ NF-κB translocation to the nucleus	[193]
	22Rv1	3β-Adiol	0.01 µM	6 well plate 6 cm dish	12 and 24 h	Survival assay	↓ Colony formation	↓ Bcl-2 ↑ Bax ↑ PUMA ↓ p53 ↑ FOXO3a	[220]
	DU145	2-ME ₂	5 µM	6 well plate 6 cm dish	1 - 6 days	Cell count in haemocytometer FACS	↓ Cell growth ↑ Cytotoxicity	Caspase-mediated	[203]

						Annexin V-FITC and PI <i>in situ</i> Caspase inhibition	G2/M cell-cycle arrest ↑ Apoptosis		
	DU145	2-ME ₂	10 µM	10 cm dish	0 - 48 h	DNA fragmentation assay TUNEL FACS	↑ Apoptosis	SAPK/JNK activation	[208]
	DU145	APVE ₂ *	0.0001 - 1 µM	75 cm ² flasks 24 and 96 well plate	4 / 5 days	MTS BrdUrd uptake assay Flow cytometry Caspase assay	↓ Cell number ↑ Apoptosis	↑ Caspase-3 activity	[251]
	DU145	2-ME ₂	0.5 - 10 µM	96 well plate	4 - 72 h	MTS Flow cytometry Cyclin B1 and A- dependent kinase assay Caspase-3 assay	↓ Cell growth	-	[231]
	DU145	2-ME ₂	≥ 5 µM	-	24 h		↑ Cells in G2/M	G2/M cell-	[231]

						Flow cytometry	phase	cycle arrest	
						Cyclin B1 and A-dependent kinase assay	↓ Cells in G1 phase	↑ Cyclin B1	
						Caspase-3 assay	↑ Apoptosis	↓ XIAP	
	DU145	2-ME ₂	5 and 10 μM	-	48 h	ELISA	↓ Cell number in G1 phase	G2/M cell-cycle arrest	
						Flow cytometry	↓ Cell number in S-phase	↑ Cyclin B1	
						Cyclin B1-cdc2 kinase activity assay	↑ Cell number in S and G2/M phases	↑ cdc2 phosphorylation	[204]
							↑ Apoptosis	↑ wee1	
								↑ cdc25C	
								ERK 2 and JNK pathways	
	DU145	2-ME ₂	1, 5 and 10 μM	75 cm ² flasks	48 h	Western Blot		MEK-ERK 2 pathways	[243]

								↓ Bcl-2 ↑ β-catenin ↓ β-catenin induced Cyclin D1	
DU145	2-ME ₂	0.5, 1, 3 and 5 μM	96 well plate 6 cm dish	24 and 72 h	MTS Apoptosis detection Colony formation assay	No effect	-	[207]	
DU145	2-ME ₂ + Eugenol	0.5 μM + 41 μg/mL	6 cm dish	24 h	Apoptosis detection Colony formation assay	No effect	-	[207]	
DU145	8β-VE ₂ *	6 μM	multichambered slides	12 h	Immunohistochemistry	↑ Apoptosis	↑ Casp-3 ↑ Casp-8	[249]	
DU145	3β-Adiol	0.001 - 0.01 nM	10 mm dish	48 h	Cell count in hemocytometer Adhesion assay Microchemotaxis assay Matrigel gel assay	↓ Proliferation	-	[221]	
DU145	2-ME ₂	5 μM	-	24 h	Clonogenic cell survival		↓ c-FLIP	[209]	

						assay	↑ Apoptosis ↓ Colony forming ability	promoter activity	
	DU145	3β-Adiol	0.001 - 0.1 μM	48-well Boyden's microchemotaxis chamber	5 and 48 h	Chemotaxis assay Western blot	↓ Migration	ERβ mediated ↑ E-cadherin	[222]
	DU145	2-ME ₂	0.5, 1, 2, 3 and 5 μM	96 well plate 6 cm dish	24 and 48 h	Trypan blue exclusion assay MTS Flow cytometry Mitotic index measurements Light microscopy TUNEL FITC-PARP assay Caspase activity assay	↓ Cell growth ↑ Cells in G2/M phase ↓ Cells in G1 phase ↓ Number of mitotic cells ↑ Apoptosis	G2/M cell-cycle arrest	[202]
	DU145	2-ME ₂	1 μM		12 - 72 h	Annexin V-fluorescein	↑ Apoptosis		[242]

						isothiocyanate (FITC)/7-amino-actinomycin D			
	PC3	2-ME ₂	5 µM	6 well plate 6 cm dish	1 - 6 days	Cell count in haemocytometer FACS Annexin V-FITC and PI <i>in situ</i> Caspase inhibition	↓ Cell growth ↑ Cytotoxicity	-	[203]
	PC3	2-ME ₂	10 µM	10 cm dish	0 - 48 h	DNA fragmentation assay TUNEL FACS	↑ Apoptosis	SAPK/JNK activation Bcl-2 phosphorylation	[208]
	PC3	2-ME ₂	1 - 1.5 µg/mL (aprox. 3 – 5 µM)	6 well plate	0 - 48h	Immunoblotting Flow Cytometry Electrophoretic mobility shift assays	↑ Apoptosis	G2/M cell-cycle arrest Fas-mediated ↓ NF-κβ/FLIP signaling	[210]

								↑ DISC formation ↓ Akt ERK-mediated	
	PC3	2-ME ₂	1, 3, 5, 10, 20 μM	24 well plate	1 - 12 days	Multicellular aggregate culture Detection of apoptotic nuclei by DAPI and TUNEL	↓ Cell aggregates growth ↑ Apoptosis	-	[211]
	PC3	APVE ₂ *	0.0001 - 1 μM	75 cm ² flasks 24 and 96 well plate	4 / 5 days	MTS BrdUrd uptake assay Flow cytometry Caspase assay	↓ Cell number	G2/M cell-cycle arrest	[251]
	PC3	2-ME ₂	0.5 - 10 μM	96 well plate	4 - 72 h	MTS Flow cytometry Cyclin B1 and A-dependent kinase assay	↓ Cell growth	-	[231]

						Caspase-3 assay			
	PC3	2-ME ₂	≥ 5 μM	-	24 h	Flow cytometry Cyclin B1 and A-dependent kinase assay Caspase-3 assay	↑ Cells in G2/M phase ↓ Cells in G1 phase	↑ Cyclin B1 and its associated kinase activity	[231]
	PC3	2-ME ₂	10 μM	-	24 h	Caspase-3 assay	↑ Apoptosis	G2/M cell-cycle arrest ↓ XIAP	[231]
	PC-3U	2-ME ₂	10 μM	10 cm dish	12 h	Detection of apoptotic nuclei by DAPI and M30	↑ Apoptosis ↑ c-Myc expression ↑ β-catenin stabilization	Smad7 expression p38 MAPK and JNK pathways	[212]
	PC3	2-ME ₂	5 and 10 μM	-	48 h	ELISA Flow cytometry	↓ Cell number in G1 phase	S-phase accumulation	[204]

						Cyclin B1-cdc2 kinase activity assay	↑ Cell number in S and G2/M phases ↑ Apoptosis	G2/M arrest	
	PC3	E ₂	0.001, 0.01 and 0.1 μM	96 well plate	24, 48, 72 and 96 h	MTT Acridine orange and ethidium bromide staining Flow cytometry	↓ Cell Viability ↓ Proliferation ↑ Apoptosis	↓ IGF-IR ↑ IGFBP-3 ↑ IGFBP-4 ↓ MMP-2 ↓ MMP-9	[191]
	PC3	E ₂ + Cisplatin	0.1 and 0.2 μM + 10, 50 and 100 μM	96 well plate 3.6 cm dish	2 h	MTT assay	↓ Cell Viability	-	[260]
	PC3	2-ME ₂	1, 5 and 10 μM	75 cm ² flasks	48 h	Western Blot	↑ β-catenin	-	[243]
	PC3	2-ME ₂	0.5, 1, 3 and 5 μM	96 well plate 6 cm dish	24 and 72 h	MTS Apoptosis detection	↓ Cell growth	-	[207]

						Colony formation assay			
	PC3	2-ME ₂ + Eugenol	0.5 µM + 41 µg/mL	6 cm dish	24 h	Apoptosis detection Colony formation assay	↓ Cell growth ↑ G2/M population ↓ G1 population ↑ Apoptosis	↓ Bcl-2 ↑ Bax ↓ MMP	[207]
	PC3	8β-VE ₂ *	6 µM	multichambered slides	12 h	Immunohistochemistry	↑ Apoptosis	↑ Casp-3 ↑ Casp-8	[249]
	PC3	2-ME ₂	0.5 - 1 µM	96 well plate 6 well plate	24 - 72 h	MTS Flow cytometry DAPI staining apoptosis assay Clonogenic assay Cyclin B1 and A- dependent kinase assay Immunohistochemistry	↓ Cell growth	G2/M cell- cycle arrest ↑ p21	[206]
	PC3	2-ME ₂ +	0.5 - 1 µM +	96 well plate	24 - 72 h	MTS	↓ Cell growth	G2/M cell- cycle arrest	[206]

		Docetaxel	0.05 - 0.1 μ M	6 well plate		Flow cytometry DAPI staining apoptosis assay Clonogenic assay Cyclin B1 and A- dependent kinase assay Immunohistochemistry	↓ Colonies formation	↑ B1-dependent kinase activity ↑ p21	
	PC3/AR	2-ME ₂	0.5 - 1 μ M	96 well plate 6 well plate	24 - 72 h	MTS Flow cytometry DAPI staining apoptosis assay Clonogenic assay Cyclin B1 and A- dependent kinase assay Immunohistochemistry	↓ Cell growth	cell-cycle	[206]
	PC3/AR	2-ME ₂ + Docetaxel	0.5 - 1 μ M + 0.05 - 0.1 μ M	96 well plate 6 well plate	24 - 72 h	MTS Flow cytometry	↓ Cell growth	cell-cycle	[206]

						DAPI staining apoptosis assay			
						Clonogenic assay			
						Cyclin B1 and A- dependent kinase assay			
						Immunohistochemistry			
	PC3	3 β -Adiol	0.001 – 0.01 μ M	10 mm dish	48 h	Cell count in hemocytometer	↓ Proliferation	-	[221]
	PC3	3 β -Adiol	0.01 μ M	6 well plate 6 cm dish	12 and 24 h	Survival assay	↓ Colony formation	↓ Bcl-2 ↑ Cleaved casp-9 ↑ PUMA ↑ FOXO3a	[220]
	PC3	8 β -VE ₂ *	0.01 μ M	6 well plate 6 cm dish	12 and 24 h	Survival assay	↓ Colony formation	↓ Bcl-2 ↑ Cleaved casp-9 ↑ FOXO3a	[220]
	PC3-Luc	3 β -Adiol	0.001 – 0.01 μ M	10 mm	48 h	Cell count in	↓ Proliferation	ER β mediated	[221]

				dish		hemocytometer	↑ Cell adhesion		
						Thymidine incorporation assays	↓ Migration		
						Adhesion assay	↓ Invasion		
						Microchemotaxis assay			
						Matrigel gel assay			
	PC3	E ₂	10 ⁻⁸ - 1 μM	96 well plate	72 h	MTT assay		↑ p-ERK levels	
						Caspase 3 assay	↓ Cell viability	↑ ROS	
						Necroptosis assays	↑ ROS	↑ JNK	
						ROS assays		↑ Phospho-p16 ^{INK4A}	[194]
								↑ Phospho-p38	
								↑ Phospho-cyclin D1	
								ERβ and	

								GPER mediated	
	PC3	DES *	10^{-8} - $1 \mu\text{M}$	96 well plate	72 h	MTT assay Caspase 3 assay Necroptosis assays ROS assays		↓ p-ERK levels ↓ JNK ↑ Phospho-p16 ^{INK4A} ↑ Phospho-p38 ↓ Phospho-cyclin D1	[194]
	PC3	2-ME ₂	3 μM		24 h	Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP)	-	↓ c-FLIP promoter activity	[209]
	PC3	3 β -Adiol	0.001 – 0.1 μM	48-well Boyden's microchemotaxis chamber	5 and 48 h	Chemotaxis assay Western blot	↓ Migration	-	[222]
	PC3-AR	3 β -Adiol	0.001 – 0.1 μM	48-well Boyden's microchemotaxis chamber	5 and 48 h	Chemotaxis assay Western blot	↓ Migration	-	[222]

	PC3	2-ME ₂	3 µM		2 - 24 h	Real-Time PCR analysis Immunoblotting Immunoprecipitation	↑ Apoptosis	Cleavage of Bid Casp-8 activation ↓ FLIP ↓ Bcl-2	[213]
	PC3	2-ME ₂	1 µM		12 - 72 h	Annexin V-fluorescein isothiocyanate (FITC)/7-amino-actinomycin D Caspase activity assay Assessment of mitochondrial membrane potential	↑ Apoptosis	↑ Bcl-2 homology 3 (BH3)-only protein harakiri (Hrk) ↑ PUMA ↑ NOXA ↑ Bim ↑ p-JNK ↑ c-Jun expression and phosphorylation	[242]

								on ↑ Cytochrome C release ↑ Caspase-3 activity	
	VCaP	8β-VE ₂ *	5 or 25 μM	96 well plate	24, 48, and 72 h	Measurements of cell survival and apoptosis induction	↓ Cell survival ↑ Apoptosis	↓ AR	[248]
	VCaP rev	8β-VE ₂ *	5 or 25 μM	96 well plate	24, 48, and 72 h	Measurements of cell survival and apoptosis induction	↓ Cell survival ↑ Apoptosis	↑ AR	[248]
	VCaP AA	8β-VE ₂ *	5 or 25 μM	96 well plate	24, 48, and 72 h	Measurements of cell survival and apoptosis induction	↓ Cell survival ↑ Apoptosis	↓ AR	[248]
	C4-2B	HE3235 *	0.01 and 0.05 μM	6 well plate	3 days	Trypan blue exclusion assay	↓ Proliferation	-	[262]
	MDA-Pca 2b	αE ₂	0.01 - 5 μM	96 well plate	3 days	MTS PSA measurements	↓ Cortisol- induced PSA	ER mediated	[169]
	MDA-Pca 2b	βE ₂	0.01 - 5 μM	96 well plate	3 days	MTS PSA measurements	↓ Cortisol- induced PSA	ER mediated	[169]
	Human primary	8β-VE ₂ *	6 μM			Flow cytometry	↑ Apoptosis	-	[250]

	epithelial cell cultures from benign regions of prostate tissue								
	Primary culture from prostatic epithelial cells of C57BL6/J wild type mice	8 β -VE ₂ *	6 μ M	12 well plate	7 days	Spheroid assay	↓ Self-renewal ability	-	[250]
<i>in vivo</i>	Wistar (Rattus norvegicus) male rats	E ₂	250 mg/ kg/ day	Intraperitoneally injected	5 days	Prostate weigh measure Fluorescent immunohistochemistry (ki-67) TUNEL Caspase-3 assay	↓ Prostate weight ↓ Proliferation ↑ Apoptosis	↑ Fas and Fas L ↑ Caspase-8 ↑ Caspase-3 activity ↓ SFC/c-KIT expression	[195]
	Copenhagen Fisher rats + Dunning R3327-PAP tumors	Oestradiol benzoate *	50 μ g	s.c. injections	12 h, 24 h, 72 h and 168 h	Tumor volume measure Immunohistochemistry for proliferating cell nuclear antigen (PCNA) <i>In situ</i> detection of	↓ Tumor volume ↓ Proliferation ↑ Apoptosis	-	[201]

						apoptotic cells			
	Copenhagen Fisher rats + Dunning R3327- PAP tumors	E ₂	50 µg / day	s.c. injections	6 weeks	Volume measurements and morphological examinations of tumors	↓ Tumor growth Tumor degeneration	-	[199]
	Copenhagen Fisher rats + Dunning R3327- PAP tumors	E ₂	50 µg / day	s.c. injections	4 h, 12 h and 24 h or 6 weeks	Morphological examination Immunohistochemistry TUNEL	↑ Apoptosis	↑ TGF-β ₁ ↑ TGF-β RI ↑ TGF-β RII	[200]
	Copenhagen Fisher rats + Dunning R3327- PAP tumors	2-ME ₂	12.5 mg/kg/day	intraperitoneally	14 days	Measure of tumor diameter TUNEL	↓ Tumor growth ↑ Apoptosis	-	[208]
	Gy/T-15 transgenic mouse	2-ME ₂	75 mg/kg	s.c. implanted slow-release tablets	21 days	Palpation	↓ Tumor weight	-	[203]
	HONDA xenograft tumor model in nu/nu mice	2-ME ₂	20 mg/kg per day	Intraperitoneally	72 h	TUNEL CD31 immunohistochemical analysis	↓ Tumor growth ↑ Apoptosis ↓ Microvessel density	-	[214]
	LAPC-4	αE ₂	2 mg plus 18 mg	s.c. implanted	4 weeks	PSA and testosterone	↓ Tumor growth	-	[192]

	xenograft tumor model in SCID mice		cholesterol	pellet		levels determination			
	LAPC-4 xenograft tumor model in SCID mice	βE_2	2 mg plus 18 mg cholesterol	s.c. implanted pellet	4 weeks	PSA and testosterone levels determination	↓ PSA levels	-	[192]
	PC3 tumor model in nu/nu mice	2-ME ₂	20 mg/kg per day	Intraperitoneally	72 h	TUNEL CD31 immunohistochemical analysis	↑ Apoptosis ↓ Microvessel density	-	[214]
	LNCaP tumor model in nude athymic mice	2-ME ₂	25 mg/kg and 75 mg/kg per day	Orally	62 days	Tumor histology TUNEL Determination of serum PSA	↓ Tumor size ↓ Necrosis ↑ Apoptosis ↓ PSA levels	-	[205]
	LuCaP35V tumor model in male CB-17 SCID mice	HE3235 *	160 mg/kg	Intraperitoneal injection	5 d/wk for 4 weeks	Determinations of intratumoral androgens levels PSA determination Immunohistochemistry	↓ Tumor growth ↑ PSA levels ↓ Testosterone and DHT levels ↓ AR expression	-	[262]

	C4-2B tumor model in male CB-17 SCID mice	HE3235 *	160 mg/kg	Intraperitoneal injection	7 d/wk for 4 weeks	Radiography Bone mineral density measurements Immunohistochemistry	↓ Weight of tumor tibiae ↓ PSA levels ↓ AR expression	-	[262]
	Balb-c/Nude mice	8β-VE ₂ *	300 µg/kg/d	s.c. injection	3 days	Immunohistochemistry	↑ Apoptosis	↑ Caspase-8 Extrinsic apoptotic pathway TNFα mediated	[249]
	BPH tissue xenografts in male NOD/SCID mice	8β-VE ₂ *	300 µg/kg/d	s.c. injection	3 days	Immunohistochemistry	↑ Apoptosis	↑ Casp-8 Extrinsic apoptotic pathway TNFα mediated	[249]
	PCa tissue xenograft in male NOD/SCID mice	8β-VE ₂ *	300 µg/kg/d	s.c. injection	3 days	Immunohistochemistry	↑ Apoptosis	↑ Casp-8 Extrinsic apoptotic	[249]

								pathway	
	FG/Tag transgenic mouse model of AI-PC	2-ME ₂	150 mg/kg	-	24 and 48 h	Flow cytometry Immunohistochemistry	↓ Primary prostate tumors ↑ Apoptosis ↓ Cell proliferation ↓ Angiogenesis	G2/M cell -cycle arrest ↑ Cleaved caspase-3	[206]
	FG/Tag transgenic mouse model of AI-PC	2-ME ₂ + Docetaxel	75 mg/kg + 2.5 mg/kg	-	24 and 48 h	Flow cytometry Immunohistochemistry	↓ Primary prostate tumors ↑ Apoptosis ↓ Cell proliferation ↓ Angiogenesis	G2/M cell- cycle arrest ↑ Cleaved caspase-3	[206]
	FG/Tag transgenic mouse model of AI-PC	2-ME ₂ + Docetaxel	150 mg/kg + 5 mg/kg	-	24 and 48 h	Flow cytometry Immunohistochemistry	↓ Primary prostate tumors ↑ Apoptosis ↓ Cell	G2/M cell- cycle arrest ↑ Cleaved caspase-3	[206]

							proliferation ↓ Angiogenesis		
	AED-stimulated LNCaP tumor model in castrated SCID mice	HE3235 *	200 ml / day	Intraperitoneal injection	21 days	-	↓ Tumor incidence ↓ Tumor growth	-	[261]
	PC3 xenograft tumor model in BALB/c nu/nu male nude mice	3β-Adiol	2.5 mg / kg / day	s.c. injection	3 weeks	BLI acquisition	↓ Proliferation of established tumors	-	[221]
	PC3 xenograft tumor model in BALB/c nu/nu male nude mice	3β-Adiol	2.5 mg / kg / day	s.c. implanted pellet	4 weeks	BLI acquisition	↓ PCa progression ↓ Metastasis	-	[221]
	LuCaP 35 xenograft tumor model in male SCID mice	E ₂	0.36 mg	s.c. implantation of slow-release	90 days	Measurements of tumor volume	↓ Tumor growth ↑ Survival ↓ Testosterone and DHT levels	-	[196]
	LNCaP xenograft tumor model in male nude mice (Harlan™)	αE ₂	2 mg αE ₂ plus 18 mg cholesterol	s.c. implanted pellet	4 weeks	Measurements of tumor size	↓ Tumor growth ↓ Tumor size	-	[169]

	LNCaP xenograft tumor model in male nude mice (Harlan™)	βE_2	2 mg βE_2 plus 18 mg cholesterol	s.c. implanted pellet	4 weeks	Measurements of tumor size	↓ Tumor size	-	[169]
	C57BL6/J wild type mice	$8\beta\text{-VE}_2^*$	6 μM		24 h	Flow cytometry Immunohistochemistry	↑ Apoptosis	-	[250]
	LNCaP xenograft animal models	αE_2	2 mg αE_2 plus 18 mg cholesterol	s.c. implanted pellet	4 weeks	Immunohistochemistry	↓ Microvessel number	-	[263]
	LNCaP xenograft animal models	βE_2	2 mg βE_2 plus 18 mg cholesterol	s.c. implanted pellet	4 weeks	Immunohistochemistry	↓ Microvessel number	-	[263]
	LAPC-4 xenograft animal models	αE_2	2 mg αE_2 plus 18 mg cholesterol	s.c. implanted pellet	4 weeks	Immunohistochemistry	↓ Microvessel number	-	[263]
	LAPC-4 xenograft animal models	βE_2	2 mg βE_2 plus 18 mg cholesterol	s.c. implanted pellet	4 weeks	Immunohistochemistry	↓ Microvessel number	-	[263]
	LNCaP xenograft tumor model in male BALB/c nude	2-ME ₂	150 mg / kg	Intraperitoneally	4 weeks	Measurements of tumor size Immunohistochemistry	↓ Tumor growth ↓ Proliferation ↑ Apoptosis ↓ Microvessel	↑ Bax ↓ Bcl-2 Caspase-3 mediated	[215]

							density	↓ pAkt	
								↓ VEGF	
	LNCaP xenograft tumor model in male BALB/c nude	2-ME ₂ + Quercetin	150 mg / kg + 75 mg / kg	Intraperitoneally	4 weeks	Measurements of tumor size Immunohistochemistry	↓ Tumor growth ↓ Proliferation ↑ Apoptosis ↓ Microvessel density	↑ Bax ↓ Bcl-2 Caspase-3 mediated ↓ pAkt ↓ VEGF	[215]
	PC3 xenograft tumor model in male BALB/c nude	2-ME ₂	150 mg / kg	Intraperitoneally	4 weeks	Measurements of tumor size Immunohistochemistry	↓ Tumor growth ↓ Proliferation ↑ Apoptosis ↓ Microvessel density	↑ Bax ↓ Bcl-2 Caspase-3 mediated ↓ pAkt ↓ VEGF	[215]
	PC3 xenograft tumor model in male BALB/c nude	2-ME ₂ + Quercetin	150 mg / kg + 75 mg / kg	Intraperitoneally	4 weeks	Measurements of tumor size Immunohistochemistry	↓ Tumor growth ↓ Proliferation	↑ Bax ↓ Bcl-2	[215]

							↑ Apoptosis ↓ Microvessel density	Caspase-3 mediated ↓ pAkt ↓ VEGF	
	DU145 xenograft tumor model in male BALB/ cA-nu castrated male mouse	E ₂	3.4 mg	s.c implanted pellets	25 - 35 days	Measure of tumor size TUNEL assay Soft-agar colony formation assay Immunohistochemistry Matrigel plus angiogenesis assay	↓ Tumor growth ↓ Angiogenesis ↓ Apoptosis	↓ FOXO1 ERβ and PDGFA, KLF5 pathway	[14]
	PC3 xenograft tumor model in male BALB/ cA-nu castrated male mouse	E ₂		s.c implanted pellets	25 - 35 days	Measure of tumor size TUNEL assay Soft-agar colony formation assay Immunohistochemistry Matrigel plus	↓ Tumor growth ↓ Angiogenesis ↓ Apoptosis	↓ FOXO1 ERβ and PDGFA, KLF5 pathway	[14]

						angiogenesis assay			
	TRAMP mice in pure C57BL/6 8-week-old (hyperplasia)	2-ME ₂	50 mg / kg	Orally in diet	16 weeks	Measurements of prostate weight Histopathologic evaluation Analysis of hormone levels in serum Immunohistochemistry Invasion assay	↓ Prostate weight ↓ Neoplastic Progression ↓ Testosterone levels	↑ TSG-6	[216]
	TRAMP mice in pure C57BL/6 24-week-old (metastasis)	2-ME ₂	50 mg / kg	Orally in diet	8 weeks	Measurements of prostate weight Histopathologic evaluation Analysis of hormone levels in serum Immunohistochemistry Invasion assay	↓ Prostate weight ↓ Carcinogenesis ↓ Testosterone levels	↑ TSG-6	[216]
	LuCaP 35 xenograft tumor	E ₂	-	s.c. implanted pellets	90 days	Measurement of tumor volume	↓ Tumor volume	-	[198]

	in 15 ovariectomized female BALB/c nu/nu mice								
	TRAMP mice (22-25 weeks)	2-ME ₂	50 mg / kg	Orally in water	25 weeks	Analysis of palpable tumors number Volume of the prostate seminal vesicle complex Histological evaluation	Tumor regression ↓ Palpable tumors occurrence ↓ Prostate weight ↓ Proliferation ↓ Apoptosis (restoring normal tissue architecture)	↓ Sp1 ↓ FLIP	[213]
Patients	Prostate Carcinoma	Polyestradiol phosphate *	240 mg	Intramuscular injections	2 years	Radioimmunological or immunoradiometric assays	↓ Bone <i>turnover</i>	↓ Osteocalcin ↓ PICP ↓ PIIINP ↓ ICTP	[264]

* - synthetic

2.4.2. Suppressive actions in tumor progression and metastization

The protective actions of estrogens in PCa can also be extended to the suppression of tumor progression and metastization. Tumor progression is a complex process encompassing several biological mechanisms that determine the molecular, morphological, and functional changes in cancer cells and tumor microenvironment. These changes result in the reorganization of the extracellular matrix, promotion of angiogenesis, inflammation, and in the modulation of the immune system [265], which altogether concurs to tumor progression, invasion and metastization.

Loss of cell-cell adhesion, which depends on the disruption of cell-to-cell and cell-matrix interactions, and the involvement of several molecular players, is critical for the occurrence of cell migration and invasion. E_2 and 2-ME₂ seem to interfere in tumor progression by up-regulating and stabilizing the β -catenin protein, an important molecule in cell-cell adhesion [212, 243, 266]. β -catenin is a key component of the cadherin/catenin complex that mediates calcium-dependent homophilic interactions between cell adhesion molecules [267]. Furthermore, 2-ME₂ was shown to reduce the migratory ability of PCa cells by increasing the interaction of β -catenin with E-cadherin [243]. E-cadherin is the most abundant adhesion molecule in epithelial adherents junctions, and its loss is frequently associated with migration, invasion and metastatic dissemination [268]. The estrogenic metabolite 3 β -Adiol increased E-cadherin expression and cell adhesion, which resulted in a reduction of migration and invasion [221, 222]. *In vivo* results confirmed that 3 β -Adiol decreased PCa progression with the ability to reduce metastasis formation [221]. Furthermore, it was reported that 3 β -Adiol control the progression of PCa through the activation of ER β [221, 222]. Accordingly, studies in CRPC cells reported that ER β activation leads to the increase of E-cadherin and β -catenin expression, and reduced expression of N-cadherin [266, 269].

Other important molecules involved in remodeling of extracellular matrix and tumor progression are the MMPs. Their action degrading the extracellular matrix, play an important role in tumor cell invasion and metastasis. Also, MMPs have been shown to be regulated by estrogens. Kanagaraj *et al.* [191] reported that E_2 was able to reduce the expression levels of MMP-2 and MMP-9. These findings are highly interesting as both MMPs are prostate gland secretion products and their higher levels were related with the aggressive behavior of PCa and metastization [270, 271].

The angiogenic process is essential for tumor cell survival, growth and metastasis by providing nutrients and oxygen to the growing tumor cells [272]. Angiogenesis comprises endothelial cell proliferation and migration, which can be

activated by tumor cells via paracrine factors or direct cell-to-cell communication [273, 274]. The vascular endothelial growth factor (VEGF) is a crucial angiogenic factor with mitogenic and anti-apoptotic effects. It regulates angiogenesis by inducing proliferation, migration and permeability of endothelial cells [275]. VEGF expression is highly enhanced in developing tumors [276]. LAPC-4 or LNCaP cells conditioned media was shown to induce the growth of a murine endothelial cell line (MEC) and VEGF was the main factor responsible for this paracrine stimulation [263]. E_2 administration inhibited the paracrine effect of DHT in stimulating MEC proliferation [263]. Accordingly, both E_2 and its stereoisomer αE_2 reduced the number of microvessels in the prostate tumor tissues of LAPC-4 or LNCaP xenograft models [263]. Also, E_2 seems to suppress angiogenesis by inhibiting the $ER\beta$ - and KLF5-mediated expression of platelet-derived growth factor subunit A (PDGFA) [14]. This factor plays an essential role in regulating cell migration and chemotaxis [277], and its overactivity can trigger carcinogenesis [278]. The E_2 metabolite 2- ME_2 also displayed anti-angiogenic properties. 2- ME_2 administration to LNCaP and PC3 xenografts reduced prostate microvessels density [206, 214, 215], which occurred by decreasing VEGF expression [215].

Estrogenic compounds also showed to control inflammation in PCa. It is the case of 2- ME_2 that inhibited the progression of PCa in TRAMP mice by increasing the expression of TNF- α -stimulated gene 6 (TSG-6), a secreted glycoprotein with anti-inflammatory action [216]. No further evidence exists of the anti-inflammatory actions of estrogens either *in vitro* or *in vivo*. Nevertheless, a study in non-cancer patients showed that circulating estrogens were inversely associated with intraprostatic inflammation [73], which suggests that these hormones can have a role in controlling prostate inflammation and PCa progression.

2.5. Natural compounds with estrogen-like activity in prostate cancer: the phytoestrogens scope

Among all the natural products that have been used in improving human health, as either preventive supplements or treatment, phytoestrogens are a class of compounds of particular interest. The relevance of phytoestrogens in human health relies on their wide distribution in several types of plants and fruits and their potent and diverse biological effects. Phytoestrogens have recognized antioxidant, anti-proliferative, anti-angiogenic and pro-apoptotic activity [34], making them highly attractive to be exploited in the context of anti-cancer therapy. Also, a substantial number of

epidemiological studies supports the protective role of phytoestrogens in malignancy. For example, the incidence of breast and PCa is much lower in Asian people when compared with Westerns [279]. The average daily consumption of phytoestrogens in Asia is estimated to range from 20 to 50 mg, whereas in the United States and Europe, it is predicted to be 0.153 mg and 0.491 mg, respectively [280]. This astonishing difference is justified by the dietary habits of the Asian population with a plant-based diet and much less consumption of meat and animal-derivative products [281], and it has been postulated to explain the lower incidence and mortality rates of PCa in Eastern countries [282, 283].

2.5.1. Classification and structure

Phytoestrogens are a group of natural biologically active phenolic compounds derived from plants and fruits, which because of their analogous structure to the principal estrogen, E₂, display estrogenic and/or anti-estrogenic activity [284]. The phytoestrogens encompass two main classes of compounds, flavonoids and non-flavonoids. The first group includes flavones, isoflavones, flavanones, flavonols and coumestans [285-287]. Stilbenoids and lignans are included in the non-flavonoids class [285, 286]. Table 2.6 resumes the types of phytoestrogens with an identified role in modifying human prostate cell fate.

2.5.2. Sources and metabolism

Phytoestrogens are abundant in nature and mainly present in fruits, vegetables and whole grains usually consumed by humans [288]. Moreover, they are also found in several edible and/or medicinal plants [288]. Plant extracts with recognized estrogen-like activity comprise soy, red clover, kudzu, hops, licorice, rhubarb, yam and chasteberry, among others [289].

After being consumed, phytoestrogens are metabolized by intestinal bacteria, absorbed, conjugated in the liver, disseminated in plasma and expelled in urine [290]. Gut metabolism has been shown to have a crucial role in determining the effectiveness of phytoestrogens actions. For some members of this class of compounds, the estrogenic effect is mainly due to the metabolites generated by digestion than by the original phytoestrogen molecule [291]. For example, daidzein is a phytoestrogen that is converted to a more active metabolite, equol, with enhanced estrogenic activity

compared with the original molecule [292]. However, the effect of equol is variable depending on individual specificities, since the ability to this conversion and the extension of this reaction appears to be restricted to approximately 1/3 of the population [292]. Moreover, the bioavailability and uptake of some isoflavones require some metabolic processes that allow absorption to the peripheral circulation, such as hydrolysis and re-conjugation to glucuronic and sulfuric acids [293, 294].

Despite some limitations on bioavailability and bioaccessibility, the potential of phytoestrogens as anti-cancer molecules is unquestionable, and continuous research would help in identifying new preventive or treatment approaches.

2.5.3. Mechanisms of action

As above-mentioned, the molecular structure of phytoestrogens is very similar to E_2 and they can bind $ER\alpha$ and $ER\beta$. However, binding affinity seems to be higher for the $ER\beta$ [295, 296]. After ligand binding, the receptors are translocated to the nucleus, where they bind to specific regulatory regions, the estrogens response elements, modulating gene transcription and, consequently, the cell protein network and activity [297]. Besides their ability to bind to ERs, phytoestrogens can modulate the activity of other transcription factors and pathways, namely, serotonergic and IGF-1 receptors signaling [298-300]. The effect of these compounds can also be exerted by binding free radicals, inducing DNA methylation and histone modification, and modulating tyrosine kinase, cAMP/protein kinase A, cGMP/NO, PI3K/Akt and MAP kinases activity [301-304].

In addition to classical ERs, phytoestrogens also have been shown to activate GPER [305].

2.5.4. Phytoestrogens actions against prostate cancer

2.5.4.1. Apigenin

Apigenin ($C_{15}H_{10}O_5$), also known as 4',5,7-trihydroxyflavone, is a nontoxic and nonmutagenic flavonoid widely present in common fruits and vegetables [306]. This phytoestrogen has proven to have anti-inflammatory and anti-carcinogenic effects in preclinical studies. Several types of cancer are sensitive to apigenin, namely, leukemia, breast, colon, lungs, skin, thyroid and prostate [307].

Apigenin effects showed to inhibit PCa growth, reducing cell proliferation and inducing cell cycle arrest at G2-M phase [308-311]. In LNCaP and DU145 cells, cell cycle inhibition by apigenin was linked to a marked decrease of cyclins D1, D2 and E expression, and their activating partners CDK2, 4 and 6 [312, 313]. This phytoestrogen also increased the expression of tumor suppressor protein p53 and cell cycle inhibitor p21 [309, 312-317], and caused the dephosphorylation and inactivation of retinoblastoma protein (Rb) [318].

The anti-proliferative effect of apigenin seems to be mediated through the activation of ER β or the suppression of the IGF axis, one of the well-known crucial pathways for cell proliferation [319, 320]. Although no studies were performed in PCa, apigenin also acts through GPER [321].

Apigenin's actions also showed to be related to the control MAPK and Akt/ PI3K several survival pathways [318]. Inactivation of the Akt pathway, and stimulation of the production of reactive oxygen species (ROS), culminated in the apoptosis of PCa cells [317, 322, 323]. Akt is known to phosphorylate the Bad protein, which renders the anti-apoptotic Bcl-2 protein free and activated and cells resistant to death. Thus, Akt inactivation promptly is linked to increased apoptosis as Bcl-2 is inactivated. Apigenin treatment was shown to shift the Bax/Bcl-2 ratio towards apoptosis by increasing Bax and reducing Bcl-2 expression [312, 314, 316, 317].

Existent reports also indicate that apigenin can induce apoptotic cell death triggered at the cell membrane. Stimulation of PCa cells with apigenin strongly induced the expression of DR5 and TRAIL death receptors [324, 325], as well as, caspase-8, caspase-10, caspase-9 and caspase-3 [313, 322-324].

Noteworthy, apigenin treatment significantly decreased the viability of cancer cells and augmented apoptosis, contrastingly with the low magnitude of effects observed in non-neoplastic cells [308, 324], indicating a selectivity over PCa cells.

Apigenin also inhibited the cellular processes related to cancer progression, such as EMT, migration and invasion. Exposure to apigenin resulted in decreased cell motility and reversion of EMT [326, 327], and the inhibition of the migration and invasive potential of PCa cells [328]. These effects showed to be dose- and time-dependent [328].

In LNCaP and PC3 cells, apigenin treatment also inhibited the expression of VEGF and HIF α , angiogenesis and hypoxia markers, respectively [329-331]. HIF α is a known driver of the hyperglycolytic phenotype of cancer cells in response to hypoxia by regulating the expression of several targets in the glycolytic flux, namely, glucose transporters (GLUTs), as is the case of GLUT1 [332]. Accordingly, in the presence of apigenin, PCa cells displayed decreased expression of HIF α accompanied by the

diminution of GLUT expression [333, 334], which likely limited nutrient availability contributing to suppress proliferative activity. Exposure of PCa cells to apigenin also diminished the activity of fatty acid synthase [333, 335], a target metabolic regulator whose expression is highly increased in PCa [336]. Overall, these findings indicate the potential of apigenin to counteract the metabolic rewiring in PCa.

The beneficial properties of apigenin could be observed in animal models [337], and a study reported that a herbal extract containing apigenin was able to enhance the chemotherapeutic effect of docetaxel in PCa [338]. Also, apigenin combined therapy enhanced the efficacy of abiraterone acetate [339] and doxorubicin [340], and sensitize the PCa cells to radiation [341]. Moreover, apigenin sensitized PCa stem cells to therapy, which is relevant as this cell population is generally resistant to therapy and is believed to be involved in the non-response of PCa to anti-cancer drugs and disease recurrence [35, 36]. Overall, the present data support the interest in this natural agent.

2.5.4.2. Chrysin

Chrysin ($C_{15}H_{10}O_4$), also known as 5,7-dihydroxyflavone, is a flavone that can be extracted from honey, propolis, and blue passion flowers [342]. This agent is considered a valuable resource for health purposes as it shows anti-cancer, anti-angiogenic, anti-inflammatory, anti-diabetic, anti-bacterial, anti-aging and anti-allergic properties [343-348]. Chrysin showed some binding activity to the nuclear ERs and GPER [349-351]; however, there is no evidence of ER β - or GPER-mediated actions of this compound in PCa. Nevertheless, chrysin suppressed proliferation and induced apoptosis of PCa PC3 cells [352-354]. One of the mechanisms by which this substance induces apoptosis is through the production of ROS [353]. In PCa DU145 cells, chrysin decreased HIF α expression by inhibiting its protein synthesis [343].

In parallel to the effects controlling PCa cell fate, a report shows that the use of chrysin as complement together with docetaxel increases the therapeutic efficacy of this anticancer drug, mitigating some side effects, such as edema [355].

2.5.4.3. Biochanin A

Biochanin A ($C_{16}H_{12}O_5$), 5,7-dihydroxy-4'-methoxyisoflavone, is an *O*-methylated isoflavone that can be found, for example, in red clover, soy, alfalfa sprouts, peanuts and other legumes [356]. This flavonoid was shown to induce a dose-dependent inhibition of LNCaP, DU145 and PC3 cells proliferation and viability [315, 357]. Furthermore, it was suggested that ER β could mediate the anti-proliferative effect of

biochanin A as this compound stimulated the receptor expression in PC3 cells [357]. Although biochanin A action through GPER has been reported [358], no studies were done in PCa.

The capability of biochanin A to suppress cell division, with the accumulation of PCa cells in G₀ phase [359], was related to the decreased expression of cyclins B and E and oncogene c-Myc, as well as with the increased expression of cell cycle inhibitor p21 [315, 357, 359]. Akt and ERK were other proliferative and survival signaling pathways associated with biochanin A actions in PCa cells [357].

In addition, this isoflavone was able to increase the apoptotic rate of LNCaP and PC3 cells [315, 357, 359, 360], which was suggested to be mediated by the TRAIL cell death receptor [360]. Also, biochanin A modulated the expression levels of mitochondrial regulators Bax and Bcl-2 governing apoptosis [357].

Administration of biochanin A *in vivo* reduced the incidence and tumor size in an LNCaP xenograft model [359]. Studies exploring the potential of biochanin A as a possible therapy in PCa are inexistent. However, a report showed that it reduced PSA production by PC3 cells [361].

2.5.4.4. Daidzein

Soybean is the major source of daidzein (C₁₅H₁₀O₄), a natural isoflavone chemically known as 7-hydroxy-3-(4-hydroxyphenyl)-4Hchromen-4-one [362]. Other legumes and fruits can also be important sources of daidzein [362].

Similarly to other flavonoids, daidzein has anti-proliferative effects in PCa cells. Exposure of PC3 cells to a soy isoflavone concentrate highly enriched in daidzein caused the accumulation of cells in the G₂/M phase of the cell cycle underpinned by the increased expression of p21 [363].

BPH-1, LNCaP and PC3 cells treated with daidzein exhibited higher apoptotic rates than the control untreated groups [364-366]. Concerning the activated apoptotic pathway, this isoflavone increased the expression of DR4 and DR5 death receptors and thus triggered TRAIL-mediated apoptosis [366]. In PC3 cells, treatment with daidzein increased the expression of the pro-apoptotic Bax protein [364], also implicating the intrinsic pathway of apoptosis.

Additionally, several studies reported that daidzein can reduce PCa growth [367-371] by suppressing migration, invasion and angiogenesis [372-374].

This isoflavone has been shown to interact with ER β with some selectivity [354, 375], and novel daidzein analogues showed anti-carcinogenic activity in PCa cells through ER β mediation [376]. Also, GPER seems to be involved in the daidzein

mechanism of action, with reports existing in other cancer types [377, 378]. Further investigation is needed to clarify if ERs mediate the daidzein effects in PCa.

The remarkable beneficial effects of daidzein counteracting the cancer hallmarks were tested in clinical trials. Men diagnosed with PCa consuming daily a phytoestrogen-enriched diet containing daidzein showed favorable evolution of PSA levels and free/total PSA ratio [379]. Other significant observed effects included the inhibition of tumor growth and a chemopreventive action in prostate carcinogenesis, and with nontoxic effects [380-393].

2.5.4.5. Equol

Equol ($C_{15}H_{14}O_3$), (3S)-Isoflavan-4,7'-diol, is a metabolite of the isoflavone daidzein produced by the activity of intestinal bacteria [394, 395]. Only about 30 to 50% of the population can convert daidzein to equol, with a much higher prevalence found in Asian countries compared with Western [396, 397]. Equol has two diastereoisomers, R-equol and S-equol, being the last one the natural form. Equol has been shown to have a modest binding affinity for ERs, though it has been suggested that it mimics estrogen's effects via ER β and GPER [398-400]. Equol was reported to have stronger bioactivity than daidzein and also genistein, namely, in what concerns the antioxidant activity [401].

The anti-proliferative activity of equol in PCa has been demonstrated in LNCaP, CxR, 22Rv1 and PC3 cells. Equol administration resulted in the inhibition of cell proliferation and diminished cell growth [402-404]. Moreover, equol increased the number of LNCaP, LAPC-4, 22Rv1, DU145 and PC3 cells arrested in Go [402, 403]. However, the mechanisms by which equol control cell proliferation and the dependency on ERs still needs clarification.

Equol also showed to increase apoptosis of PCa cells [366, 402, 403, 405]. Despite the information provided by several studies, the mechanistic underlying the pro-apoptotic effect of equol is highly unknown. However, in LNCaP cells, the presence of this compound was shown to increase the expression of death receptors, specifically, the TRAIL receptor [366].

Although the information available is very limited, equol was shown to inhibit the invasion of DU145 cells probably by downregulating the expression of MMP-2 and MMP-9 [406].

Reports in animal models and PCa patients also supported the beneficial effects of equol delaying prostate tumor growth [380, 386, 407-411]. Feeding rats with soy flour (containing daidzein that is converted to intestinal metabolite equol) inhibited the growth of Dunning R3327 prostate adenocarcinoma [380]. In the TRAMP mice, prostate carcinogenesis induced by a high-fat diet occurred concomitantly with the lowered equol serum concentrations because of the adverse effects over equol-producing bacterium [410], which demonstrates the relevance of this bioactive compound in prostate physiology. Equol's effects include the reduction of PSA levels, and the blockage of the PSA increase in response to DHT stimulation both *in vitro* and *in vivo* [409]. These findings were mirrored in human patients. Men with higher levels of equol in the diet presented reduced PSA levels [386, 412], and lower PCa risk and incidence [386, 407, 411].

2.5.4.6. Formononetin

The isoflavone formononetin ($C_{16}H_{12}O_4$), 7-hydroxy-4'-methoxyisoflavone, can mainly be found as a component of red clover plants [413]. This type of plant is a component of traditional Chinese herbal medicine and has been widely used in China for thousands of years. Formononetin seems to promote cell cycle arrest in PC3 cells by downregulating the Akt pathway and cyclin D1 and CDK4 expression [414]. Moreover, exposure of LNCaP, DU145 and PC3 cells to formononetin resulted in the induction of apoptotic cell death, which was shown to occur by several mechanisms, namely, the augment of the Bax/Bcl-2 ratio, suppression of the p38/Akt pathway through the downregulation of the IGF-1/IGF-1R signaling pathway, and the inactivation of ERK1/2 mitogen-activated protein kinase [415-418].

A recent study showed a synergistic anti-tumor effect in PCa cells by the combination of formononetin with docetaxel in one nano-sized drug delivery system [419].

Formononetin can interact with ER β [354] and GPER [420], with ER-mediated effects reported in breast cancer [421]. No evidence of this relationship exists in PCa, and further research is needed to implicate ERs in the anti-proliferative and pro-apoptotic actions of formononetin over prostate cells.

2.5.4.7. Genistein

Genistein ($C_{15}H_{10}O_5$), chemically known as 4',5,7-trihydroxyisoflavone, is the main isoflavone present in the human diet, as it is predominantly included in the

composition of soybeans, peas, lentils and other beans [422]. This compound has been shown to interact with both ER isoforms being one of the most potent phytoestrogens stimulating the transcriptional activity of both ER α and ER β [354]. However, genistein interaction with ER α showed to be one-thousandth of E₂ potency, whereas for ER β the potency was one-third of the E₂ [354]. Therefore, it is the selective binding to ER β that sustains the genistein anticancer activity [354, 423, 424]. Nevertheless, genistein action through GPER has been reported in other types of cancer except PCa [425, 426].

Multiple biological processes in PCa cells have been shown to be modulated by the activity of genistein. This compound seems to decrease the viability and proliferation of LNCaP, 22RV1, DU145 and PC3 cells [369, 427-435], which was linked to the downregulated expression of cyclin B and upregulation of the cell cycle inhibitor p21 [427, 428, 434]. Furthermore, in LNCaP, DU145 and PC3 cells, genistein arrested cell cycle in G2/M [427-430, 433, 434]. Curiously, cell growth inhibition by genistein was associated with the suppression of telomerase activity and inhibition of stemness by targeting of the hedgehog pathway [431, 432]. Another mechanism linked to the genistein anticancer activity was its ability to downregulate the expression of the oncogene *MDM2*, both at transcriptional and posttranslational level [436].

Genistein also strongly modulates the survival of PCa cells. It inhibited activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B), mediated by Akt signaling [437]. Genistein inhibited the radiation-induced activation of NF κ B pathway in PCa cells [427], and Akt phosphorylation, which resulted in increased apoptosis [433, 438]. Another study reported that this flavonoid could abrogate epidermal growth factor (EGF)-induced activation of Akt and inhibit the Akt kinase activity [437].

The reported *in vitro* findings were mirrored in human PCa cases. By analyzing radical prostatectomy specimens, it was shown that the consumption of genistein was related to the increase of apoptotic rate [383, 438], together with the upregulation of p53 [438].

Genistein can also control the metastatic spreading of PCa cells. This natural compound demonstrated to inhibit the contact-stimulated migration of PCa cells [372, 433, 439] and to suppress MMP-2 activity and cell invasion [433, 440-443]. Furthermore, it reversed the EMT in PCa cells by increasing E-cadherin expression and reducing that of vimentin, both at mRNA and protein level [444, 445]. Moreover, genistein-treated DU145 e PC3 cells displayed decreased activity of focal adhesion kinase [446], which was suggested to control the aggressiveness of PCa [447].

Also, the angiogenic process in PCa seems to be modulated by genistein, since treatment with this flavonoid suppressed VEGF expression [448, 449].

Interestingly, the stimulation of LNCaP, LAPC-4, VeCap and PC3 cells with genistein also decreased or even inhibited PSA expression [450, 451].

Genistein, as other plant-derived compounds, can induce epigenetic alterations in human cells, altering the landscape of active molecular targets [452]. In PCa, studies showed that exposure of LNCaP and LAPC-4 cells to this natural agent reduced the ER β promoter methylation, increasing the receptor expression [453], which can be an additional mechanism enhancing the estrogenic effects of genistein.

This phytoestrogen can also modulate DNA methylation in the promoter regions of retinoic acid receptor β (RAR β 2), glutathione S-transferase P1 (GSTP1), Ras association domain family 1 (RASSF1A) and ephrin B2 (EPHB2) genes, which seems to be protective against PCa [454, 455]. Moreover, this natural compound increased the expression of the tumor suppressor gene BTG3 in RWPE-1, DU145 and PC3 cells, as well as sFRP1 and Smad4 genes, via demethylation and histone modification [433, 456].

Noteworthy, genistein has also been reported to modulate the expression of regulatory microRNAs. It reduce the expression of the oncogenic miRNAs miR-1260b and miR-151 that are upregulated in PCa [433, 457]. On the other hand, genistein treatment increased the tumor suppressor miR-574-3p, which is down-regulated in PCa [458].

Studies in animal models confirmed the beneficial properties of dietary genistein suppressing the development of PCa [459-462]. A low-fat diet with soy protein and isoflavones, namely, genistein, reduced tumor growth in LNCaP xenografts [463]. Furthermore, genistein in the diet seems to reduce the incidence of poorly differentiated PCa and improve survival in TRAMP mice [464-467]. This anti-tumor role of genistein is supported by reports that showed a reduction of proliferation and increase of apoptosis in the ventral prostate of Simian Virus-40 T-antigen (SV-40 Tag) targeted probasin promoter rat model, a transgenic model developing PCa spontaneously [461], and in the cancerous prostate dorsolateral prostate of Lobund-Wistar Rats [460]. At molecular level, genistein reduced the cyclin D1 levels post-transcriptionally, disrupting cell cycle progression and thus reducing proliferation [464]. One of the molecular mechanisms related to these genistein effects is the regulation of the PTEN/Akt axis. Genistein in the diet up-regulated PTEN, inhibiting the activation of Akt and restoring the activation of GSK-3 β [460, 464]. Furthermore, genistein in the diet retained cadherin-1 expression, through the decrease of snail-1 transcription [464], which can be critical in maintaining the integrity of prostatic epithelial cells and retarding cancer progression.

Curiously, this natural compound reduced AR mRNA and protein levels [460, 468] and ER α and ER β expression in the rat prostate [468]. Moreover, genistein showed to down-regulate the AR levels in LNCaP cells through the activity of ER β [469], which highlights that besides their direct actions, estrogens could modulate the response to androgens. In addition, genistein decreased steroid receptor coactivator-3 (SRC-3) levels in the ventral prostate of SV-40 Tag rats [461]. SRC-3 is an important regulator of PCa proliferation and survival and is essential for the progression of prostate tumorigenesis in the TRAMP model [470]. Another interesting effect of genistein is its ability to reduce intraprostatic DHT levels together with diminished expression of 5 α -reductase-2 [459], corroborating the benefit of genistein to prevent prostate diseases such as PCa.

The protective role of genistein against PCa development is also supported by the reduction of IGF-1 [459, 461], a player that has been associated with increased risk of an advanced stage of PCa [471]; and the inhibition of osteopontin [465], an extracellular matrix protein secreted by infiltrating macrophages and prostate tumors cells themselves [472-474], which is related with increased tumorigenicity and metastatic ability [475].

From a therapeutic perspective, the beneficial effects of genistein, when joined to classical chemotherapy and anti-androgenic drugs, are worth noting. In fact, this isoflavonoid has been tested in clinical trials with PCa patients with promising results [386, 476-482].

The combination of genistein with polysaccharides enhanced the activity of docetaxel, bicalutamide and Src kinase inhibitors in both androgen-sensitive and CRPC cells, namely, in LNCaP, CWR22Rv1 and PC3 cells [483]. Furthermore, this natural agent increased the efficacy of cabazitaxel in C4-2, ARCaP_M and PC3 cells [484] and paclitaxel in LNCaP and DU145 cells [485]. Genistein has also been shown to potentiate the effect of radiation diminishing PCa cell proliferation and tumor growth [486-490]. The use of BIO 300, a nanosuspension of genistein sensitized human PCa xenografts to radiation therapy [491].

Additionally, several reports demonstrated that genistein combined with SB715992, an experimental inhibitor of kinesin spindle proteins that play an essential role in mitotic spindle formation, can improve the outcomes in PCa patients [492].

A limitation found in clinical trials for the use of genistein in treatment is its reduced bioavailability upon oral delivery. Using nanostructured delivers carrying genistein is a possible solution to increase its bioavailability and enhance anti-cancer action against PCa [493]. Some approaches were tested with good results, namely, a nanoliposomal formulation encapsulating celecoxib and genistein showed to inhibit the

COX-2 pathway and GLUT1 expression, preventing PCa cell proliferation [494]; other liposomal formulation, containing plumbagin and genistein inhibited proliferation and induce apoptosis of PCa cells [495, 496]; nanoparticles loaded with genistein and doxorubicin reduced the development of PCa metastasis by amplifying oxidative stress damage [497] and genistein-gold nanoparticles conjugates displayed antioxidant and anti-tumorigenic effects in PCa [498].

Despite the beneficial effects of genistein against PCa, attention should be given to reports that demonstrated a dual effect of this compound in PCa cells and *in vivo* [499-503]. Moreover, it is critical deeply understand the mechanisms underlying genistein actions in PCa and establish the ideal threshold dose to avoid dual effects before disseminating its use in treatment.

2.5.4.8. Naringenin

Naringenin ($C_{15}H_{12}O_5$), 4', 5, 7-trihydroxyflavanone, is a bioactive compound mainly found in grapefruit and orange [504, 505], which demonstrated to have anti-proliferative and pro-apoptotic activity in PCa cells [506]. Available information concerning naringenin effects and mechanism of action in PCa still is scarce. However, naringenin-induced apoptotic cell death was shown to be mediated by the PI3K/Akt and MAPK signaling pathways [506]. This biologically active substance also was able to repress the migration and invasion of PCa cells by increasing the expression of E-cadherin, and diminishing vimentin expression levels [507, 508]. On the other hand, exposure of LNCaP cells to naringenin stimulated DNA repair, preventing mutagenic changes as a consequence of oxidative damage [509], which raises curiosity about the role of this compound against early events in human prostate carcinogenesis.

Naringenin interaction with the ERs has been demonstrated, with a stronger binding affinity and higher transcriptional activity for ER β [354]. Also, it showed to modulate the receptors expression levels in breast tumors, decreasing ER α expression while increasing ER β [510]. Thus, it is reasonable to assume that the naringenin actions in PCa could be mediated by ERs, with a relevant role for ER β . Additional investigation is needed to clarify this relationship and further explore the naringenin actions.

2.5.4.9. Kaempferol

Kaempferol ($C_{15}H_{10}O_6$), 3, 4', 5, 7-tetrahydroxyflavone, is a secondary metabolite found in many plants, plant-derived foods and traditional medicines [511], which seems to have an important role against PCa. The beneficial effects of kaempferol are

demonstrated by a set of *in vitro* and *in vivo* experimental evidence [512-516]. Several studies have reported the anti-proliferative effects of kaempferol [370, 517, 518], increasing the proportion of PCa cells blocked in G2/M phase [370, 518] and its ability to inhibit the DHT-stimulated growth of LNCaP cells [517]. Moreover, the pro-apoptotic properties of kaempferol have been described [515, 517, 519, 520]. This compound augmented apoptosis of PCa cells accompanied by the increased expression of TRAIL, enhanced levels of caspase-8, caspase-9 and caspase-3, and by the inhibition of the NFκB pathway [515, 519, 520].

A study addressing the effect of a mix of flavonoid compounds, including kaempferol, in a preclinical murine model demonstrated the ability of these compounds to target AR signaling and inhibit PCa growth [516]. Despite the known selectivity of kaempferol for ERs [354], no ER-mediated effects were reported in PCa. Nevertheless, as shown in other tissues, it seems to act mainly by antagonizing ERα activity [521, 522].

2.5.4.10. Myricetin

Myricetin (C₁₅H₁₀O₈), 3,3',4',5,5',7-Hexahydroxyflavone, is a family member of flavonoids normally found in vegetables, fruits, nuts, berries, tea and red wine [523, 524]. This phytoestrogen has recognized antioxidant properties [525], and it was demonstrated to inhibit proliferation and induce apoptosis in PC3 cells [526]. Moreover, myricetin effectively suppressed the *in vivo* progression of PCa by disrupting the interaction of the protooncogene protein proviral integration site for Moloney murine leukemia virus-1 (PIM1) with the C-X-C chemokine receptor type 4 (CXCR4) [527]. PIM1 is overexpressed in PCa, playing an important role in tumorigenesis, castration resistance, and metastasis [528-532]. PIM1 promotes the phosphorylation and surface expression of CXCR4, supporting the CXCL12–CXCR4 axis responsible for cancer cell proliferation and metastasis [530, 533].

An interesting study showed that PLGA-encapsulated myricetin formulation combined with enzalutamide enhanced the effects of enzalutamide alone in a xenograft model of PCa [534].

This compound showed to bind ERs competing with E₂ and was able to reduce ERα expression in breast cancer cells [535, 536]. Nevertheless, there is no evidence of the involvement of ERs in mediating the myricetin effects in PCa.

2.5.4.11. Quercetin

Quercetin ($C_{15}H_{10}O_7$), also known as 3,3',4',5,7-pentahydroxyflavone, is a polyphenol belonging to flavonoids, and commonly found in several fruits and vegetables, namely, capers, lovage, dill, cilantro, onions, apples and berries [537]. The main biological properties of this phytoestrogen include antioxidant, antiallergic, anti-inflammatory and anti-viral activity [538-540]. Additionally, it has been proved that quercetin can be useful against several types of cancer, such as lung, liver, breast, colon, cervical, and prostate [307].

Quercetin can reduce the viability and proliferation of LNCaP, LAPC-4, DU145 and PC3 cells [518, 541-546], in part through diminishing the action of growth factors [547, 548]. Quercetin showed to arrest cell cycle progression in all phases of the cycle [541, 546, 549, 550]. In LNCaP cells, quercetin arrested the cell cycle in the S phase, significantly reducing the number of cells in G1 [546]. The control of cell cycle progression and cell proliferation was shown to depend on the regulation of several molecular targets. Quercetin strongly inhibited the expression of cyclin B1 and CDK1 and increased the levels of p21 and hypophosphorylated Rb protein in a dose-dependent manner in PC3 cells [541, 550]. Moreover, the inhibition of PCa growth by quercetin can be mediated by the reduction of AR signaling since quercetin demonstrated the ability to suppress AR expression and inhibit receptor function [543, 544, 548, 551]. Considering ERs, although quercetin has been shown to act through these receptors in other tissues [354, 425, 552], no reports exist concerning the involvement of ERs in PCa cell responses.

Research efforts have been made to clarify quercetin's actions and the molecular players involved in stimulating PCa cell apoptosis. Quercetin induced TRAIL-mediated apoptosis of LNCaP, DU145 and PC3 cells by increasing the expression of TRAIL and DR5 cell death receptors [519, 553, 554]. Moreover, the exposure of PCa cells to quercetin promoted the activation of caspase-3, caspase-8 and caspase-9 and affected the expression of several apoptosis-related proteins. It decreased the levels of anti-apoptotic proteins Bid and Bcl-2 and increased the expression of pro-apoptotic protein Bax [526, 549, 553, 555-558].

Another mechanism reported to diminish the survival of DU145 cells in response to quercetin is the induction of ROS production, which will lead to an increase in oxidative stress culminating with cell death [559].

Quercetin also affects PCa cell migration and invasion, stemness and angiogenesis [560]. It synergizes with epigallocatechin gallate to inhibit PCa stem cell characteristics, EMT, invasion and migration [561]. Accordingly, the expression of

prostate stem cell antigen is downregulated by quercetin in DU145 cells [562, 563]. Moreover, quercetin decreased the migration of PC3 cells induced by TGF- β [564]. In PC3 cells, quercetin also reversed the EGF-induced EMT and invasiveness via activation of the EGFR/PI3K/Akt pathway [565] and downregulated expression of MMP-2 and MMP-9 [566]. Furthermore, this phytoestrogen demonstrated the capability of reducing angiogenesis by targeting VEGFR-2 regulated Akt/mTOR signaling pathways [567].

Interestingly, quercetin actions also seem to target the immune response, which can be highly relevant for treatment purposes, particularly considering immunotherapy. It stimulated GM-CSF production in PC3 cells, and the conditioned medium of quercetin-treated PC3 cells increased chemotaxis of human dendritic cells, suggesting that quercetin treatment can promote the recruitment of dendritic cells to the tumor site [568].

Noteworthy, quercetin sensitized PCa cells to chemotherapy and anti-androgenic drugs. In combination with docetaxel, paclitaxel, doxorubicin or enzalutamide, quercetin increased the sensitivity of PCa cells to treatment leading to a notorious anticarcinogenic effect compared with the administration of the anticancer drugs alone both *in vitro* and *in vivo* [569, 570][42-45, 571].

Several robust findings pointed out the mechanisms that stimulated the effects of chemotherapeutics in suppressing cell proliferation and invasion, increasing apoptosis, and reducing tumor growth in the presence of quercetin, such as inhibition of PI3K/Akt and signal transducer and activator of transcription (Stat) 3 signaling pathways [42, 43], increased expression of cleaved caspase-7 [44], activation of the mitochondrial/ROS pathway [42], decreased expression of the multidrug resistance-related protein (MRP1) [43], and reduced blood concentrations of growth factors like VEGF and EGF [44].

Resistance to the next-generation antiandrogen, enzalutamide, has been proposed to depend on a large extent of the AR splice variant AR-V7 [572]. This constitutively active variant is generated by the alternative splicing of the AR involving the activity of splicing factors such as hnRNPA1 [573, 574]. Quercetin reduced the expression of the hnRNPA1 and, consequently, that of AR-V7, sensitizing resistant PCa cells to enzalutamide treatment [45]. Furthermore, quercetin can reverse docetaxel-resistance *in vitro* and *in vivo* through AR and PI3K/Akt signaling pathways, supporting its clinical use in docetaxel-resistant PCa [575]. Quercetin also increased PCa radiosensitivity by targeting the radiation-induced ARv7 [576], supporting the possibility of applying radiotherapy combined with quercetin in PCa treatment. Studies in rodents further confirmed the potential of quercetin to prevent PCa growth by

suppressing cell survival and proliferation and the expression of anti-apoptotic proteins [577, 578]. Quercetin supplementation reverted the augmented levels of proliferative and anti-apoptotic markers, as well as the decreased expression of pro-apoptotic markers resultant of N-Methyl-N-nitrosourea plus testosterone induction of prostate carcinogenesis [577]. In this carcinogen-induced PCa model, quercetin also increased the expression of antioxidant enzymes and decreased the expression of insulin-like-growth factor receptor 1 (IGF-1R), Akt and AR [577].

The beneficial use of quercetin was also reported in PCa patients [37, 38]. A study evaluated the association between serum vitamin D and dietary quercetin and PCa risk in African men. In individuals with vitamin D deficiency, the augmented levels of quercetin in diet were linked to a lower risk of PCa [38]. Also, it was reported that metastatic PCa patients receiving increasing doses of quercetin display longer PSA doubling time [37]. However, the enormous benefits of quercetin in suppressing PCa growth have some limitations in clinical practice and therapy. As other natural compounds, quercetin displays reduced water solubility and low bioavailability, which largely conditions the potential of therapeutic application. To solve this problem, few studies have evaluated the use of nanoparticles to deliver quercetin directly to PCa cells. The use of cationic PEGylated niosome-containing quercetin and quercetin-loaded nanomicelles assembled from DSPE-PEG₂₀₀₀ had promising results regarding tumor regression [579, 580]. More recently, several approaches, such as LHRH-conjugated, PEGylated, poly-lactide-co-glycolide nanocapsules [581], carboxylated graphene oxide as nanocarrier [582], chitosan-coated, quercetin-loaded PLGA nanoparticles [583] and others [584] showed promising results in controlling PCa growth and progression, opening new avenues of research for exploiting treatment approaches.

2.5.4.12. Coumestrol

Coumestrol ($C^{15}H^8O^5$), 3,9-Dihydroxypterocarp-6a(11a)-en-6-one, is a natural organic compound found in soybean, legumes, Brussels sprouts, alfalfa and spinach [585, 586]. Recently, this natural compound was shown to exhibit anticancer activity against several types of cancer [587, 588]. However, only a few studies have investigated the effects of coumestrol in PCa cells. Treatment with coumestrol inhibited the proliferation of LNCaP and PC3 cells and stimulated apoptosis, by inducing DNA damage and mitochondrial dysfunction, as a consequence of loss of mitochondrial membrane potential and increased ROS production [405, 589]. Furthermore, PCa cells treated with coumestrol showed elevated levels of the pro-apoptotic protein Bad and cleaved caspase-3 and caspase-9 [589].

The anti-proliferative and pro-survival properties of coumestrol seem to be mediated by the modulation of PI3K/AKT and ERK1/2 and JNK MAPK cell signaling pathways, which were also implicated in the decreased migration of LNCaP and PC3 cells in the presence of this phytoestrogen [589]. Furthermore, the capability of coumestrol reducing PCa progression can be related to the suppression of HIF α . In hypoxic PC3 cells, coumestrol suppressed HIF α by inhibiting ROS-mediated sphingosine kinase 1 [590], a key enzyme that converts sphingosine into sphingosine 1-phosphate [591], a sphingolipid metabolite and a lipid mediator crucial in several biologic processes of carcinogenesis [592-594].

In humans, coumestrol intake was associated with a reduced risk of developing PCa [595]. Noteworthy coumestrol's actions have been related to its ability to bind ERs, with the capability of transcriptional activation of both ER α and ER β at concentrations of 1-10 nM [354, 596]. Indeed, as genistein, coumestrol is among the phytoestrogens with higher estrogenic potency in transactivation assays compared with E₂ [354]. In PCa, evidence for the ER-mediated effects of coumestrol is scarce. Nevertheless, the intake of phytoestrogens like coumestrol substantially reduced PCa risk among men with specific polymorphic variation in the promoter region of the ER β gene [597], which is suggestive of this receptor involvement.

2.5.4.13. Resveratrol

Resveratrol (C₁₄H₁₂O₃), also known as trans-3,40,5-trihydroxystilbene is a stilbene polyphenol highly abundant in red grapes, peanuts and other fruits [598]. Generally, red wine still comprises resveratrol in amounts ranging from 0.1 to 14.3 mg/L [599].

This natural agent is considered a phytoestrogen due to its ability to bind ERs and GPER, competing with natural estrogens and altering the normal biological response, including an anti-carcinogenic effect [600-602]. Several studies showed that resveratrol targets cancer cells without producing harmful effects on non-malignant cells [46, 47]. For this reason, it has been considered an ideal molecule for anti-cancer therapy.

Resveratrol has obtained attention as a chemopreventive agent targeting multiple signaling pathways and affecting several cellular processes, namely, cell viability, proliferation, apoptosis, survival, migration and invasion, and metabolism. A large number of studies have shown that resveratrol reduces the viability and proliferation of both androgen-sensitive and CRPC cells, namely, LNCaP, 22Rv1, DU145 and PC3 cells. [253, 603-608]. It increased the number of cells in G₀/G₁ arresting phase and diminished the number of cells in the G₂ phase [606-608], likely by reducing the

expression of cyclin D1, B1 and E and increasing the expression of some cell cycle inhibitors, such as p21 and p27 [607, 609]. Interestingly, resveratrol seems to reduce PC3 and 22Rv1 cell survival without affecting non-malignant PNT1A cells [607]. In fact, the antiproliferative effect of resveratrol was associated with its ability to reduce ER α and increase ER β expression [610, 611]. Other mechanisms associated with resveratrol effects in reducing PCa cell proliferation include the inhibition of the nuclear factor NF κ B, which is also associated with cell survival, and the inhibition of the mTORC1 pathway that promotes cancer cell growth, survival, invasion and angiogenesis [612, 613].

Induction of PCa cell apoptosis by resveratrol was dependent on modulating the expression of the pro- and anti-apoptotic proteins. Resveratrol can upregulate the expression of pro-apoptotic genes *BAX*, *BID* and *BAK* and downregulate the anti-apoptotic ones, *MCL-1*, *BCL-2* and *BCL-XL* in several human and rodent PCa cell lines, namely, LNCaP, C4-2B, DU145, PC3, and TRAMP-C1, TRAMP-C2, and TRAMP-C3 [614-618]. The consequent alterations in the apoptosis-related proteins culminate with the increased activity of several caspase family members, namely, caspase-7 and caspase-3 [253, 608, 614, 616, 618, 619]. Furthermore, resveratrol sensitized LNCaP cells and PC3 xenografts to TRAIL-mediated apoptosis by increasing the expression of TRAIL receptors [620, 621]. Other mechanisms involved in resveratrol-induced apoptosis include the increased expression of p53, the generation of ROS, the downregulation of the PI3K/Akt survival pathway, and autophagy induction [614-617, 622]. Moreover, the activation of apoptotic targets was also achieved by the activation of FOXO transcription factors [620].

Concerning PCa progression, resveratrol was shown to control cell migration, invasion and EMT. Resveratrol treatment reduced migration and invasion of LNCaP, DU145, PC3, PC3M-MM2 and MAT-LyLu cells [603, 623, 624]. Moreover, this natural agent could revert EMT in LNCaP and PC3 cells by increasing the expression of the epithelial marker E-cadherin, and decreasing the expression of vimentin, a mesenchymal marker [625]. *In vivo* studies in TRAMP and SV-40 Tag rats, confirmed the results obtained in cell lines and the valuable effects of resveratrol in suppressing PCa growth [461, 611].

The effect of resveratrol extends to other features of cancer cells, such as the metabolic rewiring and the capacity to survive in hypoxic microenvironments. Resveratrol was shown to inhibit HIF α expression, repressing the progression of LNCaP xenografts tumors [626]. Furthermore, in hypoxic LNCaP cells at low androgen levels (mimicking CRPC), resveratrol inhibited hypoxia-induced nuclear accumulation of β -catenin, inhibiting its mediated AR signaling [626]. Concerning the metabolic

response, resveratrol decreased the uptake of glutamine and deregulated lipid metabolism in human (LNCaP, DU145, PC3, C4-2B) and murine (TRAMP and HMVP2) PCa cells, as well as in LNCaP and HMVP2 xenografts [616, 626, 627], which delayed the progression of PCa.

Overall, due to all the above-mentioned beneficial properties of resveratrol, several approaches have been developed to test its therapeutic effectiveness, alone or as combined therapy, together with the optimal delivery method, using different coated nanoparticles targeting PCa cells [628-633]. Alginate nanoparticles containing resveratrol and curcumin exhibited cytotoxic effects on DU145 cells [628]. Also, resveratrol-loaded poly(lactic-co-glycolic acid) nanoparticles reduced LNCaP cell viability, arrested cell cycle at G1-S transition phase, enhanced ROS levels and promoted apoptosis, causing externalization of phosphatidylserine, DNA nicking, and loss of mitochondrial membrane potential [632]. Other similar resveratrol-loaded nanoparticles based on poly(epsilon-caprolactone) and poly(D,L-lactic-co-glycolic acid)-poly(ethylene glycol) blend also displayed cytotoxicity in LNCaP, DU145 and PC3 cells [629], and resveratrol-loaded poly(2-hydroxyethyl methacrylate)-chitosan based nanoparticles also showed cytotoxicity in PC3 cells [634]. Planetary ball milled nanoparticles encapsulated with resveratrol in combination with docetaxel and conjugated with folic acid increased apoptotic cell number in PC3 and docetaxel-resistant PC3 cells, reducing the expression of NF- κ B p65, COX-2, and pro- (BAX, BAK) and anti-apoptotic (BCL-2, BCL-XL) genes and downregulating survivin, as well as increasing cleaved caspase-3 expression [630]. Moreover, functionalized mesoporous silica nanoparticles encapsulating resveratrol displayed antiproliferative effects and sensitize PCa cells to docetaxel in hypoxic cell environment [633].

The combination of resveratrol with docetaxel was also tested using liposomes and showed antitumor efficiency against PCa, namely, reducing proliferation and increasing apoptosis [635]. Resveratrol-loaded solid lipid nanoparticles was another approach tested in PCa cells as a potential carrier for delivery chemotherapeutics at the tumor site [636]. Other combinations, even without using nanocarriers, showed the enhanced anti-carcinogenic effects of cisplatin, docetaxel and doxorubicin and resveratrol in PCa cells [637-639].

Various reports also showed the resveratrol effects enhancing the response to ionizing radiation in LAPC4, DU145 and PC3 cells [607, 640-644].

The results obtained with resveratrol in preclinical studies supported testing this phytoestrogen in clinical trials concerning its safety, tolerability and effective dose in PCa patients [37, 645]. A pilot study assessing the effects of a phytotherapeutic intervention containing turmeric, resveratrol, green tea and broccoli sprouts revealed

that its use is feasible in men with biochemically recurrent PCa and a moderate PSA rise rate [39]. In a phase I study in non-metastatic biochemically recurrent PCa patients, 4000 mg of a pulverized muscadine grape skin containing ellagic acid, quercetin, and resveratrol was safe in augmenting PSA doubling time, with no serious adverse effects being reported in the study time-frame [37]. This formulation was further investigated in a randomized, multicenter, placebo-controlled, dose-evaluating phase II trial. However, no significant difference in PSA doubling time was observed between the control and treatment groups [646].

Besides the beneficial effects of resveratrol and the promising results from preclinical studies and clinical trials, it remains crucial to understand better its detailed mechanism of action and the ideal dose as a therapeutic agent against PCa.

2.5.4.14. Diosgenin

Diosgenin ($C_{27}H_{42}O_3$), also known as 25R-spirost-5-en-3 β -ol, is a steroidal sapogenin with a structure similar to cholesterol and other steroids, being used by the pharmaceutical industry in the synthesis of sex hormones and corticosteroids for application as anti-inflammatory, androgenic and contraceptive drugs [647, 648]. This natural compound is commonly found in a panoply of plants, namely, in species from the *Dioscorea*, *Trigonella*, *Costus*, and *Smilax* genera, and in particular in tubers and seeds [649-652].

This steroidal sapogenin has been shown to have antimicrobial, antioxidant, anti-inflammatory, anti-diabetic and anti-obesity activity [653]. Furthermore, diosgenin's role in cancer is well described. It has a potential anti-tumor effect in many types of cancer, namely, in leukemia [654-657] and gastric [658, 659], lung [660-662], breast [663, 664], liver [665-667], renal [668], colon [669-671], pancreas [672], bone [673], cervical [674], skin [675, 676], and oral [677] cancers.

The anti-cancer effects of diosgenin also have been confirmed in PCa [678-680]. This sapogenin reduced PC3 cell viability in a dose-dependent manner [680]. In the same line, diosgenin and a fenugreek extract containing diosgenin as the bioactive compound inhibited PCa cells proliferation [679, 681], inducing cell cycle arrest in the G2/M phase, and increasing the sub-G1 and G2/M phase cell population [681, 682].

Diosgenin also showed to augment apoptosis in DU145 and PC3 cells [679, 681], underpinned by the increased expression of caspase-9 [679]. The fenugreek extract also reduced the expression of mutant p53 protein in DU145 cells [682]. In addition, diosgenin can activate autophagy [679], a cell death process distinct from apoptosis, which contributes to the maintenance of the intracellular environment stability and the

normal physiological functions of the cell, and that has been related to tumor growth inhibition [683-685]. DU145 cells treated with diosgenin showed a large number of autophagosomes, including autophagosomes containing mitochondria [679].

The molecular mediators of diosgenin actions suppressing cell survival and growth have been explored. In PC3 cells, diosgenin increased the expression of cell cycle inhibitor p21 and inhibited the phosphorylation of EGFR [682]. Furthermore, it was confirmed that diosgenin inhibits several signaling pathways, namely, the PI3K/Akt, JNK and ERK pathways [678, 679, 686]. Exposure of PCa cells to diosgenin showed to reduce PI3K, Akt, mTOR, JNK1/2 and ERK1/2 phosphorylation [678, 679, 682, 686] by regulating TGF- β and the HGF pathways.

Concerning tumor progression, the steroid sapogenin diosgenin reduced the migration and invasion capabilities of PC3 cells [678, 681], inhibiting the expression and activation of MMP-2, MMP-7 and MMP-9, which have a crucial role in extracellular matrix degradation [678]. In DU145 cells, diosgenin inhibited HGF-induced motility, invasion, and EMT by increasing E-cadherin and decreasing vimentin and Mdm2 expression [686]. The role of diosgenin in inhibiting PCa tumor progression is also supported by its ability to suppress angiogenesis, as it blocked the expression of VEGF in PC3 cells and tube formation of endothelial cells [678].

Few studies have investigated the anti-cancer activity of diosgenin in animal models. However, a study showed that diosgenin treatment diminished the prostate-to-body weight ratio and serum PSA levels in a rat model of BPH, established by subcutaneous injection of testosterone propionate [687]. Reduced prostate size could be related to a pro-apoptotic effect of diosgenin, increasing Bax and p53 expression and reducing the expression of Bcl-2 [687]. Furthermore, diosgenin-treated rats displayed elevated activity of SOD and GPx and reduced MDA levels [687], suggesting that diosgenin can mitigate oxidative stress in the hyperplastic prostate. Moreover, diosgenin reduced tumor growth and metastasis in xenograft [688] and TRAMP mice through regulation of NF- κ B/STAT3 signaling [689].

Regarding the panoply of diosgenin effects against PCa, modulating many cellular processes, several studies evaluating the effect of diosgenin analogues and/or derivatives have emerged. The majority of these compounds revealed to have anti-proliferative and pro-apoptotic activity in PCa, namely, in DU145 and PC3 cells [690-694], with some displaying advantage relative to diosgenin.

Table 2.6. Phytoestrogens with a role in PCa.

Class	Subclass	Compound	Source	Cell line/ Animal Model	Concentration/ Dose	Effect	Activated Pathway	Ref.
Flavonoids	Flavones	Apigenin	Fruits	LNCaP	0 – 100 μ M (cell lines)	↓ Cell viability	↓ Cyclin D1, D2 and E	[308, 309, 311-320, 322-330, 333, 335, 337, 339- 341, 695- 702]
			Vegetables	DU145	Up to 50 μ g/day (animals)	↓ Proliferation	↓ CDK2, 4 and 6	
				PC3		Cell cycle arrest at G2/M phase	↑ p53	
				22RV1		Cell cycle arrest at G1 phase	↑ p21	
				PCa stem cells (PCSCs)		↑ Apoptosis	↓ pRb	
				MAT-LyLu		↓ Hypoxia	↑ ROS	
				TRAMP mice		↓ Angiogenesis	↑ Bax/Bcl-2 ratio	
				PC3 xenograft		↓ Cell motility	↑ TRAIL (DR5) receptors	
				C57BL/TGN TRAMP mice		↓ Migration	↑ Caspase-8, 9, 10 and 3	
						↓ Invasion	↑ Cytochrome c	
						↓ PCa progression		

						Reversion of EMT	↓ β -catenin	
						Modulation of glucose and lipid metabolism	↓ IGF axis ↓ HIF α	
						↑ Survival	↓ VEGF GLUT1 modulation ↓ Fatty acid synthase ↓ Akt/ PI3K ↑ MAPK ↓ NF κ B ↑ PTEN ↓ Snail ↓ MMPs 1, 7 and 9 ↓ AR ↑ ER β	
		Chrysin	Honey	LNCaP	0 – 100 μ M	↓ Proliferation	↑ TRAIL receptors	[343, 352,

			Propolis	C4-2,		↓ 3D cell growth	↓ Bcl-2	353, 702, 703]
			Blue passion flowers	DU145		↑ Apoptosis	↑ caspase-3	
				PC3		↓ Vasculogenic Mimicry Tube-Formation	↓ CDK6	
				PC3 3D cell culture			↓ Cyclin D1	
				PC-3 Xenograft in Nude Mice		↓ tumor growth	↑ ROS	
							↓ HIFα	
							↓ SPHK	
							↓ VEGF	
							↓ Akt/PI3K	
							↓ VE-cadherin	
							↓ PCNA.	
	Isoflavones	Biochanin A	Red clover	LNCaP	0 – 100 μM	↓ Proliferation	↓ Cyclins B and E	[315, 357, 359, 360]
			Soy	DU145		Cell cycle arrest at G0/G1 phase	↓ c-Myc	[361]
			Alfalfa	PC3			↑ p21	

			sprouts Peanuts Legumes	LNCaP xenografts		Cell cycle arrest at G2/M phase ↑ Apoptosis ↓ PSA	↑ Bax ↓ Bcl-2 ↑ TRAIL receptors ↑ DNA damage ↓ pAkt ↓ pERK ↑ ERβ	
		Daidzein	Soybean Other legumes and fruits	LNCaP DU145 PC3 Wistar unilever rat F344 rat R3327 rat	0 – 200 μM Up to 2000 mg/kg or 200 ng/L	↓ Cell viability ↓ Proliferation Cell cycle arrest at G2/M phase ↑ Apoptosis ↓ Angiogenesis ↓ Migration	↓ Cyclin A and B1 ↑ p53 ↑ p21 ↑ Bax ↑ TRAIL (DR4 and DR5) receptors ↓ MMP-13	[363-374, 379, 435]

						↓ Invasion ↓ PCa progression Modulation of glucose and lipid metabolism ↓ PSA levels ↑ PSA level free/total PSA ratio	↓ HIFα	
		Equol	Daidzein's metabolite	LNCaP DU145 PC3 Dunning R3327 Prostate Adenocarcinoma in Copenhagen × Fisher F1 male rats TRAMP mice	0-100 μM	↓ Proliferation ↓ Growth Cell cycle arrest at Go phase ↑ Apoptosis ↓ Migration ↓ Invasion ↓ PSA	↑ TRAIL receptors ↑ DNA damage ↓ MMP-2 and MMP-9 ↑ Antioxidant defenses ↑ Degradation of AR Affinity to ERβ	[366, 380, 386, 398, 402-410] [411, 412, 704]

				Human Patients				
				Male Long-Evans rats				
		Formononetin	Red clover plants	LNCaP DU145 PC3	0 – 100 µM	Cell cycle arrest at G ₀ /G ₁ phase ↑ Apoptosis	↓ Cyclin D1 ↓ CDK4 ↑ Bax/Bcl-2 ratio ↓ pAkt ↓ ERK1/2 ↓ IGF-1/IGF-1R	[414-419]
		Genistein	Soybeans Peas Lentils Beans	LNCaP DU145 PC3 3D culture of PC3 cells MAT-LyLu LTL1630a BALB/c nu/nu nude	0 – 200 µM 25 mg/kg to 1g/kg	↓ Cell viability ↓ Proliferation Cell cycle arrest at G ₂ /M phase ↑ Apoptosis ↑ Angiogenesis ↓ Migration	↓ Cyclin B ↑ p21 ↑ p53 ↑ ROS ↑ GSH ↑ Catalase ↓ NO	[369, 372, 383, 427-432, 437-446, 448-451, 494, 497] [453] [354, 434, 435, 485, 704-709]

				mice		↓ Invasion		
				C57BL/6 mice		Reversion of EMT	↓ MMP-2	
				BALB/c mice		↓ PSA	↑ E-cadherin	
				Lobund-wistar rat		↓ Advanced PCa incidence	↓ Vimentin	
				C17 beige SCID mice		↓ Tumor growth rate	↓ FAK	
				Sprague dawley rat			↓ VEGF	
				SV-40 TAG rat		↓ Tumor weight	↓ COX-2 pathway	
				ACI/Seg rat		↑ Survival	↓ GLUT1	
				TRAMP mice			↓ pAkt	
				SCID mice			↓ EGF-induced activation of Akt	
				Xenograft tumor mouse model established with 22RV1 cells			↓ p38 MAPK	
							↓ NFκβ activation	
							Hedgehog pathway	

							↑ERβ ↓ AR ↑ PTEN ↓ IGF-1 ↓ 5α-reductase-2 ↓ GSK-3β phosphorilation ↓ Cyclin D1 ↓ Osteopontin ↓ SRC-3	
	Flavanones	Naringenin	Grapefruit Orange	LNCaP PC3 22RV1 MAT-LyLu	0 – 75 μM	↓ Proliferation ↑ Apoptosis ↓ Migration ↓ Invasion ↓ Metastasis	↑ Bax ↓ Bcl-2 ↑ E-cadherin ↓ Vimentin ↓ MAPK	[506-508, 710]

							↑ SOD activity ↓ Glutathione reductase ↑ Lipid peroxidation	
	Flavonols	Kaempferol	Kale Beans Tea Spinach Broccoli	LNCaP 22Rv1 DU145 PC3	0 – 1000 µM	↓ Proliferation Cell cycle arrest at G1 phase Cell cycle arrest at S phase Cell cycle arrest at G2/M phase ↑ Apoptosis	↑ TRAIL receptors ↑ Casp-8, 9 and 3 ↓ Akt ↑ ERK ↓ NFκβ ↓ IGF-I ↓ Ki67	[370, 515, 517-520, 711-713]
		Myricetin	Vegetables Fruits Nuts	PC3 C4-2B C4-2B Xenograft	0 – 300 µM	↓ Proliferation ↑ Apoptosis ↓ Tumor growth	↓ CXCR4	[526, 527, 534]

			Berries			↓ PCa progression		
			Tea					
			Red wine					
		Quercetin	Capers	LNCaP	0 - 160 µM	↓ Cell viability	↑ Cells in G0 phase	[40, 41,
			Lovage	LNCaP/R (resistant to docetaxel)	Up to 200 mg/kg or 400 mg/day	↓ Proliferation	↓ Cells in G1 phase	518, 519,
			Dill	DU145		Cell cycle arrest at S phase	↓ Cyclin B	526, 541-
			Cilantro	PC3		Cell cycle arrest at sub-G0/G1 phase	↓ CDK1	546, 548-
			Onions	PC3/R (resistant to docetaxel)		↑ Apoptosis	↓ pRb	551, 553-
			Apples	SCIV mice		↓ Angiogenesis	↑ p21	561, 565-
			Berries	Sprague dawley rat		↓ Migration	↓ Bid and Bcl-2	567, 571,
				BALB/c nude mice		↓ Invasion	↑ Bax	575, 704,
				TRAMP mice		↓ EMT	↑ TRAIL and DR5 receptors	714-716]
							↓ Heat shock protein 90	

							↑ Casp-8, 9 and 3 ↑ Autophagy ↑ ROS ↓ MMP-2 and MMP-9 ↓ EGFR/PI3K/Akt pathway ↓ VEGFR-2 regulated ↓ PI3K/Akt/mTOR ↓ IL6 ↓ AR	
	Coumestans	Coumestrol	Soybean Legumes Brussels sprouts Alfalfa	LNCaP PC3	0 – 100 µM	↓ Proliferation ↓ Migration ↓ Tumor progression Modulation of	↑ p53 ↑ Caspase-3 and caspase-9 ↑ DNA damage ↓ GSK-3	[405, 589, 590]

			Spinach			glucose metabolism	↓ Akt ↑ ERK 1/2 ↓ HIFα	
Non-Flavonoids	Stilbenoids	Resveratrol	Red grapes	LNCaP	1 - 150 μM	↓ Cell viability	↑ Cells in G0/G1 phase	[253, 603-608, 610, 612-624, 627] [40, 626, 637, 704, 717-722]
			Peanuts	DU145 PC3 22RV1 MAT-LyLu rat Primary culture from human prostate tumor Primary culture from TRAMP mice LNCaP xenograft in castrated male BALB/cSlc-nu/nu mice	Up to 4g/kg or 200 μg/mL	↓ Proliferation Cell cycle arrest at G1/S phase ↓ Survival ↑ Apoptosis ↓ Migration ↓ Invasion Modulation of glucose, glutamine and lipid metabolism ↓ PSA levels	↓ Cells in the G2 phase ↓ Cyclin D1, B1 and E ↓ CDK1, CDK4 ↑ p15, p21 and p27 ↑ p53 ↑ FOXO transcription factors ↑ BAX, BID, BAK, PUMA, NOXA, BIM, BAD	

				Mouse allograft TRAP rat			↓ MCL-1, BCL-2, BCL-XL, XIAP, survivin	
				SV-40 Tag rat			↑ TRAIL receptors	
				PTEN KO mice			↑ FAS system	
				TRAMP mice			↑ Casp-7, 9 and 3	
							↑ DNA damage	
							↑ ROS	
							↑ Autophagy	
							↑ E-cadherin	
							↓ Vimentin	
							↓ HIFα	
							↑ IGF-1	
							↓ GSK-3	
							↓ PI3K/Akt	

							↓ NFκβ ↓ COX-2 ↓ mTORC1 ↑ AMPK ↑ Hedgehog signaling pathway ↓ AR ↓ ERα ↑ ERβ	
	Steroidal sapogenin	Diosgenin	<i>Dioscorea</i> <i>Trigonella</i> <i>Costus</i> <i>Smilax</i> <i>genera</i>	LNCaP DU145 PC-3 C4-2 TRAMP mice	1.4 to 100 μM	↓ Cell viability ↓ Proliferation Cell cycle arrest at G2/M phase ↑ Apoptosis ↑ Autophagy	↓ Cyclin D1 ↓ Bcl-2 ↓ Bcl-xL ↓ Mcl-1 ↓ Survivin	[678, 679, 681, 682, 686, 688, 689]

				DU145 xenograft in nude mice		↓ EMT ↓ Migration ↓ Invasion ↑ Senescence ↓ Tumor growth	↓ NEDD4 ↑ Caspase- 9 ↑ p53 ↑ p16 ↑ p21 ↓ MMP-2,-7 and 9 ↓ Mdm2 ↓ Vimentin ↑ E-Cadherin ↓ COX-2 ↓ VEGF ↓ PI3K/Akt/mTOR signaling ↓ ERK phosphorylation	

							↓ JNK phosphorylation ↓ STAT3 phosphorylation ↓ Src phosphorylation ↓ JAK2 phosphorylation ↓ NFκβ ↓ EGFR ↓ TGF-β ↑ UHRF1 degradation	

2.6. Conclusions

The female sex hormones estrogens have been shown to play a role in men's physiology. The adult prostate is one of the male reproductive organs highly dependent on estrogens' actions, which oscillate from anti-carcinogenic, supporting the use of these hormones in therapy, to pro-tumorigenic, accelerating PCa growth. However, the relationship between PCa risk and E_2 serum levels remains controversial, with most studies not founding statistically significant associations. This lack of relationship can be a consequence of the broad range of serum E_2 concentrations found in men, which is a result of a set of different variables not always controlled in the different studies. E_2 concentrations were shown to vary with age groups, ethnicity, endocrine and metabolic conditions, including adiposity and body mass index, as well as with specificities in aromatase expression and activity. The intraprostatic production of estrogens cannot be ignored in the scenario of their influences on prostate physiology and carcinogenesis. External androgen precursors or circulating testosterone can be converted to estrogens locally in the prostate by aromatase. Indeed, it was shown that increasing activity of aromatase with augmented E_2 production accompanies the development of PCa (non-cancerous prostate < primary tumors < metastatic tumors). Nevertheless, further research is needed to clarify the impact of intraprostatic estrogen biosynthesis in prostate pathology.

As mentioned, and after the discontinuity of using estrogens in PCa treatment, research efforts over the years have produced a vast amount of information establishing the dual role of estrogens in prostate carcinogenesis, balancing from guardians to guilty. The enormous amount of data herein reviewed, has detailed the biological processes and molecular events orchestrating the estrogens' actions as prostate carcinogens. Naturally occurring and synthetic hormones can augment cell proliferation, resistance to apoptosis, migration and invasion, and other events involved in tumor growth and progression. These pro-carcinogenic effects have been mainly related to the differential activation of $ER\alpha$. Contrastingly, the role of estrogens in counteracting PCa development and metastization by reducing cell growth, promoting cell cycle arrest, increasing apoptosis, and suppressing migration, invasiveness and angiogenesis, has mainly implicated $ER\beta$ as the mediator. Nevertheless, the complexity of estrogens' actions is far from being totally enlightened. For example, it remains to be disclosed whether differential responses in human patients may rely on the physiological condition, which may disrupt $ER\alpha$ and $ER\beta$ expression changing prostate cells to a cancer-like phenotype. This hypothesis gains strength considering the reports showing that ERs expression is altered in the prostate

of diabetic [723] and obese [724] patients. Noteworthy, diabetes markedly reduced ER β and GPER expression in PCa patients [723].

The revision of the literature in the *in vitro* setting also demonstrated the lability of ERs expression depending on the environmental conditions, as different studies have reported contradictory results of ER isoforms expression in the same cell line model. Therefore, it should be critical to evaluate ERs expression when defining experimental design.

More research work is also needed to clarify the role of GPER in PCa, as the last decades have witnessed the emergence of reports showing it can trigger both pro- and anti-cancer responses. Also, it will be of paramount importance to further investigate the relationship between GPER, aromatase expression and intraprostatic estrogens production.

Another relevant issue in the landscape of prostate responses to estrogens is related to the fact that different concentrations/doses of estrogens can trigger distinct and sometimes opposing effects. Generally, data support that lower concentrations/doses are associated with the pro-carcinogenic effects of these hormones, with the higher doses being anti-tumorigenic. This was the basis of estrogen therapy in PCa, with the high hormone doses causing well characterized adverse effects. A great pharmacological challenge in PCa therapy relies on developing new approaches allowing the use of reduced doses of estrogens, receptor-specific agonists or alternative estrogens-like compounds with similar properties to estrogens but fewer side effects. In the last years, many studies have investigated the effect of natural compounds with estrogen-like activity in controlling PCa. This review summarized the promising properties of a panoply of phytoestrogens against PCa, reported both *in vitro* and *in vivo*. Overall, distinct substances of this class of compounds have been shown to control PCa cell proliferation and tumor growth, reduce migration and invasion, suppress EMT, angiogenesis and metastization, and also regulate the bioenergetic metabolism of PCa cells. Several molecular targets have been discovered alongside identifying phytoestrogens actions and disclosing their mechanisms of action. These effects encompass the reduction of cyclins and CDKs expression, the increased levels of p53 and decrease of those of p21, the activation of TRAIL receptors and caspase cascades, the reduction of β -catenin and MMPs expression and the inhibition of Akt/PI3K and NF κ B pathways, whereas activating MAPK signaling. Based on their tumor-suppressor properties, phytoestrogens, such as chrysin, daidzein, genistein, quercetin, coumestrol, resveratrol and equol, have been pointed out as possible alternative therapies for PCa, and have been tested in humans. Equol, quercetin, coumestrol and

resveratrol, are among the most promising agents as when used in diet showed to reduce PSA levels and lower PCa risk and incidence.

Using natural bioactive compounds in PCa therapy is an exciting and powerful research and clinical question. Even if these compounds are not a choice as a first-line therapy, they can be used in combination with conventional treatments such as chemotherapy, radiation therapy and immunotherapy, increasing their efficacy and/or mitigating the systemic adverse effects. It is also quite challenging the demonstration that quercetin can promote the recruitment of dendritic cells to the tumor site, which opens new perspectives of research for evaluating phytoestrogens as anti-cancer molecules.

In sum, the discussion in the present review systematized the role of estrogens in prostate carcinogenesis and provided the scientific fundamentals for the evolution of PCa treatment based on the use of natural estrogenic substances.

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Chapter 3

The role of GPER signaling in carcinogenesis: a focus on prostate cancer

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3. The role of GPER signaling in carcinogenesis: a focus on prostate cancer

Abstract

The G protein-coupled estrogen receptor, GPER, also known as GPR30, belongs to the seven transmembrane receptor superfamily and is involved in the rapid non-genomic estrogenic responses. Nevertheless, GPER regulation of transcriptional activity also has been reported. GPER downstream signaling includes the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways, the stimulation of adenylyl cyclase, and the mobilization of intracellular calcium. Over the last decade, the discovery of GPER specific agonists and antagonists has been crucial to understand its physiological functions, mechanisms of action and its putative usefulness as a therapeutic target. The GPER seems to have an important role in endocrine, reproductive, immune, nervous, and cardiovascular systems, and alterations in its expression or activity have been associated with several pathological conditions such as cardiovascular diseases, obesity, diabetes, Parkinson, stroke, and cancer. GPER has been linked with the carcinogenic process, though some ambiguity exists concerning its protective or causative role in different tissues, or even in the same tissue. This chapter summarizes the existent knowledge concerning the structural and molecular aspects of GPER, its known ligands and activated pathways, as well as its role over the known hallmarks of cancer: exacerbated proliferation, resistance to apoptosis, stimulated migration and invasion, induction of angiogenesis, and the metabolic reprogramming. A special focus will be given to prostate cancer.

Keywords

GPER, GPR30, prostate cancer

3.1. Introduction

Estrogens are very important hormones playing a determinant role in mammalian biology by its wide action regulating several processes in a great variety of tissues. As all hormones, estrogens act through specific receptors that after recognizing and binding their ligands transmit the information to downstream effectors. The estrogen receptor α (ER α) was the first ER to be known in 1973 [1]; and was more than two decades after in 1996, that a second ER, the ER β , was described [2]. Since then, several isoforms of ER α and ER β have been identified known to have tissue specific functions or acting as negative regulators of full-length ERs, namely, the ER α -A-E, the ER α 36 and the ER β 1-5 [3-9]. The ER α and ER β are known as the classical ERs exerting their actions as transcription factors and regulating the gene expression network in target tissues [10]. Generally, after ligand binding in the cytoplasm, hormone–receptor complexes are translocated to the nucleus, where they dimerize and bind DNA modulating gene expression. Other alternative mechanisms of estrogens actions have been described, such as, the interaction of ERs with other transcription factors, and the hormone independent receptor phosphorylation and activation [11, 12]. In addition to the genomic actions, classical ERs also can trigger non-genomic actions. It has been described that ERs can associate with the cell membrane and lead to the activation of protein kinase signaling pathways, or cross-talk with the intracellular secondary messengers produced in response to the activation of G protein-coupled receptors (GPCRs) [11, 12].

The landscape of estrogens signaling has gained increasing interest and complexity with the report of the G protein-coupled estrogen receptor (GPER), originally known as GPR30 [13]. GPER is a member of the GPCRs superfamily, and thus, displays the typical seven transmembrane helices and predominant location at the cell membrane (Fig. 3.1) [14].

GPER expression has been found in multiple tissues and systems, including nervous, cardiovascular, gastrointestinal, immune, urinary and reproductive systems, and bone among others [15-19]. GPER is responsible for mediating the non-genomic actions of estrogens and is involved in the control of many biological processes. Its important role in several diseases including stroke, Parkinson, diabetes, cardiac diseases, bone diseases, epilepsy, autoimmune diseases, infection, and cancer has been suggested [20-32]. Concerning cancer, GPER has been detected in many types of tumors, including breast, ovarian, endometrial, prostate, testis, lung, thyroid, and others [33-38]. Also, its role in cancer development as a causative agent and associated

with tumors aggressiveness, or as a tumor suppressor has been debated in the available literature [39, 40].

In this chapter, the molecular and structural aspects of GPER, as well as, its ligands, signaling activated pathways, and downstream effectors will be presented. Moreover, the GPER expression in cancer cases, and its role modulating the typical features of cancer cells will be discussed, with a special focus on prostate cancer (PCa).

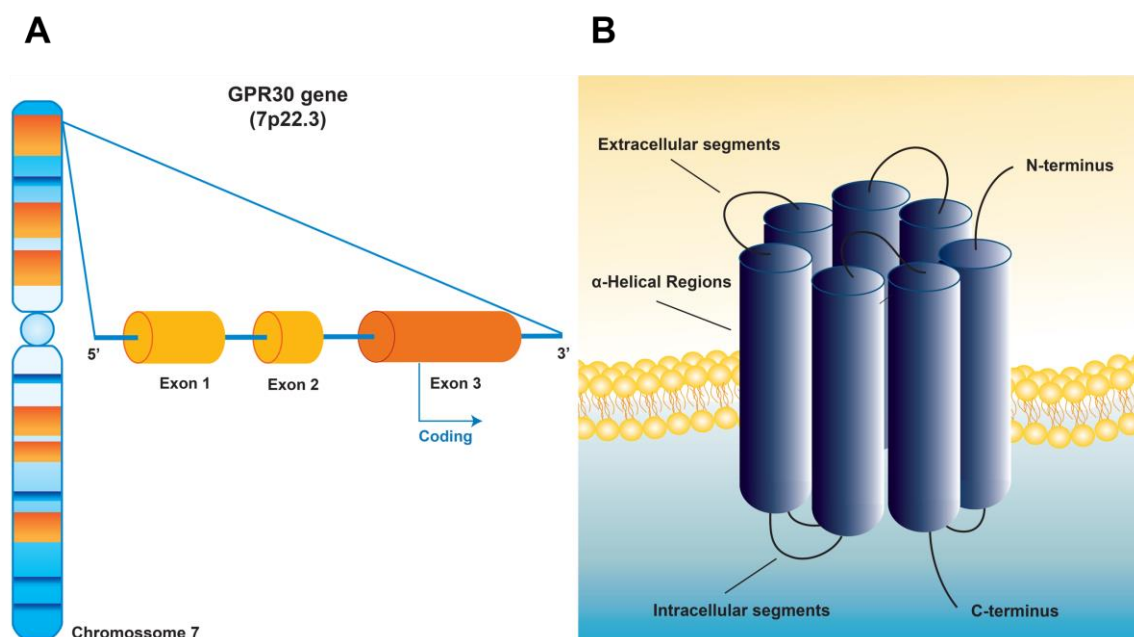


Figure 3.1. Schematic representation of the human GPER: structural and molecular aspects.

(a) Localization of GPER gene in the chromosome 7 and its structure consisting of three exons, with the coding region encoded by a single exon, the exon 3. (b) Structure of the GPER protein with the typical seven transmembrane α -helical regions, and the four extracellular and four cytosolic segments. The N-terminus is extracellular whereas the C-terminus is cytosolic.

3.2. Structural, molecular and cellular aspects of GPER

The “life history” of GPER has begun some time ago, when it aroused from the shadows, like a ghost. Before the biochemical and structural characterization of GPER, researchers just have known about the possibility of alternative estrogens’ actions via non-genomic signaling mechanisms producing rapid cell effects. Over the years, as an attempt to explain the estrogenic effects in a time-frame not compatible with gene transcription and *de novo* protein synthesis, many theories were proposed concerning the existence of a membrane-bound ER [41, 42]. Amongst other hypotheses, it was shown that the classical nuclear ERs, the ER α and ER β , were able to be translocated to

the cell membrane through several mechanisms that involve post-translational modifications, such as palmitoylation or phosphorylation, interaction with caveolin-1, binding to adaptor and co-regulator molecules, and also the association with G proteins [43-45]. It was only later on that the idea of estrogens binding a GPCR at cell membrane has emerged [46]. At the end of 90s several independent groups aimed at identifying a new GPCR characterized by its reduced homology to other GPCRs [14, 47-51], and for which no ligand was known. This orphan receptor was named GPR30, even before 17 β -estradiol (E₂) has been identified as its ligand. Indeed, it was only a few years later that the “fate” of this orphan receptor has changed after the demonstration that it binds E₂ with the activation of downstream signaling pathways [13, 52-54]. This lead the scientific community to rename the GPR30 that started to be known as GPER, a designation formally established by the International Union of Basic and Clinical Pharmacology in 2007 [55]. Even so, GPER’s “life” has been anything but consensual, and still today some groups question its role as an ER [56, 57].

The GPER is a member of the rhodopsin-like receptor superfamily highly homologous to the interleukin 8 receptor, and the angiotensin II receptor type 1 [47-50].

The human *GPER* gene is located on chromosome 7p22.3, and contains three exons (Fig. 3.1A), with the open reading frame of 1126 base pairs encoded by a single exon, the exon 3 [58]. As a GPCR, the GPER has a structure encompassing seven transmembrane α -helical regions, and four extracellular and four cytosolic segments (Fig. 3.1), which spans 375 amino acids and has a theoretical molecular mass of approximately 41 kDa [14, 15, 59]. The extracellular N-terminus has aspartic acid residues in its terminal region, which are targets for glycosylation; the C-terminal end is cytosolic [14, 15, 59]. Thus, ligand binding and receptor activation occur by interaction with the N-terminal domain, and the heterotrimeric G protein complex has been shown to bind the 3rd loop of the intracellular domain (Fig. 3.1) [60, 61]. A PDZ domain located at the C-terminal region of GPER seems to be important for receptor location at cell membrane by its interaction with other plasma membrane proteins, namely, membrane-associated guanylate kinases and protein kinase A-anchoring protein 5 [14, 15, 62]. The C-terminal region also participates in receptor desensitization and internalization depending on phosphorylation by G protein-coupled receptor kinases [14, 15, 59]. Recently, a biosensor-based approach identified four distinct calmodulin-binding domains in the GPER protein [63, 64], though the effect of calmodulin regulating GPER-dependent signaling is at the moment unknown.

Although GPER was firstly identified as a membrane receptor, the question of its subcellular localization has deserved intense discussion. Several references exist

indicating the presence of GPER in the endoplasmic reticulum, the Golgi apparatus, and also in the nucleus (Fig. 3.2) [54, 65-71]. The GPER expression in the endoplasmic reticulum and Golgi complex could be attributed at a first glance to the process of protein receptor synthesis and intracellular traffic before its translocation to the cell membrane. In fact, the GPER detected at the plasma membrane seems to be translocated from the cytoplasm 1 hour after E_2 stimulation, which also suggests that GPER activation is triggered at the cell membrane [54, 65]. However, some research groups defend that the GPER at the endoplasmic reticulum is signaling-active mediating cell responses by the mobilization of intracellular calcium (Ca^{2+}) [52], though this is not a consensual matter. Concerning the nuclear location, besides its confirmed presence at the nuclear membrane [35, 67], it was recently shown that the GPER protein has a nuclear localization signal required for its nuclear translocation by an importin-dependent mechanism [69]. Moreover, the nuclear localization of GPER also seems to be induced by E_2 , and is associated with the regulation of gene transcription and promotion of physiological effects [69, 70]. Another study reported that GPER activation at the plasma membrane with the stimulation of cAMP production is followed by receptor internalization and intracellular localization in association with the cytokeratin intermediate filaments [68].

It is also of worth note the constitutive retrograde transport of GPER from the plasma membrane towards the endosomal compartment (Fig. 3.2). Endocytosis is a common feature of GPCRs after ligand binding and activation to avoid excessive signaling. However, GPER in lysosomes, the endocytosed GPER returns to the trans-Golgi network and undergoes ubiquitin-mediated proteasomal degradation [72-74]. Recently, it was shown that the Na^+/H^+ exchanger regulatory factor (NHERF1) can improve GPER stability by inhibiting its degradation through the ubiquitin-proteasome pathway [75].

Also in opposition with other GPCRs that after endocytosis are recycled to the plasma membrane, GPER enters early and recycling endosomes, but do not follows the plasma membrane route, and instead accumulates in the endoplasmic reticulum and perinuclear region [72, 73]. Interestingly, it has been proposed that GPER may mediate endocytic intracellular signaling linked to its constitutive endocytosis activity, which may be dependent on the PDZ domain that forms receptor complexes able to influence receptor dimerization, signaling and/ or endocytosis [73]. Nevertheless, there is much to know about the factors that determine the preferred location of GPER at cell specific compartments, and its implications in intracellular signaling. The details and comprehensive analysis of the GPER signaling pathways will be explored in the following section of this chapter.

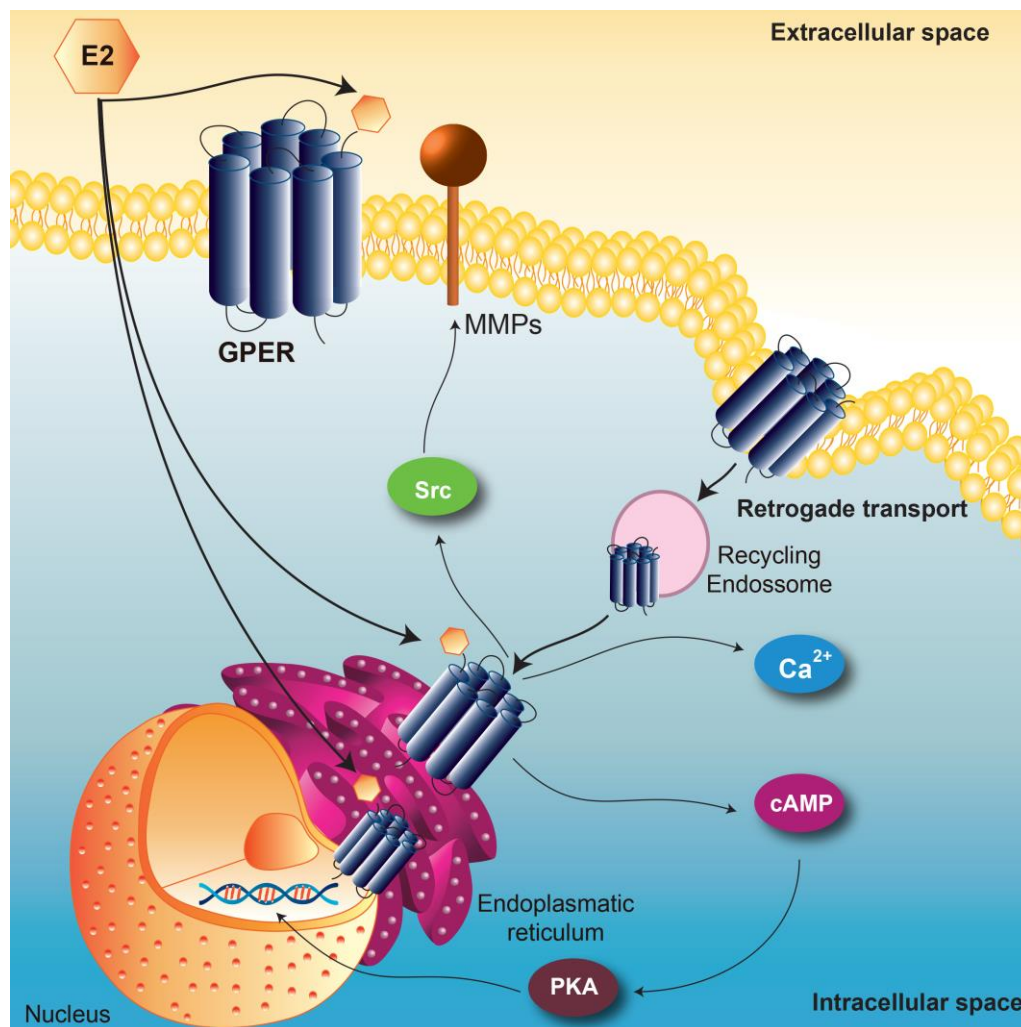


Figure 3.2. GPER subcellular localization. The receptor has been shown to be localized at the cell membrane and/or also in the endoplasmic reticulum and nucleus. Although not a consensual matter, there are reports indicating that E₂ can interact with a signaling-active GPER at endoplasmic reticulum promoting cell responses [54,65-71]. The Src, metalloproteinases (MMPs), calcium (Ca²⁺), and cAMP/protein kinase A (PKA) mediated signaling pathways are detailed throughout the text and in Fig. 3.3. The GPER, as other GPCRs, enters the constitutive endocytic pathway but it is not recycled to the plasma membrane being instead accumulated in the endoplasmic reticulum and perinuclear region [72,73].

3.3. Overview of GPER signaling pathways

GPER is widely associated with the non-genomic effects of estrogens and estrogen-like substances, which are generally characterized as being: i) very rapid, in minutes or even seconds; ii) insensitive to DNA transcription and protein synthesis inhibitors; and iii) activated by steroids coupled to high-molecular-weight molecules, such as bovine serum albumin (BSA), and thus incapable of cross the plasma

membrane. GPER-mediated responses have been described in various cell types and involve several molecular targets and signaling pathways (Fig. 3.3), including, the mobilization of second messengers, such as Ca^{2+} and nitric oxide (NO), the interaction with other membrane receptors, such as insulin-like growth factor-1-receptor (IGF-1R) and epidermal growth factor receptor (EGFR), as well as, the activation of effector molecules, namely, the Src kinase and phosphatidylinositol 3-kinase (PI3K), the serine/threonine protein kinase Akt, mitogen-activated protein kinase (MAPK) family members, and the protein kinases A and C (PKA and PKC, respectively) [76].

After ligand binding, GPER initiates intracellular signaling by activating heterotrimeric G proteins, composed of α , β and γ subunits, with the latter known as the $\beta\gamma$ -complex [77]. In general, the $\text{G}\alpha$ -subunit ($\text{G}\alpha$ s) dissociates from the heterotrimeric $\text{G}\alpha\beta\gamma$ complex and the free subunits ($\text{G}\alpha$ s and $\text{G}\beta\gamma$) activate distinct intracellular signaling pathways [78]. It has been demonstrated that both $\text{G}\beta\gamma$ [13] and $\text{G}\alpha$ s [53] participate in the GPER-mediated signaling. $\text{G}\alpha$ s activity stimulates the adenylyl cyclase producing an increase in cAMP concentration and mobilization of intracellular Ca^{2+} [52, 53, 79], whereas the $\text{G}\beta\gamma$ subunit and the downstream Src-related tyrosine kinases activate the MAPK transduction pathway [13], leading to phosphorylation of MAPKs/ERK1/2 [13]. In rat pachytene spermatocytes, the activation of GPER led to the initiation of ERK1/2 signaling cascade, correlated with an increased phosphorylation of c-Jun [80]. On the other hand, in breast cancer cell lines, the activation of MAPKs pathway influenced the activation of Smad proteins and led to the down-regulated signaling of the transforming growth factor beta (TGF- β) [81], since Smad2/3 proteins are essential signaling molecules mediating TGF- β effects [82]. The activation of ERKs signaling through GPER also was shown to activate the hypoxia-inducible factor 1 α / vascular endothelial growth factor (HIF1 α /VEGF) signaling, due to the upregulation of HIF1 α and, consequently, to the upregulation of VEGF, a well-known marker of angiogenesis [83, 84].

GPER activation of the PI3K/Akt pathway has been reported in ER-negative endometrial cancer cells and seems to stimulate cell growth [85, 86]. GPER activity, in a Src kinase and PI3K/Akt dependent mechanism with the activation NF- κ B, was also shown to up-regulate cyclooxygenase 2 (COX2) expression in oviduct epithelial cells [87]. GPER may also contribute to the pro-tumorigenic effects of endothelin-1 in hepatocarcinoma cells and breast cancer via the activation of PI3K/ERK/c-Fos/AP1 transduction pathway [88].

GPER actions were also implicated in Notch signaling, inducing both the γ -secretase-dependent activation of Notch-1 and the expression of the Notch target gene Hes-1 [85, 89]. It was reported that GPER activation leads to the association of the

intracellular domain of Notch-1 with the Hes-1 promoter inducing transcription of a Hes-1-reporter gene, as well as, to the down-regulation of VE-Cadherin and increased expression of Snail, a Notch target gene acting as a repressor of cadherin expression [89].

Interestingly, GPER activation of intracellular signaling pathways has been coupled with EGFR transactivation, through the Src-dependent stimulation of metalloproteinases (MMPs) (Fig. 3.3). MMPs have been shown to cleave the pro-HB-EGF leading to the release of free HB-EGF with resultant EGFR activation [13, 64, 90, 91]. Moreover, sphingosine 1-phosphate (S1P), a product of sphingosine kinase 1 (Sphk1), seems to be another intermediate in the GPER-mediated transactivation of the EGFR [92]. SphK1 activation stimulated by E_2 leads to the release of S1P, activating the S1P receptor Edg-3 and resulting in pro-HB-EGF cleavage [92]. A cross-talk of GPER with the IGF signaling pathway and GPER interaction with the IGFR and the ErbB receptors also has been described and associated with the progression of diverse types of cancer [93, 94].

Despite the transcriptional responses induced by estrogens are mainly associated to ER α - and ER β -mediated actions, also the rapid signaling pathways activated by GPER have been shown to regulate gene expression in broad range of mammalian tissues and cells. These includes the upregulation of expression of several genes involved in relevant biological responses, for example, the transcription factor c-fos, the connective-tissue growth factor (CTGF), the early growth response 1 (Egr-1), pS2, sirtuins, cyclins A, D1, D2 and E, and the apoptotic regulators Bcl-2 and Bax; cyclins A1 and B1 were identified as down-regulated genes by GPER [80, 95-101].

Other important aspect to consider in GPER signaling is its interaction with the nuclear steroid receptors, namely, with the classical ERs, the glucocorticoid receptors, the mineralocorticoid receptors, and the vitamin D receptor. This cross-talk was elegantly reviewed by Prossnitz *et al.* [102] and will not be further explored in this chapter.

In the end, it is important to mention the receptor activity-modifying proteins (RAMPs) that interact with the GPCRs modifying their function, and acting as pharmacological switches and chaperones regulating signaling transduction [103].

Although GPER interaction with RAMP3 has been reported and linked to the receptor presentation at the cell membrane [104], the role of this regulatory proteins in the modulation of GPER function/activity still is in its infancy and more research is needed to clarify the liaison between RAMPs and GPER.

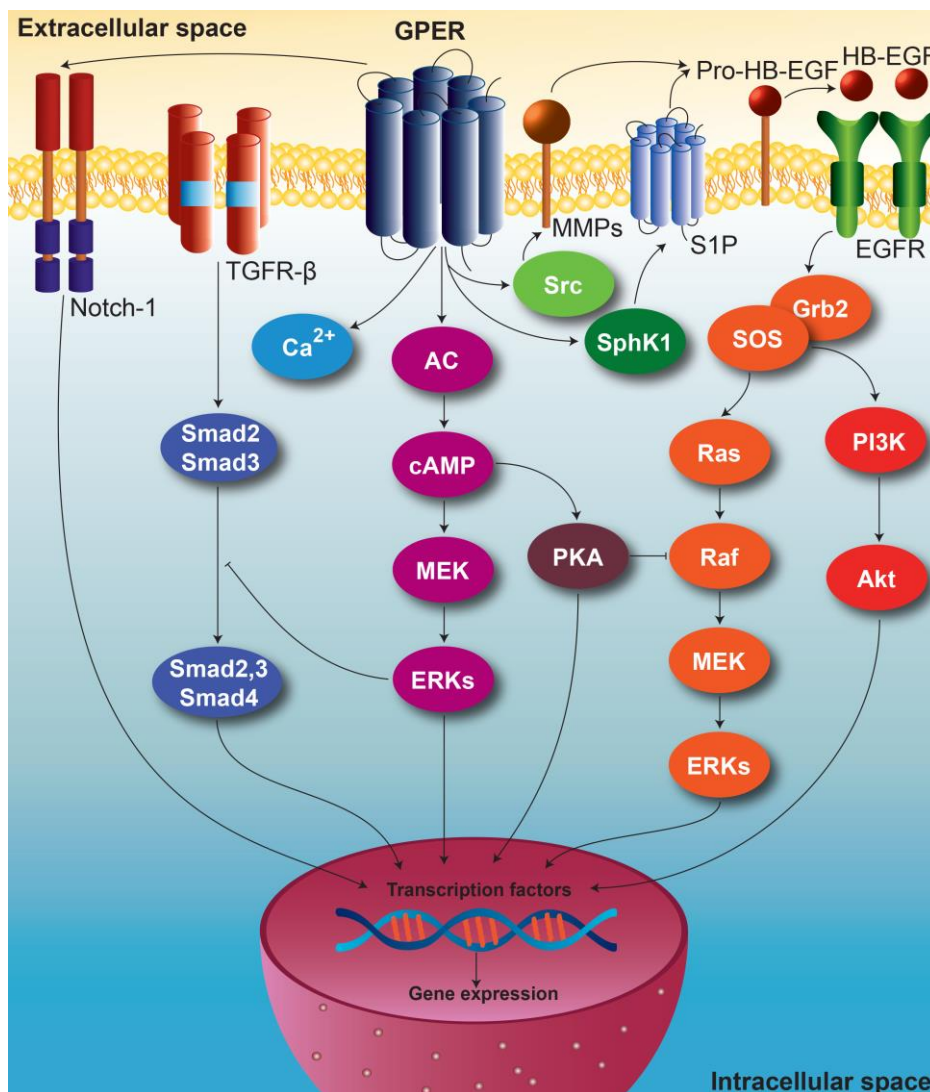


Figure 3.3. GPER signaling pathways. GPER activates several downstream signaling cascades including the PI3K/AKT pathway, the Src pathway, the MAPKs/ERK1/2 pathway and the Notch signaling. The activation of MAPKs pathway influences the activation of Smad proteins and leads to the inhibition of the TGF- β signaling. GPER activation also leads to stimulation of adenylyl cyclase (AC) with an increase of cAMP concentrations and mobilization of intracellular Ca^{2+} . GPER activation of intracellular signaling pathways also has been coupled with EGFR transactivation. GPER activation of Src-kinase stimulates MMPs that cleave the pro-HB-EGF leading to the release of free HB-EGF and EGFR activation. Alternatively, EGFR activation can be mediated by sphingosine 1 phosphate (S1P) a product of sphingosine kinase 1 (SphK1) activity.

3.4. GPER agonists and antagonists: a therapeutic approach

From the time that was considered an orphan receptor to nowadays, many compounds have been identified with the ability to bind GPER, which substantially

enriched its “molecular relationships”. Among the panoply of compounds that bind GPER, some of them are activators (agonists) whereas others inhibit receptor function/activity (antagonists). The identification of molecules able to modulate GPER activity (Table 3.1) and the disclosure of the underlying signaling pathways is an exciting field of research considering the exploitation of GPER as a therapeutic target.

Also, many studies have been performed to understand better the GPER structure and its binding characteristics. The specificity of GPER cavities and its several structural changes endows this receptor of a great ability to accept diverse ligands, some of them with large volume, and also that the same ligand can recognize different binding sites dependently on the structural conformation of GPER [105].

3.4.1. Natural hormone ligands

The first identified ligands for GPER, which gave the receptor’s name, were estrogens, particularly the E₂. Competitive binding assays in GPER-transfected COS7 cells using E₂ conjugated to the fluorophore Alexa 633 found a high-affinity for E₂ (K_d 6 nM) contrarily to the non-physiological estrogen stereoisomer 17 α -estradiol that fails to displace the fluorophore [52]. Similarly, studies with tritiated-hormone on membrane fractions also showed high-affinity of GPER for E₂ (K_d 3 nM) but low-affinity for 17 α -estradiol [53].

Table 3.1. List of compounds able to modulate GPER activity, agonists and antagonists*

Class	Compound	Effect
Hormone Ligands	17 α -estradiol	No
	17 β -estradiol-17-D-glucuronide	+
	2-hydroxyestradiol	-
	2-methoxy-estradiol	+
	7 β -OH-EpiA	-
	ACTH	?
	Aldosterone	+
	Cortisol	?
	E ₂	+
	Estriol	-
	Estrone	?
	Progesterone	No
	Testosterone	No

Synthetic Ligands	4-hydroxytamoxifen	+
	DES	No
	DPN	No
	ICI 182 780	+
	K-1	-
	MIBE	-
	PPT	+
	STX	?
	Raloxifene	+
Phytoestrogens	(-)-Epicatechin	+
	Baicalein	-
	Coumestrol	No
	Daidzein	?
	Equol	?
	Genistein	+
	Icariin	+
	Icaritin	+
	Oleuropein	+
	Prunetin	+
	Puerarine	+
	Quercetin	+
	Resveratrol	+
	SDG/ ENL	?
	Tectoridin	+
Xenoestrogens	2,29,59-PCB-4-OH	?
	Atrazine	+
	BDE-47	+
	bis(4-hydroxyphenyl)[2-(phenoxy sulfonyl)phenyl] methane	?
	BPA	+
	DDT	?
	DDE	?
	Kepone	+
	Methoxychlor	+
	Nonylphenol	+
	TDP	+
Other Compounds	Arsenite	+
	Butylparaben	+
	C4PY	-
	Cadmium	+
	CuSO ₄	?
	Methylparaben	+
	Nicotinamide	+
	Nicotinic acid/ Niacin/ Vitamin B ₃	+

Specific Ligands	Propylparaben	+
	ZnCl ₂	+
	G1	+
	G15	-
	G36	-
	GP _{ER} -L1	+
	GP _{ER} -L2	+
	PBX1	-
	PBX2	-

* Corresponding references are cited and discussed throughout the text.

+, agonist; - antagonist; ?, direct action on GPER needs confirmation; No - no effect reported on GPER activity

Nonetheless, the involvement of some E₂ metabolites in GPER activation has been reported. For example, the oxidised metabolite 2-methoxy-estradiol has been reported to act as an agonist of GPER [106] whereas the 2-hydroxyestradiol has been shown to antagonize GPER action [106, 107]. Glucuronidation and sulfonation of E₂ generate other metabolites like 17 β -estradiol-17-D-glucuronide, which also seems to be an agonist of GPER at high concentrations; 50 mM 17 β -estradiol-17-D-glucuronide increased the levels of cAMP and PKA activity [108].

Other estrogens like estrone and estriol exhibited very low affinity for GPER, whereas progesterone and testosterone were unable to bind GPER [53]. In the case of estriol, it seems to act as an antagonist of GPER [109, 110].

Considering the weak androgens dehydroepiandrosterone (DHEA) and epiandrosterone, no studies exist reporting GPER binding or activity. However, the endogenous metabolite 7 β -hydroxy-epiandrosterone (7 β -OH-EpiA) was shown to be capable of antagonizing estrogenic actions mediated by GPER [111].

Curiously, aldosterone, a mineralocorticoid steroid hormone, also seems to have affinity for GPER, with agonistic activity increasing the ERK phosphorylation in rat aortic endothelial cells and the migration of renal cancer cells [112-114]. Moreover, it was shown that the non-genomic effect of aldosterone causing rapid sodium intake involves GPER [115]. However, other studies were unable to detect aldosterone binding to cell membrane fractions [116, 117], suggesting that aldosterone could act through GPER, but without direct receptor binding [102].

Stress related hormones such as cortisol or adrenocorticotrophic hormone (ACTH) seem to modulate GPER expression, although their ability to bind the receptor remains to be evaluated [53, 118].

3.4.2. Synthetic ligands

Tamoxifen, a synthetic non-steroidal anti-estrogen is a recognized selective estrogen receptor modulator (SERM) with demonstrated efficacy as an adjuvant therapy for ER-positive and metastatic breast cancer [119]. It is a substituted triphenylethylene with low binding affinity for ERs compared with E_2 , but with a similar binding affinity for both ER subtypes displaying tissue-dependent ER antagonist or partial agonist activity. The 4-hydroxy metabolite (4-hydroxytamoxifen) is a more potent ER ligand and its active metabolite [120, 121].

Another synthetic compound, the estrogen derivative ICI 182 780 binds ERs with high affinity and has anti-estrogenic activity, blocking the ER transactivation, impairing its dimerization and inducing its degradation [120]. Interestingly, both ERs antagonists, 4-hydroxytamoxifen and ICI 182 780, can bind GPER acting as agonists [122, 123]. Moreover, the tamoxifen analog STX (a diphenylacrylamide) was shown to have rapid neurological responses through a mechanism unrelated to the classical ERs and dependent on a Gq protein, which suggests the involvement of GPER [124]. More recently, another SERM raloxifene also was shown to activate GPER in cells devoid of ER α , mediating Akt activation [35, 125].

The knowledge that the classical ER antagonists induce agonistic effects via GPER has stirred their application in cancer treatment. On the light of this information, extreme caution has to be placed on the application of anti-estrogens therapies when GPER is present in tumors, which can be overcome by the introduction of GPER analysis in clinical diagnosis. This would be particularly relevant in estrogen-related cancers, such as breast, endometrium and ovary.

The stilbene estrogen diethylstilbestrol (DES) a potent estrogenic compound binding both ER subtypes with an affinity similar to that of E_2 , do not display affinity for GPER [13, 52, 53, 122, 123, 126], though weak binding capability able to induce activation of CREB, and of the downstream targets PKA, Src, and ERK1/2 has been reported [53, 127]. Also, DES seems to inhibit GPER expression [128].

Interestingly, the widely used ER α -selective agonist propylpyrazole triol (PPT) has shown agonistic activity for GPER in a range of 10 to 100 nM concentrations, whereas diarylpropionitrile (DPN) the ER β -selective agonist had no activity through GPER [35]. Therefore, the use of PPT to ascertain ER α specific actions in different tissues and physiological conditions may have confounding effects triggered by GPER.

Also there are reports of compounds disrupting the GPER action transactivating EGFR (Fig. 3.3). The anti-estrogenic benzopyran derivative 2-[piperidinoethoxyphenyl]-3-[4-hydroxyphenyl]-2H-benzo(b)pyran (K-1) showed to

interfere with GPER mediated-EGFR activation, decreasing phosphorylation of MEK, ERK and of its downstream effectors such as c-jun and c-fos. Moreover, K-1 decreased the expression of β -catenin, proliferating cell nuclear antigen (PCNA), Cdk4, cyclin D1, c-Myc, p-CREB and Bcl-xl and increased the expression of apoptotic markers like NOXA, PUMA α , p21, p27 and Bax [129]. The newly synthesized compound MIBE (ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyl)-1-methyl-1H-indol-3-yl] but-2-enoate) prevented the GPER-mediated EGFR and ERK activation, with consequent effects on gene transcription and cell proliferation, and was, thus, indicated as a GPER antagonist [130].

3.4.3. Membrane-impermeable ligands

The development of ligands with membrane impermeable properties, through steroid association with large proteins (e.g. BSA), oligomeric/polymeric dendrimers (PAMAM) or cyclodextrins, as well as the ionic estrogen derivatives (e.g. NMe³⁺), have allowed studying membrane-associated ERs [131, 132], and to ascertain the existence of GPER effects triggered at cell membrane.

Revankar *et al* [133] evaluated the properties of a set of 17 α -substituted estrogen derivatives with differential cell permeability. For example, the cationic E₂-NMe³⁺ with a high-affinity for GPER, but membrane impermeable, at least for short periods of incubation and at low concentrations, was incapable of generating Ca²⁺ rise responses, whereas the related neutral carboxamide E₂-NB with membrane permeability similar to those of E₂ rapidly activated both the ER α and GPER [133]. These observations were quite relevant to support the intracellular location of GPER and its functionality driven by the endoplasmic reticulum. Nevertheless, this matter is far away from being solved and widely accepted among the scientific community. Moreover, these novel ligands are important to evaluate the subcellular location of GPER in healthy and pathological tissues and cells, and to decipher whether it varies with different environmental conditions, tissue properties, and/or developmental or disease stage. Further research is needed to clarify all these issues.

3.4.4. Phytoestrogens and xenoestrogens

In the last years, with the increasing relevance of compounds mimicking estrogens and the concerns about endocrine disruption, many studies have identified some phytoestrogens and xenoestrogens as possible GPER ligands.

Phytoestrogens mainly encompass phenolic compounds, which are structurally divided into flavones, isoflavones, lignans, coumestans, and stilbenes [134]. The potential of phenolic compounds as preventive or therapeutic agents in several human diseases has been under intense investigation, and a large variety of these compounds also has shown to be able to modulate GPER activity.

(-)-Epicatechin, a flavonoid present in cacao and many other food products, increases the activity of ERK1/2 through GPER activation [135]. Icariin is a prenylated flavonol glycoside isolated from plants of the genus *Epimedium*, which is used as a food supplement; icaritin is its hydroxylated derivative. Both icariin and icaritin seem to induce cellular effects through GPER, also inducing c-fos expression and EGFR-MAPK signaling activation [136, 137]. Baicalein, the main flavonoid derived from the root of *Scutellaria baicalensis* Georgi, a plant widely used in traditional Chinese medicine, also was indicated to interfere with GPER signaling [138]. In breast cancer cells, baicalein does not directly affect GPER expression, but it can inhibit GPER signal transduction pathways, namely, by suppressing the phosphorylation of ERK1/2, Akt, and Src, as well as the transcription of GPER-regulated genes, like c-fos, CTGF, CYR61, and Egr-1 [139].

Genistein, an isoflavone found in soy products, has shown high-affinity for GPER, with agonistic activity leading to MAPKs activation via a G β γ -associated pathway and requiring Src-related and EGFR kinase activity. Moreover, genistein seems to upregulate c-fos expression [95, 140-142]. Quercetin is a genistein related flavonoid that naturally occurs as the aglycone form of other flavonoid glycosides. At 1 mM concentration, quercetin showed the same behavior of genistein, acting as a GPER agonist with stimulation of c-fos expression [95]. The isoflavone Daidzein present in many plants and fruits exhibited both estrogenic and anti-estrogenic actions, despite no GPER binding affinity has been reported. However, it has been shown that daidzein mediates glutamate-induced effects via GPER [143]. Intestinal flora can metabolize daidzein to equol, which also do not have GPER binding ability described, but it has been suggested that it is involved in the generation of mitochondrial reactive oxygen species via GPER-mediated EGFR transactivation [144]. Nevertheless, puerarine an 8-C-glucoside of daidzein, was able to activate and modulate GPER activity and expression [145]. Similar findings were observed with prunetin, an isoflavone found in several agricultural foods, which seems to bind GPER with activation of ERK/MAPK

through adenylyl cyclase and cAMP [146]. Tectoridin, the 7-glucoside of the isoflavone tectorigenin, also exerted estrogenic effects mediated through GPER, whereas it binds ER α poorly [147].

Coumestrol is a phenolic phytoestrogen of the class of coumestans known to activate ER-mediated signaling pathways, but its ability to bind and activate GPER was not described yet [140].

Resveratrol, a stilbene derivative with important biological actions and indicated as an anti-cancer compound [148] had its activity inhibited in the presence of GPER antagonists, which suggests that its role depends on GPER, likely by promoting PKC activation [149].

Oleuropein, the glycosylated conjugate of elenolic acid present in olives, also has been included in the group of GPER agonists [150].

The flaxseed derivative secoisolariciresinol diglucoside (SDG), upon metabolism by mammalian cells, originates the metabolite enterolactone (ENL), that shares the similar binding site of GPER specific agonist G1 as indicated by docking simulations, suggesting that it can bind GPER [151].

Xenoestrogens include a wide variety of nonsteroidal chemicals including nonylphenols, polybrominated diphenyl ethers, organophosphates, chlorinated hydrocarbons, biphenyls and phthalates that can be found in daily-life products such as detergents, surfactants, resins, lubricants, plastics, fire retardants, and pesticides [152].

The nonylphenols used in plastic industry, despite having the low affinity for classical ERs, showed a higher binding affinity for GPER [140]. Both bisphenol A (BPA) and nonylphenol, are strong competitors of E₂ displacing the tritiated-hormone from GPER-containing membrane fractions [140]. BPA induced a rapid activation of ERK1/2 and the transcription c-fos likely through an AP1-mediated pathway [153-157]. BPA also stimulates the upregulation of X-linked inhibitor of apoptosis protein (XIAP), with accompanying cell migration and angiogenesis [154, 158-160]. Moreover, BPA at 100 μ mol/L seems to be able to increase GPER protein levels in the nucleus, but had no influence in the cytoplasmic fractions [161]. On the other hand, low doses of BPA did not seem to have stimulatory effects via GPER [162]. The 4,4'-thiodiphenol (TDP), a BPA derivative that exhibits more potent estrogenic activity than BPA does, can increase the GPER expression and activate the GPER-PI3K/Akt and ERK1/2 pathways [163].

Commercial dichlorodiphenyltrichloroethane (DDT) products consist of a mixture of compounds with the p,p'- and o,p'-isomers, both of which showed binding affinity for GPER, although it was higher in the case of p,p'-isomer [164, 165]. The degradation of DDT produces isomers of dichlorodiphenyldichloroethylene (DDE),

p,p'-DDE and o,p'-DDE, which also exhibit binding affinity for GPER [140]. The 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), a flame retardant frequently used in furniture, infant products, and electronics, also seems to have effects as a GPER agonist [166].

Several pesticides also have been indicated as activators of GPER. Methoxychlor showed ability to bind GPER and activate the GPER/EGFR signaling transduction pathway [140], whereas atrazine, induced ERK1/2 activation and the expression of c-fos and other E₂-target genes. Atrazine and others chlorotriazines, including cyanazine and simazine were also shown to regulate GPER expression in breast cancer cells [167].

Polychlorinated biphenyls (PCBs) are another group of environmental xenoestrogens, including the phenolic derivative 2,2',5,5'-PCB-4-OH, which exhibited low binding affinity for GPER [140]. Contrastingly, the high-affinity binding of GPER to another chlorinated endocrine disrupting chemicals, such as the insecticide kepone was indicated [140].

Also, the lipophilic bis(4-hydroxyphenyl)[2-(phenoxy sulfonyl)phenyl] methane, present in cell culture medium as an impurity of phenol red (phenolsulfonphthalein) used as a pH indicator, exhibits estrogenic properties, however, its interaction with GPER remains unclear [164, 168].

In an exhaustive study of Peter Thomas' group [140] comparing the estrogenic activity of a panoply of phytoestrogens and environmental contaminants, it was possible to hierarchize several of the compounds mentioned above concerning its ability to bind GPER. The phytoestrogen genistein was the most effective competitor for GPER, and BPA, and nonylphenol also displayed relatively high binding affinity. Kepone, DDT, 2,2',5,5'-PCB-4-OH and o,p'-DDE had lower affinity for GPER whereas o,p'-DDT, p,p'-DDE, methoxychlor and atrazine caused less than 50 % displacement of tritiated-estrogen from GPER-containing membrane fractions [140]. Moreover, it was shown that genistein, BPA, nonylphenol and kepone act as GPER agonists sharing the mechanistic of adenylyl cyclase activation [140].

3.4.5. Other compounds

Calixpyrroles belong to the category of macrocyclic compounds and are made up of pyrrole units assembled by quaternary carbon atoms at their 2,5-positions [169, 170]. A derivative of these compounds, the calix[4]pyrrole derivative [meso-octamethylcalix[4]pyrrole (C4PY)], acts as an antagonist of GPER preventing the ERK activation by E₂ although without trigger ERK phosphorylation. Moreover, C4PY

inhibited the E₂ and GPER agonist-induced expression of c-fos and Egr-1 and Akt phosphorylation [171].

The parabens, methyl-, propyl- and butylparaben, also seem to activate GPER, inducing the activation of the ERK1/2 pathway, an effect mediated by the G protein $\beta\gamma$ dimer. No activation was perceived concerning the cAMP/PKA pathway, and propylparaben was the only paraben able to activate Akt [172].

Reports also exist indicating that metallic compounds actions are mediated by GPER. Copper, an essential trace element naturally occurring in soil, water and air, has been implicated in tumor initiation and progression [173]. A recent study showed that GPER (together with HIF-1 α) is required for the copper sulfate (CuSO₄)-induced VEGF expression and proliferation of breast cancer cells [174]. Nevertheless, the ability of copper to directly activate GPER signaling requires confirmation. Arsenite and cadmium, environmental contaminants acting as endocrine disruptors were shown to activate GPER leading to MAPK activation [175], with cadmium directly activating the receptor [176]. Cadmium-induced effects mediated by GPER included the rapid activation of ERK1/2 and Akt and stimulation of breast cancer cells proliferation [177-179]. Zinc is essential in the regulation of several cellular functions, and a recent study also demonstrated that the stimulatory effects of zinc chloride (ZnCl₂) in breast cancer cells and cancer associated fibroblasts (CAFs) with the activation of ERK and Akt pathways are dependent on GPER [180].

Nicotinic acid, also designated niacin or vitamin B₃, and nicotinamide were reported as agonists of GPER, inducing GPER-mediated ERK activation and gene expression, stimulating cell proliferation and migration [181].

It is important to refer that, despite the high homology of GPER amino acid sequence with the chemokine receptor subfamily of GPCRs, chemokines did not seem to activate GPER [47].

3.4.6. GPER specific ligands

The majority of endogenous and synthetic compounds with affinity for GPER also has the ability to bind the classical nuclear ERs. For this reason, it was necessary to find out specific ligands for GPER that would allow separating the contribution of each receptor subtype to the physiological effects of estrogens.

Virtual and biomolecular screening for chemicals with similarity to estrogens in a library with thousands of compounds, retrieved one hundred strong candidates to be tested for GPER binding activity. One of these compounds presented agonist activity

for GPER and was inactive for the classical ERs. It was called G1 (GPR30-specific compound 1, a substituted dihydroquinoline, with a tetrahydro-3H-cyclopenta[c]quinoline core structure [182] that still today is the true selective GPER agonist being widely used in *in vitro* and *in vivo* studies.

Binding affinity studies demonstrated a high-binding affinity of G1 for GPER yielding a K_i of 11 nM whereas for E_2 the K_i was 5.7 nM. No significant binding of G1 was perceived for $ER\alpha$ and $ER\beta$ at concentrations up to 1 μ M [182]. Moreover, functional studies showed that the G1 effects inducing Ca^{2+} mobilization and PI3K activation were exclusive of cells harboring GPER, being absent in cells expressing either $ER\alpha$ or $ER\beta$ [182].

More recently, an antagonist of GPER closely resembling G1 but lacking the ethanone moiety was identified, the so-called G15 [183]. Competitive binding assays demonstrated that G15 binds GPER with an affinity of, approximately, 20 nM. Similarly to G1, G15 poorly binds $ER\alpha$ or $ER\beta$ at concentrations up to 10 μ M [183]. G15 seems to be able to reduce Akt phosphorylation, and NF- κ B signaling [184].

However, some low-affinity cross-reactivity of G15 with the classical $ER\alpha$ was described. For this reason, efforts were made to identify an antagonist with improved selectivity for GPER. In this way, an isosteric G1 derivative, the G36 was synthesized. This new GPER antagonist showed diminished capability of activating $ER\alpha$, while maintaining its GPER antagonist activity. Moreover, G36 was shown to selectively inhibit GPER activation of PI3K but not of $ER\alpha$. Also, it inhibited estrogen and G1 effects on Ca^{2+} mobilization and ERK1/2 activation, with no effect on EGF-mediated ERK1/2 activation [185]. Thus, the identification of G36 as a GPER antagonist with improved ER counter selectivity represented a significant step towards the development of new highly GPER selective therapeutics for cancer and other diseases. Indeed, both G1 and G36 have been envisaged as therapeutic tools [35, 186-188].

Other two original compounds, GPER-L1 and GPER-L2, were developed as GPER selective agonists [189], representing also valuable tools to disclose the pharmacologic actions of GPER and to discriminate more accurately the actions driven by each particular ER subtype. These compounds showed high affinity for GPER and were also capable of upregulating the expression of GPER target genes with higher efficacy than G1 or E_2 [189]. More recently, as a strategy to find out a new GPER antagonist, two novel compounds with a structure based on pyrroloquinoxaline were synthesized; PBX1 and PBX2, which were described as selective antagonists having the ability to suppress GPER-dependent signaling [190].

The development of new and selective agonists and antagonists of GPER significantly advanced the current knowledge about the physiological roles of this

receptor, controlling biological processes in healthy and pathological conditions. Also, this allowed and reinforced the consistency of targeting GPER as a potential therapeutic strategy, which could be achieved by activating or inhibiting its activity, depending on the deregulation of GPER identified in each particular disease.

3.5. Expression pattern of GPER in cancer tissues

GPER is expressed practically in all organs and tissues of the human body and has several important physiological functions in healthy conditions. However, GPER deregulated expression has been associated with several diseases, such as cancer, which may include either upregulated or downregulated expression levels.

Cancer-related expression of GPER has been described both in male and female cancers, with a great representation in hormone-dependent cancers, but reports in other oncological conditions also exist (Table 3.2). However, GPER has been mainly associated with female cancers, particularly breast cancer, but also with the endometrium and ovary cancers. In man, testicular and prostate cancers are important targets of this estrogen receptor.

GPER was firstly identified in human breast cancer cases, and cell lines and, thus, not surprisingly, its function in carcinogenesis is intimately related to breast cancer. Several studies revealed that GPER is strongly expressed in normal breast tissue, with a variable expression among tumor specimens. However, studies show that GPER is detected in the majority of invasive breast tumors whereas only 42 % of intraductal tumors were GPER-positive [191, 192]. Indeed, GPER association with breast cancer aggressiveness has been reported [39].

Table 3.2. Summary of GPER expression and subcellular localization in different human cancers*

Tissue	Tumor Subtype	Expression	Localization
Breast	Invasive tumors	++	Membrane
	Intraductal tumors	+	Cytoplasm
	Inflammatory breast cancer	+	Nucleus
	ER-positive	+	
	ER-negative	+	
	Triple-negative	+	
Ovary	-	+	?

Endometrium	ER-negative endometrial carcinoma	+	Cytoplasm
	ER-positive endometrial carcinoma	+	
	Carcinosarcoma	++	
Myometrium	Leiomyomas	++	Cytoplasm Nucleus
Testis	Sertoli cell tumor	+	Membrane
	Leydig cell tumor	+	Cytoplasm
	Seminomas	++	
	Embryonal carcinoma	+	
	Teratomas	+	
Lung	Adenocarcinomas	++	Cytoplasm
	Squamous cell carcinomas	++	Nucleus
	Non-small cell carcinoma	++	
Thyroid	Papillary carcinoma	++	Membrane
	Follicular carcinoma	++	Cytoplasm
Blood	T-cell leukemia	+	?
Bladder	Urothelial carcinoma	+	?
Pancreas	Adenocarcinoma	+	?
	Mucinous neoplasm	+	
Cervix	Carcinoma	+	?
Kidney	Adenocarcinoma	+	?
	Carcinoma	+	?
Pituitary	-	+	Cytoplasm
Prostate	Androgen sensitive	+ / ++	Membrane
	Castration resistant	++ / +	Cytoplasm Nucleus

* Corresponding references are cited and discussed throughout the text.

+, GPER expression; ++, high GPER expression; ?, unknown

Concerning subcellular location (Table 3.2), GPER seems to be found in the cytoplasm [193], around the nucleus and specifically at the plasma membrane in breast cancer cases [194]. Moreover, the cytoplasmic location of GPER in breast cancer seems to be correlated with the less advanced tumor stages, better histologic differentiation, non-ductal histologic subtypes, and enhanced overall patient survival whereas GPER nuclear expression is associated with poorly differentiated carcinomas [195]. Also, GPER overexpression and plasma membrane localization were indicated as key events in breast cancer progression [194]. Oppositely, the absence of GPER at the plasma membrane is indicative of a better long-term prognosis of primary breast cancer [194], suggesting that membrane-triggered GPER signaling may favor tumor progression.

A significant association between GPER and ER expression, as well as a positive correlation of GPER with HER-2/neu, EGFR, C-erbB2 and CD133 expression, tumor size, metastasis, peri-nodal invasion, and lymph node status, were also described [39,

178, 192, 196-198]. Not consistently, other study found no correlation between GPER expression and ER α [193]. In the case of triple-negative breast cancers (TNBCs), which are defined by the absence of classical ER α , progesterone receptor (PR) and HER-2/neu, GPER expression is maintained [199-201] showing a nuclear location [195]. However, GPER expression seems to be restricted to a group of younger women and possibly associated with recurrence of disease since it happened in 22.2 % of patients in the GPER-positive group against only 9.5 % in the GPER-negative group [199]. This is further supported by findings showing that the *in vitro* inactivation of GPER reduces growth of TNBC cells, preventing the activation of the kinase Src [202]. Moreover, the phosphorylation of the EGFR was almost abolished, and *c-fos* induction was inhibited with GPER inactivation [202].

Other evidence exists implicating GPER in recurrence of breast cancer [198]. A study evaluating GPER expression in local and distant metastatic lesions found that almost 70% of specimens express GPER, and also identified a relationship between GPER positivity and resistance to tamoxifen because recurrent tumors displayed increased GPER expression [196, 203]. Accordingly, a more recent study evaluated 77 breast cancer tissues with 53 recurrent breast cancer specimens after tamoxifen treatment and found that GPER expression was increased in 73.58 % of cases [204].

On the other hand, studies are proving that GPER expression decreases with the progression of breast cancer, in consequence of a reversible promoter inactivation by methylation [205, 206]. In addition, GPER down-regulated levels were suggested contributing to breast carcinogenesis and development of lymph node metastasis [34, 207]. Thus, GPER also has been proposed as a tumor suppressor gene [34, 207]. In line with these reports, the protective role of GPER in TNBC cells was described since its activation lead to inhibition of cancer cells growth [208]. Also, it was found that GPER expression in TNBC cases is negatively associated with high-grade tumors and lymph node metastasis, whereas displaying a significant and positive correlation with overall survival [209].

Concerning uterine tissues, GPER was described to be overexpressed in patients with endometriosis comparatively to the normal endometrium, suggesting the involvement of this receptor in disease onset [210, 211]. Endometriosis has been considered a risk factor for endometrial cancer, and in this way, it would be expectable a role for GPER in the endometrium cancer. Expectation confirmed by a study demonstrating that GPER modulates endometrial tumor growth by activation of its characteristic signaling pathways [35]. Besides that, GPER expression is augmented in endometrial carcinoma comparatively with non-neoplastic tissues, with 70 % of endometrial carcinomas expressing GPER, against only 26.67 % of normal

endometrium [212]. However, contradictory reports also exist indicating a decreased expression of GPER in endometrial carcinoma comparatively to the normal endometrium [213, 214]. Moreover, GPER was found to be expressed in ER-negative endometrial carcinoma and in ER-positive and ER-negative endometrial cancer cell lines [212].

Endometrial cancers display GPER expression in the stroma and glandular tissue with protein expression being mainly cytoplasmic [215]. GPER expression was correlated with the grade of endometrial carcinoma but failed any association with FIGO stage, myometrial invasion, or peritoneal cytology [212]. On the other hand, others described that loss of GPER expression was significantly associated with more aggressive features of endometrial tumors and poor prognosis. This included high FIGO stage, non-endometrioid histology, presence of lymph node metastasis, aneuploidy, and ER α loss, which suggests a link between the absence of GPER and the development of an aggressive tumor phenotype [215].

In uterine carcinosarcoma, namely, in the glandular component, GPER expression is higher than that in the normal endometrium [216]. Moreover, GPER expression was higher in advanced stages of carcinosarcoma and seemed to be correlated with the expression of ER β [216]. On the other hand, GPER is also highly expressed in uterine leiomyomas compared with their matched myometrium and, in this case, ER α also was overexpressed. Furthermore, GPER expression in uterine leiomyoma is detected in the nucleus, but in the myometrium, GPER localization is cytosolic [71].

GPER is expressed in ovarian cancer cell lines [217] and ovarian granulosa cell tumors [218, 219], and seems to be preferentially expressed in “high risk” epithelial ovarian cancer than in less aggressive ovarian tumors [40, 220]. Others also defended that GPER expression increases with the development and progression of ovarian cancer [40]. Accordingly, the high frequency of GPER expression in malignant ovarian endometriotic cysts comparatively with the benign forms was reported [221]. Moreover, GPER expression seems to be positively correlated with clinic pathological parameters, such as tumor stage, size, and lymph node metastasis, and also correlates with matrix MMP-9 expression, mainly in higher stage tumors [221]. In general, increased GPER expression is associated with aggressiveness of ovarian tumors and lower survival rates [40, 220, 221].

Testicular germ cell tumors (TGCTs) are the most common testicular solid cancer accounting for 90 % of primary tumors and mainly affecting young men [222]. TGCTs are in majority seminomas, which were shown to overexpress GPER [37, 223-225]. The overexpression of GPER in seminomas comparatively to non-seminomas could be due

to two polymorphisms frequently found in the promoter region of GPER in seminomas, rs3808350 and rs3808351 that cause a switch of the homozygous ancestral genotype GG to the homozygous AA genotype [223]. Thus, GPER has been considered a possible biomarker predictive of TGCTs [226]. It was also shown that GPER location in seminoma-derived cells was found at the cell membrane and cytoplasm, with a ratio of approximately 20 % : 80 % [223]. The augmented expression of GPER was described for TGCTs, but GPER was also detected in Sertoli and Leydig cells tumors, embryonal carcinomas, and teratomas [224, 225].

The expression pattern of GPER and its biological roles in PCa will be discussed in a separate topic of the chapter.

Although GPER has been highly related to cancers of reproductive tissues, likely by their hormonal dependency, there are other cancer types displaying altered expression patterns of GPER.

In lung cancer, namely, adenocarcinomas, squamous cell carcinomas, and non-small cell lung carcinomas, GPER expression was significantly increased relatively to the surrounding non-tumor tissue, both at mRNA and protein level [36]. Also, non-small cell lung cancer cell lines display consistently higher expression of GPER relatively to immortalized normal lung bronchial epithelial cells [36]. Moreover, GPER expression in non-small cell lung cancer cells was shown to be nuclear and cytoplasmic, with cytoplasmic location following tumor stages IIIA–IV, lymph node metastasis, and poorly differentiated non-small cell lung cancer cells [227].

Although the expression pattern of GPER in human oral cancers needs to be fully characterized, this receptor was detected in non-neoplastic oral tissues, such as the submandibular and parotid glands, and tongue, and its functional role in oral squamous carcinoma cells also was suggested [184, 228].

In the case of thyroid cancer, a high expression of GPER comparatively to the nodular hyperplasia tissues was found, and associated with lymph node metastasis in papillary thyroid carcinoma [229]. Moreover, GPER, in cooperation with EGFR and CXCR1, seems to have an important role in the diagnosis screening to differentiate between malignant follicular thyroid carcinoma and benign follicular thyroid adenoma because it is highly expressed in malignancy [230].

GPER expression was also reported in other human cancers (Table 3.2), namely, leukemia [231], urothelial carcinoma [16, 232], pancreatic adenocarcinoma and mucinous neoplasm [233], cervical carcinoma [234], kidney carcinoma and adenocarcinoma [235], and pituitary tumors [236], which indicates a broad action of GPER in the control of cell fate and tissue homeostasis contributing to malignant

transformation. Also, it is highly indicative that GPER expression is probably altered in other cancers that have not been evaluated yet.

3.6. The role of GPER controlling cell fate and metabolism

3.6.1. GPER actions in cell proliferation and apoptosis

Abnormalities in the control of cell-fate decision commonly lead to the unbalance between proliferation and apoptosis being major drivers of carcinogenesis. GPER, the new actor in the estrogens' "drama" that is expressed in a broad range of neoplastic and non-neoplastic tissues, seems to be one of the molecular protagonists in the regulation of cell fate, influencing cell proliferation, apoptosis and malignant features, such as, cell migration and invasion (Table 3.3).

Table 3.3. Summary of GPER actions controlling cell fate in different human cancers.

Tissue	Tumor Subtype	Proliferation	Apoptosis	Invasion and Migration
Adrenal cortex	Carcinoma	↓	↑	?
Bladder	-	↓	?	?
Breast	ER-negative	↑↓	↑	↑
	ER-positive	↓	↑↓	↑
	Triple-negative	?	↑	↑↓
Cervix	-	↓	?	?
Endometrium	-	↑	?	↑
Miometrium	Leiomyoma	↑	?	?
Blood	T-cell leukemia	?	↑	?
Kidney	Renal cell carcinoma	?	?	↑
Lung	-	↑	↑	↑
Lymphatic ganglions	Mantle cell lymphoma	↑	?	?
Nervous tissue	Neuroblastoma	?	?	↑

Oral mucosa	Squamous carcinoma	?	↓	?
Ovary	-	↑↓	↑↓	↑
	Granulosa cell tumors	?	?	↓
Prostate	Androgen-dependent	↑↓	↑↓	?
	Androgen-independent	↑↓	↑↓	?
Testis	Leydig cell tumor	↓	↑	?
	Seminoma	↑	?	?
Thyroid	Papillary carcinoma	↑	?	?

↑, increase; ↓, decrease; ?, unknown action

Some studies have reported that GPER is able to stimulate cell growth, increasing the proliferative activity. Estrogens and the GPER selective agonist G1 were shown to increase the mitotic index in the epithelial non-neoplastic MCF10A breast cells, and also the proportion of dividing cells in normal and malignant human breast and breast cancer explants [237]. The dependency of GPER to promote breast cells growth was further confirmed by the estrogenic effects observed on ER-negative breast cancer cells [178]. Moreover, GPER activation in MCF10A cells led to the activation of ERK in a process requiring EGFR transactivation [237]. Interestingly, in breast CAFs, E₂ seems to cause an interaction between GPER and phosphorylated EGFR, recruiting them to the cyclin D1 gene promoter [70]. In this CAFs, G1 activation of GPER resulted in transient increases in cell mitotic index, intracellular Ca²⁺, and ERK1/2 phosphorylation, linked with the promotion of proliferation and cell-cycle progression [238]. This supports the idea that GPER is involved in the functional connection between breast tumor cells and CAFs [239]. GPER activation in breast and endometrial cancer cells also was associated with the upregulation of Egr-1, a transcription factor involved in the promotion of cell proliferation. It was reported that G1 induces transcription of Egr-1 through GPER/EGFR/ERK signaling and induces the recruitment of Egr-1 to the CTGF and cyclin D1 promoter sequences [98].

In endometrial cancer cells and leiomyoma cells GPER increased cell proliferation also through the MEK/ERK MAPK pathway [126, 212, 240-242]. Pretreatment of endometriotic cells with G1 stimulated cell proliferation accompanied by rapid Akt phosphorylation, which was reversed by the GPER antagonist G15 [243]. Accordingly, a recent study showed that targeting GPER by the overexpression of the regulatory miR-424, decreased E₂-induced cell proliferation by inactivation of the PI3K/Akt signaling [244].

In the case of ovarian cancer cells, an augmented proliferation was observed upon G1-stimulation and GPER activation, which was underpinned by an increased number of cells in S-phase, and upregulated levels of c-fos, cyclin D1, cyclin E, and cyclin A proteins [96, 245].

In primordial germ cells, precursors of both sperm and eggs, GPER activation by estrogens induced cell proliferation through G $\beta\gamma$ -subunit protein- and matrix metalloproteinase-dependent transactivation of the EGFR [17]. EGFR signaling activated the PI3K/Akt/ β -catenin pathway, increasing c-fos, c-Myc, and cyclin D1/E expression [17]. The proliferative effects of GPER over germ cells were confirmed at post-natal stages. G1 induced proliferation of the spermatogonial GC-1 cell line, with the upregulated expression of cyclin D1 and through a cross-talk between GPER and ER α , and activation of the EGFR/ERK/fos pathway [246].

Also in thyroid cancer cells, GPER activation caused transcriptional activity of c-fos promoter with increased expression of c-fos, cyclin A, and cyclin D1 [247].

The GPER also contributed to the proliferation and survival of mantle cell lymphoma cells, and its expression levels were correlated with Akt and MAPK phosphorylation, as well as, with cyclin D1 expression [248]. The activation of MAPK pathway by GPER also seems to be involved in the induction of proliferation in lung cancer cells [227].

There are also reports of GPER stimulation of cell proliferation through its activation by other compounds than estrogens or G1. It is the case of the estrogen-mimicking compound used in plastic production BPA that activates GPER with enhanced proliferation of breast cancer cells and augmented levels of c-fos, Egr-1, and CTGF proteins [98, 249]. BPA also was shown to increase proliferation of spermatogonial cells and the proliferative activity in testicular seminomas, effects mediated by the GPER [155, 156, 250, 251].

Also, the ER antagonist 4-hydroxytamoxifen, known to act as an agonist of GPER [126], stimulated breast [97, 98] and thyroid [247] cancer cells growth. Regarding thyroid cancer cells, similar effects were seen with the phytoestrogen genistein [247].

Overall, the facts described above argue for the “villain role” of GPER in tissue homeostasis by its ability to promote cell growth and proliferation. However, the other face of GPER as the “good guy” is sustained by many other studies that demonstrate its suppressive effects on cell proliferation, including in tissues where the pro-proliferative activity was described.

The activation of GPER inhibited the growth of ER-negative breast cancer cells with the cell cycle arrested at G2/M [252]. These effects were underpinned by the downregulated expression of G2-checkpoint regulator cyclin B and increased

expression of p53, as well as, by enhanced p53 phosphorylation at serine 15, which promotes its nuclear translocation and inhibits ubiquitylation. The augmented levels of p53 were also accompanied by increased expression of cell cycle inhibitor p21 [252]. GPER also inhibited proliferation of ER-positive breast cancer cells, blocking cell cycle at G1, and decreasing the population of cells in S-phase, since both p53 and p21 proteins were upregulated by G1 administration [178]. G1 suppression of cell growth and arrest of cell cycle lead to caspase activation and apoptosis. Moreover, it was shown that G1 could bind the colchicine binding site of tubulin, inhibiting tubulin polymerization and the subsequent assembly of mitotic spindle apparatus in breast cancer cell mitosis [253].

In human cervical cancer cells, GPER activation induced G2/M cell cycle arrest and down regulated cyclin B expression, inhibiting cell proliferation through ERK1/2 and EGFR signaling [234]. Also, in human bladder-derived T24 carcinoma cells, E₂ inhibited cell growth via GPER mediated phosphorylation of ERK [232].

In ovarian cancer cells, it was shown that G1 blocks tubulin polymerization hampering the assembly of microtubules, which leads to the cell cycle arrest and suppression of proliferation [254]. Similar G1 effects were observed in endothelial cells, with inhibition of DNA synthesis and accumulation of cells in S and G2 phases of cell cycle [255], and adult female rats hippocampus that displayed significantly diminished cell proliferation [256].

The activation of GPER was also associated with the decreased proliferation of adrenocortical carcinoma cells [186], and GPER-positive endometrial adenocarcinoma cell lines HEC-1A and RL95-2, but not in GPER-negative endometrial adenocarcinoma HEC-1B cells [213].

In the other plate of the balance of tissue homeostasis, there is apoptosis, the fundamental biological process of programmed cell death that also seems to be regulated by the GPER. Curiously, and as discussed for cell proliferation, GPER actions in the control of apoptosis are the two faces of the same coin. Studies exist reporting that GPER activation diminishes apoptosis, while others defend the opposite.

The apoptotic process may be triggered by two distinct and interrelated pathways, the intrinsic (or mitochondrial) and the extrinsic (or death receptor) pathway that converge at the activation of apoptosis effector caspase-3 [257]. Nevertheless, the GPER effects have been mainly related to the mitochondrial pathway and altered expression or activity of the Bcl-2 protein family of apoptosis regulators [258]. The antiapoptotic effect of GPER was stated, for example, in the ER α -negative ovarian cancer cells OVCAR5, by diminishing the expression of cleaved-caspase-3 [245]. In rat Sertoli cells, GPER seems to decrease apoptosis, by increasing the

expression of Bcl-2 and decreasing Bax levels [259], respectively, anti- and pro-apoptotic members of the Bcl-2 family [258]. The same effect of GPER activation elevating Bcl-2 levels and reducing Bax with the consequent inhibition of apoptosis was seen in myocardial cells following ischemia/reperfusion injury [260]. The GPER activation through the ERK pathway also was seen to be cardioprotective by inhibiting the opening of mitochondria permeability transition pore mPTP [261]. Furthermore, GPER stimulation enhanced mitochondrial function and decreased oxidative stress in cardiac muscle [262]. Moreover, decreased TNF- α levels were reported following ischemia-reperfusion and GPER activation [260]. Accordingly, E₂ enhanced Bcl-2 expression and CREB phosphorylation, preventing oxidative stress-induced apoptosis in keratinocytes by phosphorylating cAMP response element-binding protein via cAMP/PKA pathway, an effect mediated via membrane GPER [99]. The GPER also showed antiapoptotic and protective effects in spinal motor neurons after injury through the activation of PI3K/Akt pathway [263].

Other mechanism associated with the GPER inhibition of apoptosis was reported in the ER-positive MCF7 breast cancer cell line, and involves FOXO3a inactivation [264]. FOXO3 is a transcription factor that specifically induces the transcription of proapoptotic genes, such as Bim, p21, and p27 [265]. The activation of GPER leads to rapid FOXO3a translocation to the cytoplasm, and this process seems to be achieved by the p110 α catalytic subunit of PI3K as a result of EGFR transactivation. Additionally, G1 stimulation of MCF7 cells resulted in decreased caspase activation under proapoptotic conditions [264]. In this report, the SERMs tamoxifen and raloxifene, as well as ICI 182 780, also were able to mediate FOXO3a inactivation in a GPER-dependent mechanism [264].

The antiapoptotic action of GPER was also evidenced by the use of GPER antagonist G15. Administration of this compound induced apoptosis of human oral squamous carcinoma cells, indicating that GPER activation is needed to sustain cell survival and diminish apoptosis [184].

Nevertheless, a substantial number of studies report the proapoptotic nature of GPER. In ER-positive MCF7 breast cancer cells, and contrastingly with the stated above, the proapoptotic role of GPER also was described. Knockdown of receptor was linked to decreased basal expression of tumor suppressor protein p53, which is linked to increased apoptosis and decreased cell-cycle progression [266]. This proapoptotic action of GPER was also found in the ER-negative SK-BR-3 breast cancer cells, concomitantly with enhanced expression of Bax, Bim, and cleaved-caspase-3, and diminished expression of Bcl-2 [205, 252]. Similar effects were seen in the triple-negative MDA-MB-231 and MDA-MB-468 breast cancer cells [208] that, despite

devoid of classical ERs, are known to express GPER [208], which demonstrates the importance of GPER inducing apoptosis.

Also in other tumor types, the GPER effects inducing apoptosis were indicated. It is the case of tumor Leydig cells, in which GPER activation led to apoptosis, with decreased Bcl-2 and increased Bax expression, cytochrome c release, and activation of caspase-3 and poly (ADP-ribose) polymerase 1 (PARP-1) [267]. In ovarian cancer cells, the GPER agonist G1 caused DNA fragmentation, increased the expression of cell-cycle inhibitor p21, and decreased the expression of Bcl-2 and cleavage of PARP and fodrin, two important markers of apoptosis [254]. Enhanced caspase-3 activation and apoptosis in response to G1 stimulation and on the dependency of GPER actions were also reported in human ovarian endometriotic stromal cells [268], lung cancer cells [269] and adult T-cell leukemia cells [231].

Moreover, also in the adrenocortical H295R carcinoma cells, G1 treatment was shown to cause morphological changes in cell nuclei, DNA damage and apoptosis by the activation of the intrinsic pathway [186]. GPER agonist treatment increased Bax expression whereas decreasing Bcl-2, and increased cytosolic cytochrome c levels decreasing its content in the mitochondrial compartment. Accordingly, activation of the initiator caspase-9, as well as, the executioners caspase-3/7 was detected [186].

There is also the example of rat pachytene spermatocytes, in which GPER activation by E₂ or G1 up-regulates the expression of proapoptotic factor Bax whereas downregulating the cell cycle regulators cyclin A1 and B1 [80]. Moreover, it was demonstrated that the rapid EGFR/ERK/c-Jun pathway modulates gene expression towards the balance between cellular proliferation and apoptosis [80]. Similarly, in the mouse spermatocyte-derived cell line GC-2, GPER activation caused rapid ERK, c-Jun and p38 phosphorylation, Bax upregulation, Bcl-2 downregulation, cytochrome c release, caspase-3 and PARP activation, DNA damage and increased expression of cell cycle inhibitor p21 [270].

GPER regulation of cell proliferation and apoptosis has also been linked with the regulation of other associated processes, namely, oxidative stress and Ca²⁺ homeostasis. In lung cancer cells, it was shown that GPER regulates the NO levels, and superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) activity, contributing to decreased cell proliferation and increased apoptosis [269, 271]. GPER was able to increase T-type Ca²⁺ channels currents in trigeminal ganglion [272], and the activity of L-type Ca²⁺ channel α 1D subunit in endometrial carcinoma cells and breast cancer cells, which was required for the E₂-stimulated Ca²⁺ influx associated with the promotion of cell proliferation, and development of endometrial cancer [273, 274].

In the scenario of cell-fate decision and maintenance of tissue homeostasis, it became evident the opposite effects of GPER in the control of cell proliferation and apoptosis. This duality of action most likely depends on a panoply of factors that may include the type of tissue and physiological context, the dose of activators and time of exposure, as well as other unforeseeable variables. The different effects of GPER also would be explained by a regulatory role of GPER shaped by the tissue specificity in particular environmental and biological conditions.

3.6.2. GPER influence over cell migration and invasiveness

Cell migration and invasiveness are coordinated biological processes that play a major role in cancer progression being closely related to metastization. The loss of cell-cell adhesion is a driven event for malignant cells evasion from their primary sites, which also depends on the degradation of the extracellular matrix, acquisition of an invasion phenotype, and finally, invading and metastasizing to other organs/tissues [275]. Indeed, the great majority of cancer deaths (> 90 %) are caused by metastasis rather than by the primary tumors [276], which renders cell migration a therapeutically relevant target point. Both migration and invasion seem to be promoted by GPER in ER-negative breast cancer cells through the activation of ERK and Akt pathways, nuclear translocation of NF- κ B and increased expression of interleukin-8 (IL-8) [277]. The role of this cytokine in the progression and metastasis of a variety of human cancers has been discussed [278]. GPER activation also leads to migration and invasiveness of TNBC cells and inflammatory breast cancer cells, responses mediated by the activation of the ERK1/2 pathway [279, 280]. Furthermore, GPER seems to upregulate β 1-integrin expression, through EGFR/ERK signaling pathway, which promotes migration of CAFs and epithelial-mesenchymal transition of tamoxifen-resistant breast cancer cells [204], contributing to tamoxifen resistance via interaction with the tumor microenvironment [204, 281]. Moreover, GPER activation can lead to the increased expression of IL1 β in CAFs and IL1 receptor 1 (IL1R1) in breast cancer cells, promoting the interaction between these two cell types, the upregulation of inflammatory target genes, inducing migration and invasion of breast cancer cells [282]. Accordingly, GPER inhibition with G15 seems to increase the sensitivity of epithelial breast cancer cells to doxorubicin by preventing epithelial-mesenchymal transition [283].

GPER actions prompting cell migration and invasion were also described in ER-positive breast cancer cells, through the activation of ErbB2-ERK signaling

transduction pathway [284]. In this breast cancer cell type, GPER enhanced migration also by mediating the dramatic proteolysis of cyclin E, with the involvement of EGFR signaling [281]. Other results showed that E₂ and ICI 182 780 enhanced adhesion of MCF7 breast cancer cells to matrigel, with increased autolysis of calpain 1 and proteolysis of focal adhesion kinase (FAK), with calpain activation through the ERK1/2 pathway [285]. Also, the xenoestrogen BPA stimulated migration of breast cancer cells and CAFs via GPER [249] by FAK, Src and ERK2-dependent pathways [286].

Interestingly, antagonizing GPER activity by natural compounds, like baicalein, diminished the expression of GPER target genes, including cysteine-rich 61 (CYR61) and CTGF, and suppressed E₂-stimulation of migration and invasion of breast cancer cells [138].

The pro-invasiveness effects of GPER were also identified in ovarian cancer cells OVCAR-5 and SKOV3 with modulation of expression and activity of MMP-2 and MMP-9, extracellular matrix proteins with a determinant role in cancer cell invasion and metastasis [287, 288]. Similar effects dependent on increased MMP-2 and MMP-9 production were seen in the endometrial Ishikawa and KLE cancer cells [241]. In the endometrial cancer cell line RL95-2, GPER mediated carcinogenesis and invasion via the activation of MEK/ERK MAPK pathway [240]. Moreover, the phosphorylation of FAK, a tyrosine kinase with a key role in tumor cells invasiveness [289], also was involved in the migration of Ishikawa and RL95-2 cells induced by GPER activation [290]. The association of MAPK pathway with cell migration and invasion in response to GPER activation also was described in lung cancer cells (200). Also in lung cancer and mesothelioma, GPER contributes to the chemotaxis and migration, through IGF-I actions [291].

In renal cell carcinoma, GPER activation mediated cell invasiveness via PI3K/Akt/MMP-9 signals [235].

The environmental pollutant BDE-47 increased migration and invasion of neuroblastoma SH-SY5Y cells in a mechanism dependent on GPER [166]. The associated molecular events included the downregulated expression of E-cadherin and zona occludin-1, and the upregulated expression of MMP-9, via activation of PI3K/Akt signaling pathway [166].

Nevertheless, contrary evidence exists depicting the role of GPER suppressing cell migration and invasion (Table 3.3). A recent study has shown that the activation of PI3K/Akt and ERK1/2 pathways by the GPER specific agonist G1 suppress migration and invasion of TNBC cells. Other responses included the inhibition of the epithelial mesenchymal transition by reducing the phosphorylation, nuclear localization, and

transcriptional activity of NF- κ B, [209]. There is also evidence that E₂-effects mediated by the membrane GPER in human metastatic breast cancers lead to decreased cell adhesion, through a PKA-dependent mechanism requiring the activity of voltage-gated sodium channels (VGSCs) [292].

In granulosa cell tumors, E₂ decreased cell migration and matrix invasion, effects accompanied by GPER inhibition of ERK1/2 signaling through a non-genomic mechanism [219]. Other studies with ovarian cancer cells suggested that E₂ inhibits tumor invasion, by inhibiting EGF-induced cell migration and the expression of urokinase plasminogen activator receptor (uPAR) expression, effects mediated by GPER [293]. Also in lung, and contrarily to the studies mentioned above, the activation of GPER showed to inhibit the migration of non-small cell lung cancer cells, suppressing the activity of IKK- β and NF- κ B [294].

3.6.3. Angiogenesis, inflammation and the GPER

Angiogenesis and inflammation are other important hallmarks in tumor progression fueling cancer cell growth within the “hostile” tumor microenvironment. GPER activation increased VEGF levels in ER-negative breast cancer cells and CAFs via upregulation of the HIF1 α in consequence of activation of the EGFR/ERK/c-fos signaling pathway [83]. Moreover, the receptor seems to be involved in the formation of human endothelial tube, enhancing angiogenesis and tumor progression [83].

The *GPER* knockout mice display a pro-inflammatory phenotype with augmented levels of the pro-inflammatory and immunomodulatory cytokines IL-1 β , IL-6, IL-12, TNF α , monocyte chemotactic protein-1 (MCP-1), interferon γ -induced protein 10 (IP-10) and monokine induced by γ interferon (MIG), concomitantly with the decreased expression of the adipose tissue-specific cytokine adiponectin [295]. Moreover, the anti-inflammatory role of GPER was shown in endothelial cells, in which, receptor activation counteracted the TNF effects inducing upregulation of pro-inflammatory proteins, namely, intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [296]. Moreover, airway inflammation was suppressed by the GPER agonist G1 in a mouse model of asthma, apparently via IL-10 [297]. In human breast cancer cells, GPER inhibited the TNF α -induced IL-6 expression, probably through blockage of NF- κ B promoter activity [298]. Consistent with those findings, a recent study in a *GPER* knockout mouse model, showed accelerated development of liver tumors, with immune cell infiltration, fibrosis, and

production of inflammatory factors, including IL-6. More studies are warranted to establish the role of GPER in angiogenesis and inflammation.

3.6.4. GPER as a regulator of cancer cell metabolism?

In the last years, the ability of tumor cells to reprogram metabolism, sustaining their high energy requirements, proliferation, and survival, has been indicated as a characteristic of malignant transformation and recognized as a hallmark of cancer [299]. However, the most well-known adaptation of cancer cell metabolism was described in the 30s by Otto Warburg and is known as the “Warburg effect” that consists in the increased glucose consumption followed by the augmented production of lactate instead of the oxidation of pyruvate in mitochondria, even under aerobic conditions [300]. In the light of available literature, GPER has “something to say” in the control of the metabolic process. *GPER* knockout female mice showed increased plasma glucose levels, leading to hyperglycemia and glucose intolerance, associated with the decreased expression of insulin in isolated pancreatic islets and diminished insulin release both *in vitro* and *in vivo* [301]. Likewise, glucose intolerance and insulin resistance were reported in the *GPER* knockout male mice [295]. In agreement, GPER seems to induce insulin secretion in pancreatic β -cells under low- and high-glucose conditions, through the activation of EGFR, ERK and PI3K/Akt pathways [302]. Curiously, insulin resistance is closely related to endometrial oncogenesis, and it seems to up-regulate Ten-Eleven Translocation 1 (TET1) that can up-regulate the GPER expression, activating PI3K/AKT signaling pathway and promoting cell proliferation [303]. Available data revealed an important role of GPER in glucose metabolism though in the particular case of cancer cells, information relating GPER with glycolytic metabolism is scarce, being an issue that deserves attention of direct studies. Nevertheless, Yu *et al* [304] first reported the interplay between breast cancer cells and CAFs concerning glucose metabolism. This study showed that the activation of the PI3K/AKT signaling pathway in breast cancer cells induces the cytoplasmic translocation of GPER in CAFs, leading to the activation of GPER/cAMP/PKA/CREB signaling and to the aerobic glycolysis switch in CAFs. In turn, the glycolytic CAFs feed breast tumor cells with extra pyruvate and lactate augmenting mitochondrial activity. Moreover, cytoplasmic GPER expression in stromal fibroblasts predicted high tumor metabolic activity and potent energy transfer between the stroma and cancer cells [304].

Besides glycolysis, other energy routes are involved in the metabolic adaptation of cancer cells, namely, glutamine metabolism, and its resulting product glutamate [305]. The glutamate transporter-1 (GLT-1) is an essential player removing the extracellular excess of glutamate. GLT-1 expression was shown to be increased by GPER activation triggered by G1 in rat primary astrocytes, which was accompanied by the increased uptake of glutamate [306]. The expression of glutamate aspartate transporter (GLAST), other transporter involved in glutamate uptake, was also increased in response to G1 [307]. Moreover, the mechanistic under this regulation was outlined with effects reported to occur via the MAPK/ERK, PI3K/Akt, TGF- α /EGFR, Src, NF- κ B and CREB pathways [307]. Further studies are warranted to confirm whether GPER actions regulate glutamine and glutamate metabolism in cancer cells, and if it has impact on disease progression and aggressiveness.

GPER also seems to have a major role in the regulation of lipid metabolism, another energetic pathway widely altered in cancer cells. Besides having a glucose intolerance phenotype, *GPER* knockout male mice display an altered lipid profile with increased cholesterol and triglyceride levels [295], which was corroborated by *in vitro* findings. GPER activation decreased triglyceride accumulation in cultured rodent β cells and reduced lipid synthesis in pancreatic islets by reducing the expression and activity of fatty acid synthase (FAS), the master effector of *de novo* lipogenesis [308]. This study also showed that the β cell transcription factor pancreatic and duodenal homeobox 1 (Pdx1) and the downstream target genes, such as proinsulin 1 (Ins1), glucose transporter 2 (GLUT2), and glucokinase (Gckr) were downregulated by E₂ via GPER action [308]. Furthermore, the FAS is the target of lipogenic transcriptional regulators, namely, the sterol regulatory element-binding protein 1c (SREBP1c), the carbohydrate response element binding protein (ChREBP), and the liver X receptor (LXR) [309-311], which were suppressed in response to GPER activation [312].

However, in breast (SkBr3), colorectal (LoVo), and hepatocarcinoma (HepG2) cancer cells and CAFs, GPER seems to up-regulate FAS expression and activity, through the EGFR/ERK/c-fos/AP1 transduction pathway [313]. Other studies reported that the GPER action increased the expression of low-density lipoprotein (LDL) receptor, and that G1 stimulation induced the uptake of LDL in liver cells [314, 315]. Curiously, GPER promoted adipogenesis *in vitro* and seems to be involved in the development of obesity in female mice exposed to a high-fat diet since *GPER* knockout mice were resistant to diet induced-obesity, glucose intolerance and insulin resistance [316].

Malate dehydrogenases (MDHs) are metabolic enzymes that function as transcriptional factors to regulate the expression of oncogenes and tumor suppressor

genes. The expression of MDH2, one of the isoforms of MDHs involved in citric acid cycle in mitochondria [317], was shown to be increased in endometrial cancer cells in response to GPER activation [318]. Moreover, the augmented expression of MDH2 lead to enhanced cell proliferation, migration and invasion but inhibited apoptosis of endometrial cancer cells by suppressing PTEN expression [318].

In sum, GPER has important roles in insulin sensitivity, glucose tolerance, adiposity and energy balance in many cell types, which ignites the curiosity about the activity of GPER in the metabolic reprogramming of cancer cells. Also, it is liable to speculate that GPER selective ligands would have therapeutic value for cancer treatment targeting metabolism.

3.7. GPER in prostate cancer

PCa is one of the most common oncological disorders in men, being highly frequent in men over the age of 50 [319]. Indeed, aging is the main risk factor for PCa, which raises the concern about its incidence in the next years considering the population aging in consequence of the increased expectancy of life. The vast majority of PCa patients (90 %) develop castration-resistant PCa (CRPC), an advanced metastatic form of disease, characterized by its aggressiveness and resistance to the classical anti-androgen therapies, which remains incurable with an average survival time of only 16–18 months [320, 321]. The search for new and effective approaches for better management and treatment of both localized and metastatic PCa remains crucial and urgent.

It is well established that the development and progression of PCa are strongly regulated by sex steroid hormones, with the male hormones androgens highly implicated in the regulation of prostate functions from the embryonic development to adulthood, but also in the onset of prostate malignancy [322]. Estrogens, classically viewed as female hormones, also seem to have a relevant role in PCa with some studies reporting their actions as causative factors in prostate carcinogenesis [323, 324]. On the other hand, estrogens have been used to treat PCa by its negative feedback actions at the hypothalamus and pituitary reducing circulating androgens levels [325]. However, it has also been shown that estrogens have beneficial direct effects on PCa cells [326]. Independent studies have indicated that these steroids can be protective against PCa by their proapoptotic and antiproliferative actions over prostate cells [326, 327].

Estrogenic actions through the interaction with the classical nuclear estrogen receptors, ER α and ER β , and involving genomic responses characterized by changes in gene transcription and protein *de novo* synthesis have been reported in both non-neoplastic and neoplastic prostate cells [328]. However, the activity of these receptors in PCa relies on the paradigm that ER α is responsible for the proliferative effects of estrogens being oncogenic whereas ER β is considered protective, anti-carcinogenic and related with the activation of apoptosis [329].

Also, the GPER has been identified in normal human prostate, and benign and neoplastic conditions. The prostate gland mainly encompasses two compartments: the fibromuscular stroma and the glandular tissue. In the glandular epithelium, it is possible to distinguish two histological layers, the secretory luminal layer consisting of tall columnar cells responsible for the production of prostatic secretions, and an underpinning basal layer of cuboidal epithelial cells. The population of basal epithelial cells has shown to be heterogeneous and containing adult prostate stem cells, which have been proposed as cancer stem cells in the origin of PCa [330]. In the human benign prostate gland, a strong GPER expression was observed in the cytoplasm of basal epithelial cells [331]. Therefore, it is liable to assume that GPER may be the mediator of estrogenic actions in this proliferative prostatic compartment. Accordingly, GPER expression was found in prostaspheres derived from human normal adult prostate stem cells [332].

GPER also was found in human prostate epithelial cells, displaying both membrane and cytoplasm localization [332-334]. Furthermore, a weak staining in the cytoplasm of stromal cells of benign prostate was also detected, suggesting the possible involvement of this receptor in estrogen signaling between stromal and epithelial prostatic compartments [331]. Accordingly, GPER was found to be expressed in human primary prostate stroma cells, derived from fresh surgical prostate specimens of benign prostate hyperplasia (BPH), and in the normal human prostate stroma cell line, WPMY-1 [335].

The reports characterizing the GPER expression in neoplastic conditions are relatively scarce, but a study found that GPER was expressed in 97.5 % of all prostate tumor cases evaluated [336]. It was described that pre-neoplastic lesions (high-grade prostatic intra-epithelial neoplasia) show higher GPER expression comparatively with non-neoplastic tissues, and that GPER expression decreases from moderately to poorly differentiated PCa [331]. Moreover, GPER expression was correlated with the Gleason score of prostate adenocarcinoma being intense in Gleason patterns 2 and 3, and weak to moderate in Gleason pattern 4 [331], which indicates an inverse relationship between GPER expression and neoplastic cell differentiation. Despite the predominant,

and sometimes nearly exclusive, cytoplasmic expression of GPER in PCa cases, areas with membrane and nuclear staining were also found [331, 336] [337].

Considering the androgen-sensitive stage of disease and CRPC, GPER seems to be more expressed in CRPC cases; 80 % of metastatic CRPC highly express GPER, against only 54 % of the primary PCa cases [338]. However, no correlation was found between GPER expression and, age, the Gleason score of primary cancer or PSA level. The type of androgen deprivation therapy, or duration of treatment also were not associated with GPER [338]. These results are not totally in agreement with the findings obtained in PCa cell lines. GPER expression levels were markedly higher in the normal progenitor prostate cells relatively to the androgen-sensitive PCa cell line LNCaP [332]. In CRPC cell line models PC3 and DU145, GPER expression was higher in PC3 cells, which mimic a more aggressive metastatic stage of disease [334]. Recent findings from our research group also demonstrated that GPER expression tends to be lost with the acquisition of the castration phenotype and aggressiveness of PCa cell line models; GPER expression in LNCaP > PC3 > DU145 cells (results to be published elsewhere).

As previously discussed for other cancer types, also in PCa, the GPER dual-mode of action has been described with opposite effects arising from separate independent studies. Some authors argue that GPER is involved in PCa development and progression based on the assumption that GPER may mediate the estrogen-initiated transformation of prostate epithelium derived from normal human prostate stem-progenitor cells, and promote the progression to invasive adenocarcinoma [332]. These data were obtained in a novel chimeric mice prostate model containing human-rat prostate tissues. Human prostate progenitor cells cytodifferentiated in chimeric prostate tissue originating epithelial hyperplasia, prostate intraepithelial neoplasia and PCa after exposure of nude mice to elevated concentrations of testosterone plus E₂ [332]. The pro-tumor action of GPER was also supported by the fact that its activation by the specific agonist G1 induced growth of normal stromal prostate cells, but not of BPH-derived cells [339]. Furthermore, GPER activation induced ERK1/2 phosphorylation, which was suggested contributing to PCa progression and hormonal independence in the PC3 cell line [333].

On the contrary, a substantial body of evidence defends the protective role of GPER in PCa. Treatment of LAPC-4 (androgen-sensitive) and PC3 (castration-resistant) PCa cells with E₂ or DES identified the growth inhibitory activity of GPER triggered by different mechanisms [340]. Also, G1 administration showed to inhibit growth of both androgen-sensitive (LNCaP) and CRPC (DU145 and PC3) cell line models [334]. The agonist G1 induced a persistent cell-cycle arrest at the G2/M phase in LNCaP and PC3 cells, which resulted in enhanced apoptotic activity, as indicated by

the increase in the Annexin V–positive cell population [334]. Moreover, G1 effects mediated by GPER induced the activation of ERK1/2 and c-jun/c-fos dependent upregulation of p21, which was accompanied by the downregulated expression of G2-checkpoint regulators, such as cyclin B1, cyclin dependent kinase 1 (cdc2) and its phosphorylated proteins, as well as by the diminished levels of cdc25C and cyclin A2 [334]. The mechanism involved in the GPER sustained activation of ERK1/2 was disclosed recently being shown that it depends on the activation of Gai1 proteins, which are highly expressed in PCa cells [341].

Interestingly, the low GPER expression described in poorly differentiated PCa was associated with an increased expression and activity of Akt, and the transcription factor CREB [331], indicating that loss of GPER may promote PCa cell survival and proliferative activity.

Other relevant study in this field, using LNCaP (androgen-sensitive cell line) and PC3 (castration-resistant cell line) xenografts as PCa models, reported that GPER activation by G1 inhibited growth of castration-resistant, but not of androgen-sensitive tumors with no observable toxicity [338]. Growth inhibition of castration-resistant tumors by G1 was underpinned by the increased apoptosis, demonstrated by the increased number of cleaved caspase 3-positive cells [338]. Other study, similarly described the *in vivo* anti-cancer role of GPER in PC3 xenografts. G1 administration significantly suppressed tumor growth having no effect on growth and histological features in the prostates of intact mice [334]. Moreover, this study showed that G1 treatment only inhibited the growth of PCa and actively proliferating BPH cells without affecting growth of quiescent cells.

In agreement with the *in vitro* and *in vivo* anti-tumorigenic actions of GPER mediated by estrogens and G1, a study with natural compounds also found decreased proliferation and increased apoptosis of PCa cells in response to GPER activation. The SDG, a lignan extracted from flaxseed was shown to suppress the development of BPH in a rat model [151]. ENL, the metabolite of SDG inhibited prostate cell growth, which significantly restricted the enlargement of rat prostate [151]. Mechanistically ENL activated the ERK pathway causing cell cycle arrest with upregulation of p53 and p21 and downregulation of cyclin D1 [151].

The available literature unquestionably places GPER as a modulator of PCa cells growth regulating the expression of cell cycle-regulators and other cellular elements, but its influence over the control of metastatic features also was reported. G1 treatment inhibited migration and the invasion properties of both PC3 and DU145 cells by reducing the formation of filopodia and stress fibers [341]. G1 administration *in vivo* was also capable of reducing the intratumoral microvessel density in a castration-

resistant xenograft tumor model [338], suggesting the anti-angiogenic role of GPER in PCa.

Other described effect of GPER in prostate is related with inflammation, with its activation leading to increased expression of COX-2 and augmented secretion of IL-8 by human prostate cells [342]. The pro-inflammatory actions of GPER were further supported by the observed marked intratumoral infiltration of neutrophils and upregulated expression of neutrophil-related chemokines and inflammation-mediated cytokines in a CRPC model after treatment with G1 [338].

In what concerns cancer cells metabolism, there are no reports implicating estrogens and GPER actions in their metabolic reprogramming. However, the emergent impact of metabolism as a therapeutic target together with others and ours recent findings highlighting the role of sex steroid hormones as metabolic regulators [343-345] strongly encourage opening this “avenue” of research.

Overall, the high expression of GPER in PCa, particularly in CRPC, and the demonstrated beneficial effects of G1 agonist counteracting the malignant behavior of PCa and BPH cells, without affecting non-proliferating cells, confer GPER the “status” of a therapeutic target. However, and despite some important advances characterizing GPER activity in prostate cells and tissues, much more investigation is needed to fully resolve the multiple GPER’ actions in PCa and its therapeutic potential.

3.8. Conclusions

GPER is a seven-transmembrane receptor with a wide mode of action activating several intracellular signaling pathways involved in the genomic and non-genomic effects of estrogens. In addition to E₂ and G1, the most potent activators of GPER, several other compounds with the ability of binding GPER acting as agonists or antagonists have been pointed out. The astonishingly increasing list of these compounds, encompass hormone ligands, synthetic compounds and environmental phytoestrogens and xenoestrogens, which places GPER in the physiological context of normal and oncological conditions, but also as a target of endocrine disruption. The diversity of biological processes under GPER control mainly includes cell proliferation, apoptosis, cell migration and invasion, angiogenesis, inflammation, and metabolism.

The multifaceted GPER is expressed in a broad range of neoplastic and non-neoplastic tissues, which further extends its broad scope of action. The development of GPER specific agonists and antagonists, as well as cell and animal knockout models have contributed greatly to ascertain its role in cancer and other diseases. GPER was

associated with the development and progression of breast, ovarian, endometrial and testicular cancers. However, other strong evidence of GPER actions counteracting known hallmarks of cancer as exacerbated proliferative activity and resistance to apoptosis support its role as a tumor suppressor.

In prostatic tissues, GPER is expressed both in normal gland and PCa. Also in this case, a duality in the GPER role has been reported. Studies arguing the causative role of GPER in prostate carcinogenesis are available in the literature, co-existing with others defending the protective action of GPER in PCa development. Reports indicating the high expression of GPER in PCa cases, together with its effects suppressing cell growth, also allow speculating about its usefulness as a therapeutic target. Nevertheless, future research is needed to deeply clarify the role of GPER in PCa and establish its potential as a therapeutic point of intervention.

Overall, we can discuss that the distinct functions of GPER in different tissues, or even within the same tissue, may depend on the specific physiological/environmental conditions in each tissue at a given moment, and/or of the experimental design including the type of ligand and the concentrations tested. The question of concentration would be quite relevant. For example, in the context of testicular apoptosis and male infertility, it was shown that at low concentrations estrogens act as survival factors whereas at high concentrations have the opposite effect inducing cell death [346, 347].

Finally, GPER can be viewed as a “cell guardian”, with their functions modulated by tissue specificity and environmental conditions, i.e., GPER would act protecting cells that need to be protected from harmful stimulus, or destroying those that are “dangerous” for tissue homeostasis.

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Chapter 4

The receptor tyrosine kinase c-KIT and its ligand (stem cell factor, SCF) in PCa: a target of estrogenic regulation?

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4. The receptor tyrosine kinase c-KIT and its ligand (stem cell factor, SCF) in PCa: a target of estrogenic regulation?

4.1. Brief overview of the structure and function of SCF/c-KIT system

The cytokine SCF is a potent growth factor firstly identified in 1990 [1]. The SCF protein contains three distinct regions (Fig. 4.1): the intracellular domain, the hydrophobic transmembrane domain, and the extracellular domain responsible for recognizing and binding c-KIT [2]. Besides the full-length membrane-bound SCF (mSCF), soluble forms of SCF have also been identified (Fig. 4.1). The proteolytic cleavage of an alternative spliced variant originates a soluble SCF (sSCF) that also binds and activates c-KIT. However, sSCF promotes transient activation and faster degradation of c-KIT whereas mSCF induces persistent activity and prolongs the life span of receptor [3].

The c-KIT receptor was first described in 1986 as the transforming gene of the Hardy-Zuckerman 4 feline sarcoma virus and identified as the proto-oncogene *v-KIT* [4]. The main product of *c-KIT* gene is a single 5 kb transcript encoding a transmembrane glycoprotein with approximately 145–160 kDa that belongs to the type III RTK family [4]. This class of receptors is structurally characterized by the presence of three main functional regions [5]: an intracellular domain, containing proximal and distal kinase domains separated by an interkinase domain, that is involved in signalling transduction; a transmembrane region constituted by a short hydrophobic chain of amino acids that anchors c-KIT at cell membrane; and an extracellular domain comprising five immunoglobulin-like domains, which participate in recognition of c-KIT ligand and receptor dimerization. Distinct c-KIT protein variants have been identified over the years (Fig. 4.1). The use of an alternative 5'-donor splice site produces c-KIT isoforms that differ by the presence or absence of the tetrapeptide Gly-Asn-Asn-Lys (GNNK) in the juxtamembrane region of the extracellular domain [6]. Recently, it was demonstrated that the GNNK peptide is an important regulatory element for fine tuning receptor activation and downstream signalling since GNNK negative c-KIT variants displayed increased tyrosine phosphorylation and activity [7]. In other words, the juxtamembrane region by the presence of GNNK peptide acts as a negative regulator of c-KIT activity. A mechanism of alternative promoter usage

originates a 30–50 kDa truncated c-KIT protein (tr-KIT, Fig. 4.1) that lacks the extracellular domain and the transmembrane region [8]. tr-KIT is also devoid of the first part of the kinase domain (Fig. 4.1) and, thus, do not display kinase activity [8]. However, tr-KIT seems to retain signalling transduction capability by interacting with other RTKs, or with other receptor types [9] as a scaffold protein at the cytoplasm. Proteolytic cleavage of c-KIT releases the receptor from the cell membrane given rise to a soluble isoform (Fig. 4.1) that only contains the extracellular domain. This protein variant binds SCF with the same affinity as the full-length c-KIT, and it was suggested that it might play a role modulating the bioactivity of ligand [10].

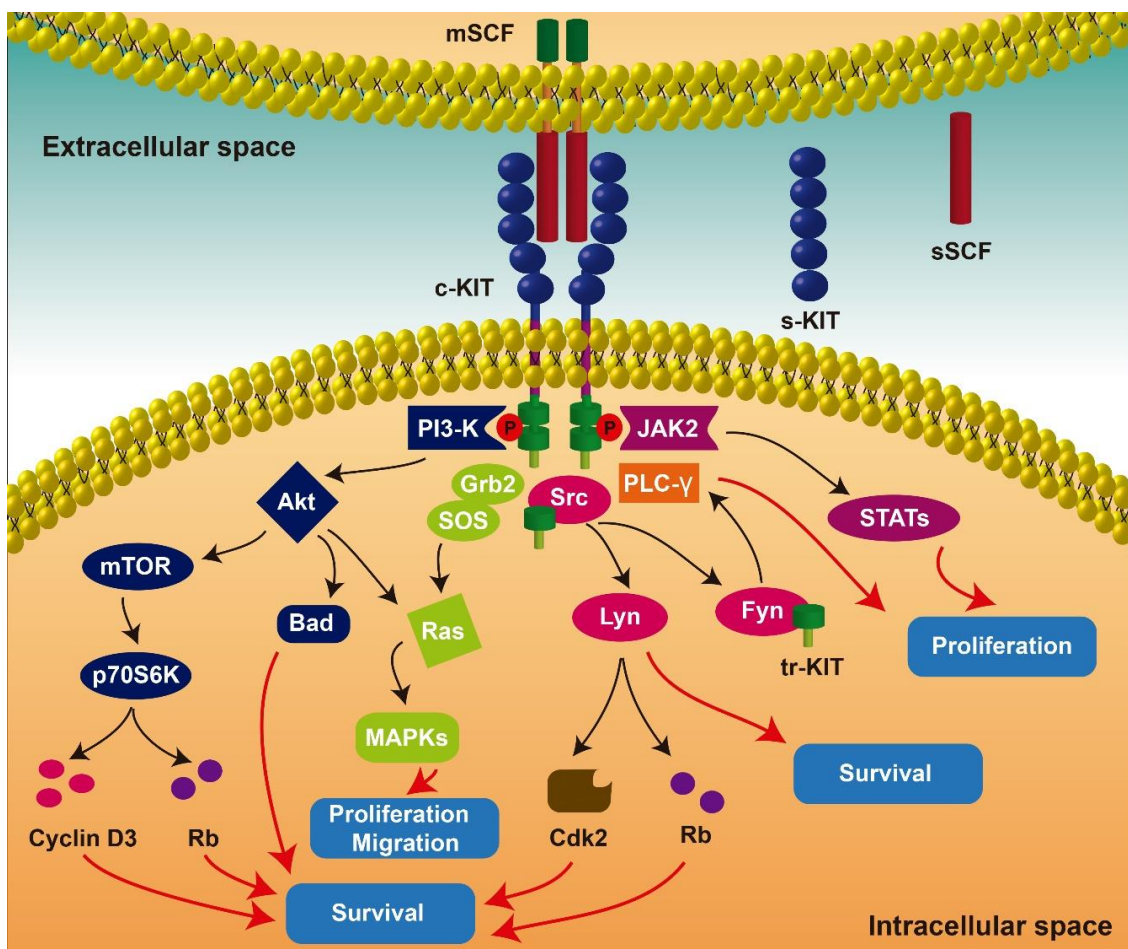


Figure 4.1. Structure of SCF/c-KIT proteins and downstream signaling pathways. Membrane-bound SCF (mSCF) contains an extracellular domain (red) that is responsible for recognizing and binding to c-KIT, a transmembrane domain (orange), and an intracellular domain (dark green). SCF also exists as a soluble protein (sSCF) secreted to the extracellular space. The five immunoglobulin-like domains (dark blue) in the extracellular domain of c-KIT are involved in ligand-binding and receptor dimerization. The transmembrane domain (violet) anchors c-KIT at the cytoplasm membrane and the intracellular region (green) is responsible for signal transduction. The receptor can be proteolytically cleaved and released from cell membrane, giving rise to a soluble c-KIT (s-KIT) consisting only of the extracellular domain. A truncated form of c-KIT (tr-KIT) residing at cytoplasm lacks the extracellular and transmembrane domains but retains part of the kinase domain. Binding of SCF homodimer to c-KIT induces receptor dimerization

and auto-phosphorylation, and consequent activation of downstream signaling cascades. The cell survival PI3K/Akt pathway (dark blue) depends on the phosphorylation of p70 S6 kinase (p70S6K) via mammalian target of rapamycin (mTOR), which leads to increased expression of cyclin D3 and phosphorylation of retinoblastoma protein (Rb). SCF/c-KIT/PI3K/Akt signaling also contributes to cell survival by inactivation of the pro-apoptotic factor Bad. The Src pathway (pink) controls proliferation, cell-cycle progression, and migration by distinct actions of Akt, Fyn, and Lyn kinases. c-KIT activates PLC- γ (orange), that can also be activated by tr-KIT through SRC member Fyn, which induces cell proliferation. SCF/c-KIT signaling has also been linked with the activity of JAK/STAT pathway (purple) through the activation of JAK2 and consequent phosphorylation of STAT transcriptional regulators, which enhance proliferative activity. Activation of the MAPK pathway (light green) occurs upon binding of adaptor proteins, such as growth-factor receptor-bound protein-2 (Grb2) and guanine nucleotide exchange factor, son of sevenless (SOS), which determines the activation of the small G-protein RAS and the propagation of kinases signaling cascade.

SCF is a noncovalent homodimer composed of two protomers; an hydrophobic crevice with a charged region on the tail of each protomer functions as the potential receptor binding site [11]. Thus, SCF binds simultaneously two molecules of c-KIT, inducing a conformational change that exposes a key dimerization site located in the fourth immunoglobulin-like domain of c-KIT [12]. Receptor dimerization allows its autophosphorylation [13], and triggers the initiation of multiple signal transduction pathways [5, 14], namely, the phosphatidylinositol 3-kinase (PI3-K), the Src, the Janus kinase/signal transducers and activators of transcription (JAK/STAT), the phospholipase-C (PLC- γ) and the mitogen-activated protein kinase (MAPK). The physiological actions of c-KIT controlling cell survival, proliferation, differentiation, and migration depend on the activation of specific or overlapping pathways [15] (Fig. 4.1).

PI3-K activation in response to c-KIT is followed by the phosphorylation of downstream signaling molecules in the PI3-K cascade (Fig. 2), as is the case of cell survival regulator Akt [16]. Akt seems to mediate SCF/c-KIT/PI3-K signaling by phosphorylating the p70 S6 kinase (p70S6K) via mammalian target of rapamycin (mTOR) kinase [17]. Phosphorylation of p70S6K leads to increased expression of cyclin D3 and phosphorylation of retinoblastoma (Rb) protein [17], allowing cell cycle progression and cell division. The pro-apoptotic factor Bad is also a target of Akt upon SCF activation of the PI3-K pathway. Phosphorylation of Bad by Akt leads to its inactivation and, thus, contributes to cell survival [18].

Studies in different types of cells have demonstrated that the SCF/c-KIT system activates several Src family members (Fig. 4.1), such as Src, Lyn and Fyn [19, 20], promoting cell proliferation. It was demonstrated that SCF activates Lyn before the G1 to S phase transition [21]. Lyn activation by SCF triggered cell cycle progression by increasing the activity of cyclin dependent kinase 2 (Cdk2) and phosphorylation of Rb

protein. c- KIT signal transduction triggering the Src pathway is also important in the regulation of cell migration. The tr-KIT is a stronger activator of Src kinases, comparatively with the full-length protein [22, 23].

The JAKs are cytoplasmic tyrosine kinases activated by ligand, which lead to phosphorylation and transcription of STAT proteins [24]. After being phosphorylated, STATs dimerize and are translocated to the nucleus, where they regulate the transcription of target genes [24]. Signal transduction by SCF and its receptor was shown to be related to the JAK/STAT pathway (Fig. 4.1) by the activation of JAK2 [25, 26]. SCF induced the association of c-KIT with JAK2 and the consequent phosphorylation of JAK2 [27]. The transient activation of JAK2 in response to SCF was associated with the downstream activation of STAT1 and STAT5 [25, 28]. Moreover, SCF also increased the expression of JAK2 and STAT1 [29]. It was also observed an increase in differentiation but not in the self-renewal of stem cells in the testis of the fruit fly, *Drosophila* sp., in the absence of JAK/STAT signaling [30]. Thus, the activation of JAK2/STAT1 pathway seems to underpin the SCF/c-KIT actions regulating stem cell proliferation. Although the enhanced renewal of testicular stem cells has been linked with the development of germ-cell tumors [31], the involvement of SCF/c-KIT and JAK/STAT signaling in testicular carcinogenesis remains to be clarified. In the case of prostate cancer, the idea that a population of cancer stem cells implicated in the ontogeny of disease and therapeutic resistance also has been gaining consistency in the last years [32, 33]. These cancer stem cells seem to be associated with the basal layer of prostate epithelium; a region reported to have high expression levels of c-KIT [34, 35]. Moreover, it was demonstrated that tumorigenesis induced by prostate cancer stem cells is accompanied by the increased expression of c-KIT [36]. Interestingly, several signaling pathways were shown to be deregulated in prostate cancer stem cells and accounting for its tumorigenic potential, which included the JAK-STAT pathway [32, 37, 38].

The PLC- γ pathway is one of the less characterized relatively to the activity of the SCF/c-KIT system. The activation of c-KIT by SCF leads to c-KIT autophosphorylation and its association with PLC- γ 1 via the phosphorylated Tyr728 residue [39]. Interaction of tr-KIT with the PLC- γ pathway has also been described, though independently of SCF since this c-KIT variant is devoid of the extracellular domain [8, 9]. It was proposed that the action of tr-KIT phosphorylating and activating PLC- γ 1 is mediated by the interaction with the Src member Fyn. The physical association of tr-KIT with Fyn promotes the catalytic activity of Fyn and the consequent phosphorylation and activation of phospholipase [40].

Cell signaling via c-KIT activates several MAPKs such as extracellular-signal-regulated kinase (ERK) 1/2, p38, c-Jun N-terminal kinase (JNK), and ERK5. As expected, the MAPK pathway is linked with the proliferative activity of SCF upon activation of c-KIT. Membrane-bound and soluble isoforms of SCF seem to have specific MAPKs targets. The mSCF effectively induced cell proliferation through the ERK whereas the sSCF proliferative effects were achieved by the activity of p38 [41]. SCF/c-KIT effects mediating cell migration also depend on the MAPK pathway via phosphorylation and activity of p38 [42]. Interestingly, it has been shown that the SCF/c-KIT activation of MAPK pathway is dependent on PI3-K signaling. The use of PI3-K inhibitors decreased the activation of RAF and ERK, but did not affect Ras activation [43]. The crosstalk between PI3-K/Akt and Ras/MAPK pathways has been described at multiple levels in distinct cell types endowing the cells with incredibly higher response patterns to the combinatorial variety of external stimuli [44].

4.2. Role of c-KIT in prostate carcinogenesis

The expression of the SCF/c-KIT system has been described in both normal prostate tissues and PCa cases [45]. In the non-neoplastic prostate, c-KIT protein is detected in mast cells and stromal compartment but not in the epithelium [29]. SCF can be found in epithelial and stromal cells [29, 45]. c-KIT and SCF transcripts are present at low levels in basal epithelial cells [45]. In opposition to what happens in normal tissues, in cases of benign prostatic hyperplasia (BPH) c-KIT is expressed in epithelial cells [45]. Regarding the SCF, its expression follows that of healthy prostate tissue, being detected in fibromuscular stromal cells, and in basal and suprabasal epithelial cells [45]. It was also found that the c-KIT and SCF expression was significantly higher in BPH when compared to the normal prostate [29]. Moreover, the SCF/c-KIT interaction and the activation of the c-KIT seems to induce the prostate cell proliferation and prostatic growth in BPH [29]. In prostate adenocarcinoma, c-KIT expression was strongly identified in the cytoplasm and membrane of epithelial tumor cells [45] and stromal cells [46]. Considering the progression of prostatic disease, an elegant study performed by Weisner and co-authors [47] revealed that c-KIT was expressed in 5 % of BPH cases, 14 % of primary prostate cancer, and 40 % of bone metastasis. This indicated that the SCF/c-KIT system would have a significant role in the pathophysiology of PCa and supported its association with the severity of disease and appearance of skeletal metastasis. Indeed, c-KIT has been identified as an upregulated gene in the reactive stroma of PCa [48, 49], a condition that is associated

with poor outcome in clinically localized PCa. It was also demonstrated that c-KIT, among other up-regulated genes in the reactive stroma, is responsible for promoting prostate cancer progression *in vivo* [49]. More recently, it was shown that c-KIT can promote migration and invasion of PCa cells derived from bone metastasis [50]. Moreover, the inhibition of bone-induced c-KIT expression in PCa cells reduced intraosseous tumor incidence and growth, suggesting that c-KIT expression and activation in prostate cancer cells by the bone microenvironment are key contributors to bone metastasis [50]. The expression of the cytoplasmic truncated variant of c-KIT originated by a mechanism of alternative promoter usage [8, 51], the tr-KIT, also appears to be associated with the more advanced stages of prostatic tumors. Although tr-KIT was not expressed in BPH, it was expressed in 28 % of low Gleason grade tumors and in 66 % of more advanced tumors [23]. tr-KIT expression was restricted to the cytoplasm of the epithelial cells of neoplastic prostatic glands [23]. Considering the positive association between tr-KIT expression and tumor progression, it was hypothesized that the aberrant expression of nuclear factors able to bind to the human tr-KIT gene promoter may be an early event in prostate cell transformation [23]. The implication of tr-KIT in prostate malignant transformation and/or metastization is further corroborated by the fact that its expression is positively associated with the increased activity of the SRC pathway, which has been implicated in survival, angiogenesis, proliferation, invasion and aberrant tumors growth [23]. Although devoid of kinase activity, the tr- KIT is a stronger activator of SRC kinases comparatively with the full-length c-KIT [22, 23]. Considering the expression of c-KIT in cell line models of prostate cancer, discrepancies exist among different studies. Some reports revealed that c-KIT mRNA is not expressed in LNCaP, DU145 or PC3, whereas others have shown its expression [47, 52, 53]. Comparing the expression levels of c-KIT mRNA among cell lines, PC3 and DU145 cells displayed, respectively, the lowest and highest expression [52]. At the protein level, c-KIT could be detected both in androgen responsive (LNCaP), and androgen non-responsive (DU145 and PC3) prostate cancer cells [52, 53], a result not confirmed by others [47]. Besides the wild-type c-KIT, also the tr-KIT could be identified in LNCaP DU145 and PC3 cells [23, 54]. All the aforementioned cancer cell lines express the SCF transcripts [45, 53], being the highest expression found in DU145 cells [47]. Also, the sSCF isoform was detected in DU145 and PC3 cells though at low levels [53].

4.3. The SCF/c-KIT system as a target of hormone regulation in hormone-dependent cancers

With a role in hormone-dependent cancers, the system of SCF and its receptor investigated as a target for hormones in these tumors.

Oogenesis and folliculogenesis are interdependent biological events extremely well regulated by the coordinated action of pituitary and ovarian hormones [55]. On the other side of the coin, a substantial body of evidence has placed both androgens and estrogens as causative agents in the emergence of ovarian cancer [56-58]. Considering the pituitary hormones, several findings have shown that follicle-stimulating hormone (FSH) and luteinizing hormone (LH) regulate positively the SCF expression in granulosa cells [59-61], allowing the interaction of ligand with the c-KIT receptor present in germ cells. This interaction is crucial to promote oocyte growth and the normal follicular development, though, the chronically augmented levels of FSH and LH in post-menopausal woman were suggested to cause the increased expression of autocrine factors like SCF contributing to the overactivation of c-KIT and cancer appearance [61]. Androgens and estrogens effects regulating the ovarian expression of SCF and c-KIT were also reported [62-64]. The non-aromatizable androgen 5 α -dihydrotestosterone was shown to increase the SCF expression in mice ovaries [63]. Accordingly, the expression of SCF and c-KIT was diminished upon treatment with the androgen receptor inhibitor flutamide, which affected the follicular transition from primordial into primary follicles [62]. Concerning the typical female hormones, estrogens, they were able to induce or decrease mRNA SCF expression in mice ovary cultured *in vitro* dependently on the experimental concentration used [64].

There is no available data demonstrating the hormonal influence over regulation of c-KIT expression and function in endometrial cancer. However, taking into account the information in other reproductive organs and the fact that the proliferative ability of endometrial epithelial cells is regulated by estrogens [65, 66], it is highly likely that these female hormones would control c-KIT expression levels in the endometrium. Despite its much more modest association with endometrium physiology and cancer, also androgens may have a role in regulating c-KIT in this tissue (141, 163).

The expression of c-KIT in breast cancer cases has been correlated with the expression of estrogen or androgen receptors though also with results somehow contradictory. If on one hand c-KIT expression is higher in estrogen receptor-positive breast cancer cases than in estrogen receptor-negative breast cancer [67], on the other hand, c-KIT expression is even higher in cases of triple-negative breast cancer [68]. In

what concerns the androgen receptor, it was found a negative correlation with c-KIT expression [69]. Considering that the androgen receptor is more expressed in estrogen receptor positive tumors [70, 71] and the sole steroid receptor expressed in triple-negative breast cancers [70, 71], it becomes quite interesting to investigate the regulation of SCF/c-KIT system by sex steroid hormones in breast cancer.

Regarding the male reproductive system, the testis is an organ strongly influenced by the hormonal action, being either a target or a major source of hormones in men. The normal testicular function, and a determinant of male fertility, is under the master control of hypothalamus-pituitary axis, which ensures the adequate hormone production, the proper development of male phenotype, and the normal course of spermatogenesis. Although testicular cancer is not a classical hormone-dependent cancer, several pieces of evidence have implicated the genetic alterations in steroidogenic enzymes and key players of the hypothalamic–pituitary–testicular axis, namely, estrogen and androgen receptors, in testicular cancer [72, 73]. On the other hand, it has been established that the tissue levels of the SCF/c-KIT system are hormonally modulated [52, 74], being likely to assume that its disturbed expression due to hormonal deregulation or hormonal treatments may have a consequence in testis pathogenesis. For instance, treatment with gonadotropin-releasing hormone (GnRH) agonists showed to increase the mSCF levels in the testis [75]. In addition, the FSH gonadotropin was also related with the increased expression of SCF in Sertoli cells and of c-KIT in testicular germ cells [76, 77]. Moreover, it was suggested that is the stimulatory effect of FSH on Sertoli cells inducing the secretion of paracrine factors that upregulates the expression of c-KIT in germ cells, in turn, augmenting the responsiveness to SCF and promoting their proliferation [76]. Regarding the action of sex steroid hormones, 17 β -estradiol (E₂) treatment was shown to increase the testicular expression of SCF [78, 79], which was accompanied by the enhanced proliferation of primordial germ cells [78]. Also, it was demonstrated that the stimulation of primordial germ cells with E₂ led to c-KIT phosphorylation and activation of its downstream molecular targets [80]. Although testosterone alone did not seem to regulate the SCF expression [77], testosterone in combination with FSH, showed to increase the mRNA levels of SCF in pubertal Sertoli cells [81]. The expression of c-KIT proteins throughout the spermatogenetic process involves the replacement of the full-length c-KIT, by the tr-KIT, which has been indicated as having a role in post-meiotic stages and sperm motility [82, 83].

In prostate, androgens are major regulators of its physiology with an important role in gland development and function, and being also implicated in prostate carcinogenesis. At the initial stages of advanced prostate cancer, the survival and

growth of cancer cells are dependent of androgens, which allows the effective use of androgen deprivation therapies. However, prostate cancer cells tend to become resistant to hormonal deprivation and tumors progress independently of the androgen circulating levels, reaching the stage known as castration-resistant prostate cancer [84]. c-KIT expression, as well as that of other putative stem/progenitor cell markers, is significantly increased in castration-resistant PCa, suggesting that proliferation of cancer initiating cells can be mediated by c-KIT [85]. Also, it was described that androgen deprivation in DuCaP cells significantly increases the c-KIT mRNA levels [85], which indicates that c-KIT actions may be involved in cancer reappearance after anti-androgen therapies. Despite the established role of androgens as stimulating agents in prostate cell growth, the classical female hormones estrogens also have been implicated in the onset and progression of prostate cancer [86, 87]. However, no supportive studies exist implicating the SCF/c-KIT in the estrogenic actions over prostate carcinogenesis. Despite c-KIT expression seems to increase in rat prostate after treatment with estrogens, the augmented expression of c-KIT resulted from the increase in the interstitial cell population, the prostatic cell type responsible for the maintenance of the smooth muscle tone, with no effect on cell proliferation in rat ventral prostate promoted by the estrogenic supplementation [88]. On the other hand, and following other pieces of evidence indicating estrogens as protective agents against prostate cancer [89, 90], it was shown that estrogens down-regulated the SCF and c-KIT levels in rat prostate cells and in human prostate cancer cells lines [52]. Moreover, the downregulation of SCF/c-KIT system was accompanied by the diminished proliferation and augmented apoptosis of prostate cells [52], suggesting that the anti-cancer effects of estrogens on prostate may depend on the down-regulation of the SCF/c-KIT system.

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Chapter 5

Aim and Outline of the Thesis

5. Aim and Outline of the Thesis

The development and progression of PCa depend on complex cellular and molecular mechanisms that ensure sustained proliferation, resistance to apoptosis, cell migration and invasion, and the metabolic reprogramming of tumor cells. All these processes are strictly regulated by hormone actions, namely, of androgens, which are undoubtedly recognized as the drivers of PCa development. Conversely, the sex steroid estrogens have been indicated as protective agents in PCa and were used in therapy for decades, only discontinued by the adverse side effects. Nevertheless, strong evidence exists implicating this class of steroid hormones in prostate carcinogenesis.

The effects of estrogens are mediated by the classical nuclear estrogen receptors, ER α and ER β , and by the membrane receptor GPER. Based on the available literature, ER α is considered an oncogene, mediating the damaging effects of estrogens inducing prostate cells proliferation and inflammation, whereas ER β seems to have an anti-cancer role. Concerning the estrogenic effects mediated through GPER, these were associated with suppressed cell proliferation and activation of apoptosis in several types of cancer. However, the role of GPER in PCa cells is much less clear. Moreover, GPER action as a metabolic regulator was recently proposed, which raises the curiosity about its relationship with PCa cells metabolism and the control of this cancer hallmark.

On the other hand, the molecular partners that are involved in the estrogens' actions regulating prostate cells fate are not fully identified. The SCF/c-KIT is a hormone-regulated ligand-receptor system that has been shown to play a pivotal role in cell fate decision, controlling cell proliferation, differentiation, survival and apoptosis. It is known that estrogens regulate the expression levels of SCF/c-KIT in several neoplastic and non-neoplastic cell types, but its action over prostate cells remain unexplored.

Another rationale in the context of estrogens' actions is related to the existence of molecules that can target ERs, promoting or inhibiting their activity with an impact on cell physiology, which ultimately could have interest in therapy. A panoply of natural compounds is known to mimic estrogens' actions by binding and activating the classical nuclear ERs and GPER also. Several of these compounds have been reported as protector agents in several types of cancer, including PCa. Diosgenin is a phytosteroid sapogenin with estrogen-like properties, which has demonstrated anti-cancer effects in different cancer types. Nevertheless, the diosgenin effects and its mechanism of action remain poorly studied in PCa.

Overall, there is a substantial amount of contradictory information concerning the actions of estrogens in PCa, with the role of GPER remaining to be disclosed. The present thesis aims to answer some of the existent questions on the estrogens/GPER actions in PCa cells, which would lay the foundations for the development of new and safe estrogen-based therapies. With this purpose beyond, the following specific objectives were delineated:

1. To investigate the action of estrogens as regulators of apoptosis and proliferation in prostate cells in the interplay with the SCF/c-KIT system;
2. To determine the effect of GPER activation on PCa cell fate: proliferation, apoptosis, migration, invasion and EMT, and associated intracellular signaling pathways;
3. To establish the role of GPER as a regulator of energy metabolism and metabolic rewiring in PCa cells;
4. To evaluate how natural compounds such as diosgenin can influence the behavior of PCa in the interplay with GPER.

After a general overview of PCa, from aetiology to treatment (**Chapter 1**), a state-of-art of the role of estrogens in PCa is presented in **Chapter 2**, which exhaustively revises their duality of actions, and presents the panoply of phytoestrogens with interesting activity in suppressing PCa cell growth. Among the different classes of these compounds, diosgenin was selected as a target based on the enormous amount of anti-cancer properties reported in several types of cancer and its ability to activate ERs. The fact that it is being used in the pharmaceutical industry, by the similar structure to cholesterol and other steroids, also weighed the decision.

Chapter 3 characterizes the GPER structure and function, and its role in carcinogenesis. A brief report of the SCF/c-KIT system and its relationship with PCa is presented in **Chapter 4**.

The thesis objectives are established in **Chapter 5**, and **Chapters 6-9** were organized as follows:

Chapter 6 determines the role of E₂ in regulating the expression of SCF/c-KIT in human prostate cell lines and rat prostate, and the consequent effects on prostate cell proliferation and apoptosis.

Chapter 7 establishes the significance and functional role of GPER in PCa. The effect of GPER activation by its specific agonist G1 is investigated concerning cell proliferation, apoptosis, migration and invasion, EMT and metabolic reprogramming.

Chapter 8 addresses the action of diosgenin in non-neoplastic and PCa cells concerning cell fate and glycolytic metabolism on the dependency of GPER activation.

Finally, **Chapter 9** integrates all the thesis results and discusses the potential impact of the obtained findings in the context of PCa prevention and treatment.

Chapter 6

Estrogens down-regulate the stem cell factor (SCF)/c-KIT system in prostate cells: evidence of antiproliferative and proapoptotic effects

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6. Estrogens down-regulate the stem cell factor (SCF)/c-KIT system in prostate cells: evidence of antiproliferative and proapoptotic effects

Abstract

The development of prostate cancer (PCa) is intimately associated with the hormonal environment, and the sex steroids estrogens have been implicated in prostate malignancy. However, if some studies identified estrogens as causative agents of PCa, others indicated that these steroids have a protective role counteracting prostate overgrowth. The tyrosine kinase receptor c-KIT and its ligand, the stem cell factor (SCF), have been associated with the control of cell proliferation/apoptosis and prostate carcinogenesis, and studies show that estrogens regulate their expression in different tissues, though, in the case of prostate this remains unknown. The present study aims to evaluate the role of 17 β -estradiol (E₂) in regulating the expression of SCF/c-KIT in human prostate cell lines and rat prostate, and to investigate the consequent effects on prostate cell proliferation and apoptosis. qPCR, Western Blot, and immuno(cito) histochemistry analysis showed that E₂-treatment decreased the expression of SCF and c-KIT both in human prostate cells and rat prostate. Furthermore, the diminished expression of SCF/c-KIT was underpinned by the diminished prostate weight and reduced proliferation index. On the other hand, the results of TUNEL labelling, the increased activity of caspase-3, and the augmented expression of caspase-8 and Fas system in the prostate of E₂-treated animals indicated augmented apoptosis in response to E₂. The obtained results demonstrated that E₂ down-regulated the expression of SCF/c-KIT system in prostate cells, which was associated with antiproliferative and proapoptotic effects. Moreover, these findings support the protective role of estrogens in PCa and open new perspectives on the application of estrogen-based therapies.

Keywords

Estrogens, prostate cancer, c-KIT, SCF, proliferation, apoptosis

6.1. Introduction

The prostate gland is highly dependent on the circulating and intraprostatic hormonal milieu and the sex steroids androgens play a crucial role in its normal development from the embryonic period to adulthood [1]. Moreover, androgens' actions are positively associated with augmented rates of cell proliferation [2] and restricted apoptosis [3], and thus intimately associated with the onset and progression of prostate cancer (PCa). Nevertheless, also the classical female hormones estrogens have been gaining importance in the context of the normal and pathological status of the prostate. The existence of estrogenic activity in the regulation of cell proliferation, apoptosis, and differentiation of the prostate cells has been reported [4, 5]. However, a duality of the role of estrogens in prostate gland has emerged over the years. If some studies defend that estrogens are potential causative agents of PCa other strong evidence indicate that these steroids may be protective against this human disease. In fact, the prevalence of PCa in older men is coincident with an augmented estrogens/androgens ratio in parallel with the decrease in testosterone levels, which argues for the role of estrogens in prostate carcinogenesis [6-8]. Also, it was found that patients with high levels of 17 β -estradiol (E₂) show increased risk to develop PCa [9], and that estrogens are associated with the promotion of cell proliferation, invasion, migration and malignant transformation of prostate cells, as well as with augmented PSA levels [2, 8, 10-13].

On the other hand, high doses of E₂ showed growth inhibitory effects in prostate cells both *in vitro* and *in vivo* [14, 15]. Also, E₂ treatment was able to suppress PCa growth and to delay mortality in castration resistant xenograft models [16]. Moreover, estrogen-based therapies have been currently used to treat human PCa though with a strong discussion associated [17, 18].

The c-KIT is a type III receptor tyrosine kinase activated by its specific ligand, the stem cell factor (SCF) [19]. The SCF/c-KIT system plays a relevant role in cell fate decision, and its activity is widely known to control cell proliferation differentiation, survival and apoptosis [20, 21]. SCF and c-KIT are expressed in a broad range of male reproductive tissues including prostate [22] and their role in prostate carcinogenesis also has been indicated [23-25]. Interestingly, the expression of the SCF/c-KIT system seems to be hormonally regulated, and estrogens are important agents in this process modulating the tissue levels of both receptor and ligand in neoplastic and non-neoplastic tissues (recently reviewed by [26]).

The present work aims to evaluate the role of E₂ in regulating the expression of SCF/c-KIT both in human prostate cell lines and in rat prostate *in vivo*. The

consequent effect of estrogenic stimulation on proliferation and apoptosis of prostatic cells *in vivo* was also investigated. The obtained findings will contribute to elucidating the molecular mechanisms underpinning the estrogens' role in the prostate, and represent a valuable information to define better the action, safety and efficacy of estrogen-based therapies against PCa.

6.2. Materials and methods

6.2.1. Chemicals

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

6.2.2. Prostate cancer cell lines and hormonal treatment

The human prostate non-neoplastic epithelial cell line PNT1A and the human PCa cell lines LNCaP, DU145 and PC3 were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK).

PNT1A, LNCaP, DU145 and PC3 cells were maintained in RPMI 1640 medium (Gibco®, Invitrogen, Paisley, Scotland), supplemented with 10 % fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany) and 1 % penicillin/streptomycin (Invitrogen, New York, NY), at 37 °C in an atmosphere equilibrated with 5 % CO₂. At 60 % confluence, culture medium was replaced by phenol red-free RPMI 1640 medium containing 5 % charcoal-stripped FBS (CS-FBS) (Gibco®, Invitrogen) free of steroid hormones, and cells were maintained for additional 24 h. Then, cells were exposed to 100 nM of 17β-estradiol (E₂), which is in the range of supraphysiological concentrations [27, 28], or vehicle for 12, 24, or 48 h.

6.2.3 Animals and hormonal treatment

A total of 32 adult Wistar (*Rattus norvegicus*) male rats (250-300 g; approximately 3 months old) were housed at constant temperature and humidity, under a 12 h light–dark cycle with food and water available *ad libitum*. Research was

conducted according with the National and European Union guidelines for the care and handling of laboratory animals (Directive 2010/63/EU).

Rats were divided into two groups (n=16) and daily injected (intraperitoneally) with vehicle (control) or with E₂ (250 µg/day/Kg) for 5 days. This E₂ dose and the duration of treatment were previously shown to be effective in the regulation of gene expression and produced effects in rat prostate [29]. After treatment, animals were euthanized under anesthesia and whole prostates were removed, weighted and either fixed in 4 % paraformaldehyde (PFA) (n=8) or snap frozen in liquid nitrogen (n=8) and stored at -80 °C.

6.2.4. Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the rat prostates using the TRI reagent (5 Prime, Hilden, Deutschland) according to the manufacturer's instructions. The quantity and integrity of total RNA were determined by spectrophotometric analysis at 260 and 280 nm (NanoPhotometer; Implen) and agarose gel electrophoresis. cDNA was synthesized from 1 µg of total RNA using the First Strand cDNA Synthesis Kit (NZYtech, Lisboa, Portugal).

qPCR was carried out to determine the expression of SCF and c-KIT in rat prostate and in human prostate cell lines. Primer sequences for rat SCF and c-KIT, and β-actin housekeeping gene and cycling conditions were previously described [30]. Human SCF and c-KIT specific primers sets were, respectively, sense: GTTGCCGATTAGTGATATGG; antisense: ACTTCTTCCTGCGATGAC; sense: CCGTCTCCACCATCCATC; antisense: ATTCATTCTGCTTATTCTCATTCG. GAPDH (sense: CGCCAGCCG AGCCACATC; antisense: CGCCCAATACGACCAAATCCG) and β2-microglobulin (β2M) (sense: ATGAGTATGCCTGCCGTGTG; antisense: CCAACCTCCATGATGCTGCTTAC) housekeeping genes were used as internal controls for normalization of human gene expression. qPCR reactions were performed in the iQ5 system (Bio-Rad, Hercules, CA, USA), and the efficiency of the amplification was determined for all primer sets using serial dilutions of cDNA (1, 1:3, and 1:9). The specificity of the amplicons was determined by melting curve analysis. Each qPCR reaction consisted of iTaq Universal SYBR Green Supermix (Bio-Rad), sense and anti-sense primers (5 µM for all primer pairs), and 1 µL of cDNA in a final volume of 20 µL. A no-template control was included for each reaction, and all reactions were performed in triplicate. Normalized expression values were calculated using the mathematical model proposed by Pfaffl ($2^{-(\Delta\Delta Ct)}$) [31].

6.2.5. Total protein extraction

Human prostate cells and rat prostate tissues were homogenized in the appropriate volume of radioimmunoprecipitation assay buffer (RIPA) (150 mM NaCl, 1 % Nonidet-P40 substitute, 0.5 % Na-deoxycholate, 0.1 % SDS, 50 mM Tris, 1 mM EDTA, 1 % protease inhibitor cocktail and 10 % PMSF), and total proteins were recovered after a 14,000 rpm centrifugation for 20 min at 4 °C. Protein concentration was determined by the Bradford assay (Bio-Rad).

6.2.6. Western blot (WB)

Total proteins were resolved by SDS-PAGE on 12.5 % gels and electrotransferred to a PVDF membrane (Bio-Rad). Membranes were incubated overnight at 4 °C with rabbit anti-SCF (1:500, H-189: sc-9132; Santa Cruz Biotechnology, CA, USA), rabbit anti-c-KIT (1:500, C-19: sc-168; Santa Cruz Biotechnology), rabbit anti-Fas (1:500, A-20: sc-1023; Santa Cruz Biotechnology), rabbit anti-FasL (1:500, C-178: sc-6237; Santa Cruz Biotechnology), rabbit anti-Bax (1:500, no. 2772; Cell Signaling Technology, MA, USA), rabbit anti-Bcl-2 (1:1000, no. 2876; Cell Signaling Technology), rabbit anti-caspase-9 p35 (H-170:sc-8355; Santa Cruz Biotechnology) or mouse anti-cleaved caspase-8 (1:1000, no. 9746, Cell Signaling Technology). A mouse anti- α -tubulin monoclonal antibody (1:5000, T9026) was used for protein loading control in all WB analyses. Goat anti-rabbit IgG-AP (1:5000, NIF1317; GE Healthcare, Buckinghamshire, UK) or goat anti-mouse IgG+IgM-AP (1:5000, NIF1316, GE Healthcare) were used as secondary antibodies. Membranes were revealed with ECF substrate (GE Healthcare) for 5 min and immuno-reactive proteins were visualized with the Molecular Imager FX Pro plus MultiImager (Bio-Rad). Band densities were obtained using the Quantity One Software (Bio-Rad) and normalized with the respective α -tubulin band density.

6.2.7. Fluorescent immunohistochemistry

Formalin-fixed paraffin sections (5 μ m) of rat prostate were deparaffinized in xylene and rehydrated through a series of graded ethanol solutions. After heat-induced antigen retrieval in citrate buffer (10 mM, pH 6.0), sections were permeabilized with 1 % Triton X-100 for 15 min at room temperature. Unspecific staining was blocked by

incubation with PBS containing 1 % (w/v) BSA (PBA) and 0.3 M glycine for 30 min at room temperature. Sections were incubated overnight at 4 °C with rabbit anti-Ki67 (1:50, ab16667; Abcam), rabbit anti-SCF (1:500, H-189: sc-9132; Santa Cruz Biotechnology) or rabbit anti-c-KIT (1:500, C-19: sc-168; Santa Cruz Biotechnology) primary antibodies. Sections were then incubated with Alexa Fluor 546 goat anti-rabbit IgG or Alexa Fluor 488 goat anti-rabbit IgG secondary antibodies (1:500, Invitrogen) for 1 h at room temperature. Specificity of the staining was assessed by the omission of primary antibody. Cell nuclei were stained with Hoechst 33342 (10 µg/mL, Invitrogen) for 5 min. Cell membrane was stained by incubation with wheat germ agglutinin (5 µg/ml, Invitrogen) for 10 min before the permeabilization step. Sections were washed with PBS and mounted in Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany). The proliferation index was determined by the percentage of Ki67-positive cells out of the total number of Hoechst stained nuclei in 20 randomly selected 40 magnification fields in each section.

6.2.8. Fluorescent immunocytochemistry

PNT1A, LNCaP, DU145 and PC3 cells were fixed with 4 % PFA and permeabilized with 1% Triton X-100 for 5 min at room temperature. Unspecific staining was blocked by incubation with PBS containing 0.1 % (w/v) Tween-20 (PBS-T) and 20% FBS (Biochrom AG) for 1 h. Then, cells were incubated with rabbit anti-SCF (1:500, H-189: sc-9132; Santa Cruz Biotechnology) or rabbit anti-c-KIT (1:500, C-19: sc-168; Santa Cruz Biotechnology) primary antibodies for 1 h at room temperature. Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 546 goat anti-rabbit IgG were used as secondary antibodies (Invitrogen). Specificity of the staining was assessed by the omission of primary antibody. Cell nuclei were stained with Hoechst 33342 (5 µg/mL, Invitrogen) for 10 min. Lamellae were washed with PBST and mounted with Dako fluorescent mounting medium (Dako). Images were acquired using the Axio Imager A1 microscope (Carl Zeiss).

6.2.9. Caspase-3 activity assay

Caspase-3 activity assay was performed as previously described [32]. Concisely, 25 µg of total protein extracted from rat prostate were incubated with reaction buffer

(25 mM HEPES, 0.1 % 3-[(3-holamidopropyl)dimethylammonio]-1-propanesulfonate, 10 % sucrose, and 10 mM dithiothreitol, pH 7.5) and 200 μ M of caspase-3 substrate (Ac-DEVD-pNA) overnight at 37 °C. Upon caspase cleavage of substrate, p-nitro-aniline (pNA) is released producing a yellow color, which is measured spectrophotometrically at 405 nm. The amount of generated product was calculated by extrapolation of the standard curve of free pNA.

6.2.10. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)

TUNEL analysis was performed using the *In Situ* Cell Death Detection Kit, TMR red (Roche) following the manufacturer's instructions. Briefly, prostate sections (5 μ m) were deparaffinized in xylene, rehydrated in ethanol solutions with increased water content, rinsed in 0.1 M PBS (pH 7.4), and permeabilized with 0.25 % Triton X-100, for 30 min at room temperature. Afterwards, sections were incubated in a humidified atmosphere with TUNEL reaction mixture (enzyme solution equilibrated in label solution), for 1 h at 37 °C, protected from the light. Then, sections were washed with PBS, the cell nuclei were stained by incubation with Hoechst 33342 (10 mg/mL; Invitrogen) for 5 min. Sections were then washed with PBS for 10 min and mounted in Dako fluorescent mounting medium (Dako). Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany).

6.2.11. Statistical analysis

Statistical significance of differences between experimental groups was evaluated by unpaired T-test with Welch's correction, using GraphPad Prism v5.00 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant. All experimental data are shown as mean $\pm$ standard error of the mean (SEM).

6.3. Results

6.3.1. Estrogens regulate the expression of SCF/c-KIT system in non-neoplastic and neoplastic human prostate cell lines

Non-neoplastic (PNT1A) and neoplastic (LNCaP, DU145 and PC3) human prostate cells were exposed to 100 nM of E₂ for 0, 12, 24 and 48 h, and the expression of SCF and c-KIT was studied at mRNA and protein level by means of qPCR and WB, respectively. Three cell line models of PCa were included in this study because LNCaP, DU145, and PC3 cells are widely recognized to represent metastatic stages of PCa with different degrees of aggressiveness. LNCaP cells were derived from lymph nodes metastasis and present low metastatic potential [33, 34], DU145 cells were derived from brain metastasis and have moderate metastatic potential [35, 36], whereas PC3 cells were derived from bone metastasis and have high metastatic potential [37]. Also, it is known that LNCaP, DU145 and PC3 cells display important differences in the expression of estrogen receptors (ERs) subtypes, which has been associated with distinct proliferative ability in response to E₂. The prostate epithelial PNT1A cells were included as a non-neoplastic cell line model to allow comparison of E₂-response with the PCa cell line models.

E₂-stimulation for 24 h significantly increased the mRNA expression of c-KIT in PNT1A, DU145 and PC3 cells (respectively, 1.86 ± 0.31 , 2.11 ± 0.34 and 3.19 ± 0.93 fold variation relatively to control, $P < 0.05$, Fig. 6.1A, E and G). On the contrary, in LNCaP cells, E₂ exposure decreased the mRNA expression of c-KIT (0.53 ± 0.14 fold variation comparatively with the control group, $P < 0.05$, Fig. 6.1C). Considering the estrogenic effect on the expression of c-KIT proteins it was shown that treatment with 100 nM E₂ for 24 h significantly down-regulated the expression of all c-KIT isoforms in DU145 cells (0.87 ± 0.01 , 0.94 ± 0.01 and 0.92 ± 0.02 fold variation relatively to control, respectively for the full-length c-KIT, 50 kDa and 30 kDa variants, Fig. 6.1F). In LNCaP cells E₂-treatment for 24 h also decreased the expression of 50 kDa and 30 kDa c-KIT truncated proteins (0.85 ± 0.02 and 0.83 ± 0.02 fold variation relatively to control, respectively; $P < 0.01$, Fig. 6.1D) though no changes were observed on the expression of full-length c-KIT. Curiously, for the same time of stimulation, an opposite effect was seen in PC3 cells, which showed increased expression of the full-length and 30 kDa truncated variant of c-KIT (respectively, 1.37 ± 0.16 and 1.32 ± 0.08 fold variation comparatively with the control group; $P < 0.05$, Fig. 6.1H).

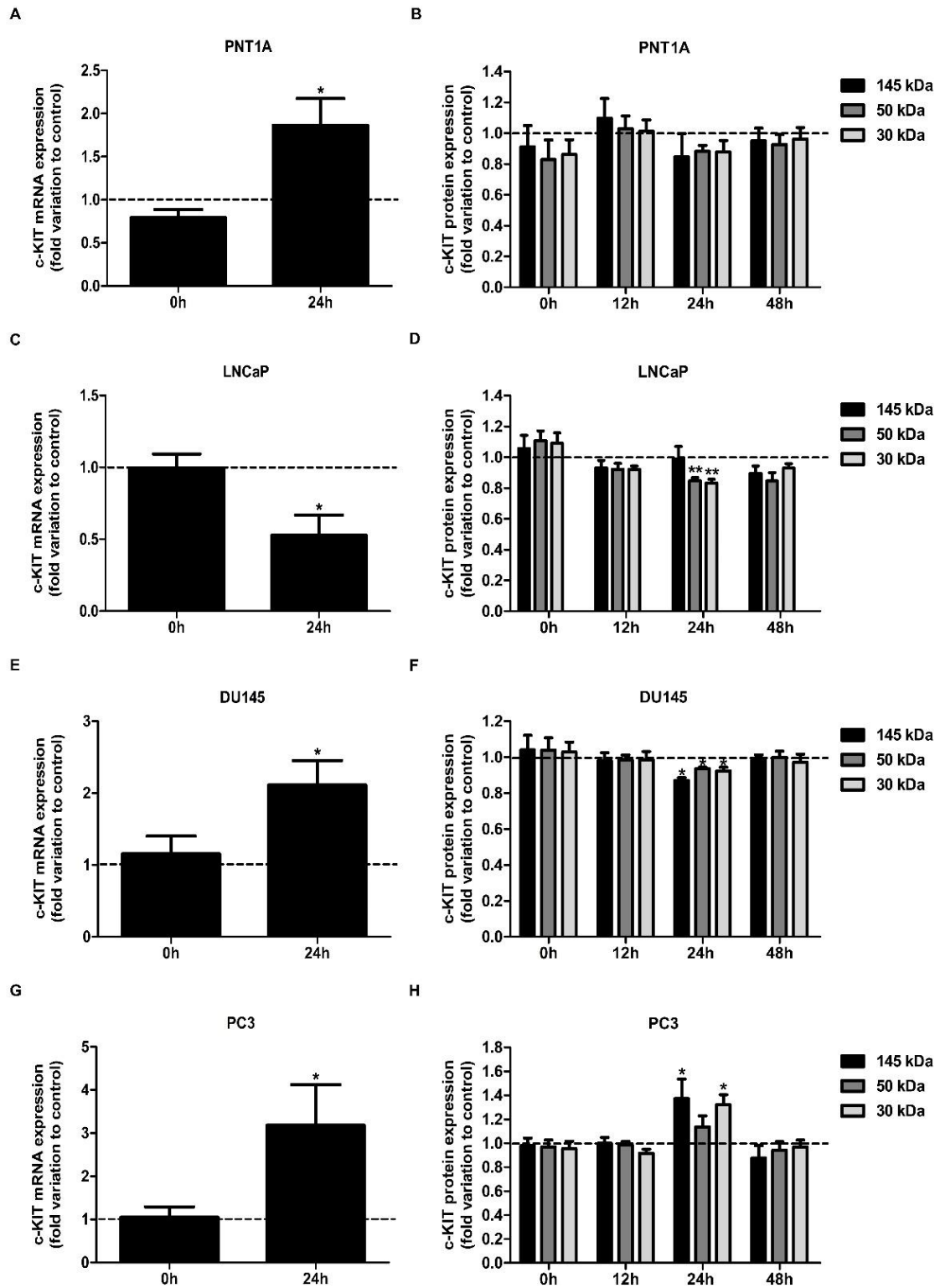
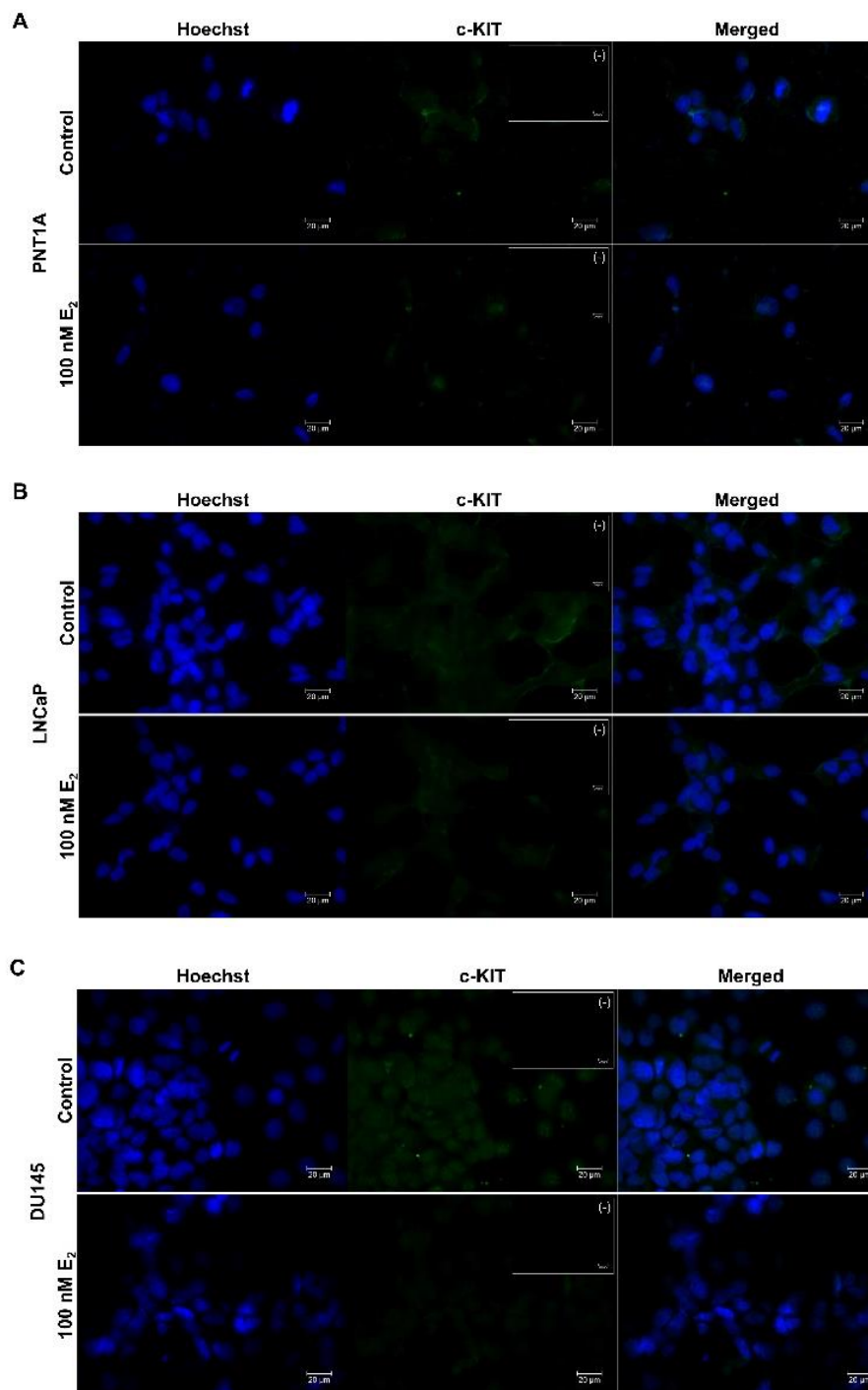


Figure 5.1. Effect of E₂ (100 nM) on the mRNA and protein expression of c-KIT in non-neoplastic (PNT1A) and neoplastic (LNCaP, DU145 and PC3) human prostate cell lines. mRNA expression (**A, C, E and G**) was determined by qPCR after normalization with GAPDH and β 2M housekeeping genes. Protein expression (**B, D, F and H**) was determined by WB analysis after normalization with α -tubulin. Results are expressed as fold variation relatively to the control group (0 nM E₂, dashed line). Error bars indicate mean $\pm$ S.E.M (n $\geq$ 5). *P < 0.05, **P < 0.01.

No changes on the expression of c-KIT proteins were perceived for PNT1A cells under E_2 -stimulation, or for LNCaP, DU145 and PC3 cells at other experimental time-points. Immunofluorescent labeling of c-KIT in PNT1A, LNCaP, DU145 and PC3 cells stimulated with E_2 for 24 h (Fig. 6.2A, B, C, D, respectively) followed the results obtained by WB analysis.



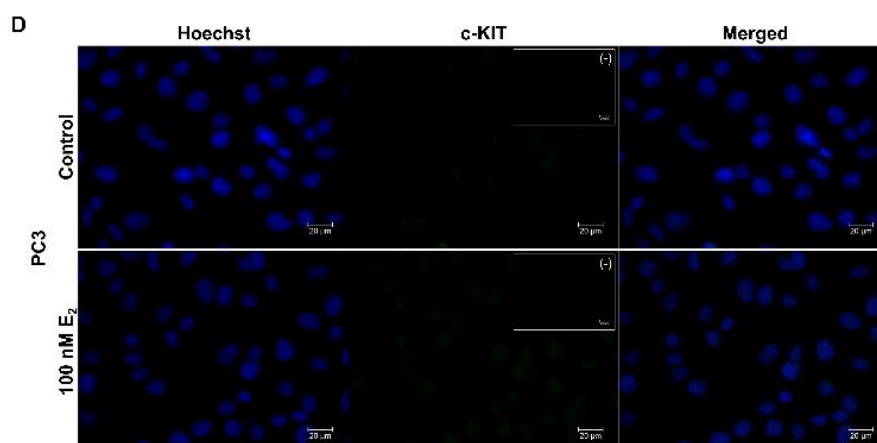


Figure 6.2. Representative images of c-KIT immunofluorescent labeling in non-neoplastic (PNT1A) and neoplastic (LNCaP, DU145 and PC3) human prostate cell lines treated with 100 nM E₂ for 24 h and controls. Images were obtained in the Axio Imager A1 microscope with an AxioCam MRc (Zeiss) under 630 x magnification. Nuclei are stained with Hoechst 33342 (blue) and c-KIT-positive staining is green. Negative controls for c-KIT obtained by omission of the primary antibody are provided as insert panels (-).

Regarding the mRNA expression of c-KIT ligand, SCF, a significant increase was found in all cell lines 24 h after E₂ administration (Fig. 6.3). The most pronounced effect was detected in PC3 with 2.75 ± 0.49 fold variation relatively to control ($P < 0.05$, Fig. 6.3G), which was almost the double of the effect seen in LNCaP cells (1.49 ± 0.13 fold variation, $P < 0.05$, Fig. 6.3C). Noteworthy, the SCF protein expression under estrogenic stimulation displayed a similar pattern to that found for c-KIT. E₂-treatment for 24 h down-regulated the SCF levels in PNT1A (Fig. 6.3B), LNCaP (Fig. 6.3D) and DU145 cells (Fig. 6.3F), respectively, 0.82 ± 0.07 , 0.85 ± 0.05 and 0.69 ± 0.09 fold variation comparatively with the control group ($P < 0.05$). In contrast, the results obtained in PC3 cells showed approximately a two-fold up-regulation of SCF protein expression (1.98 ± 0.06 fold variation relatively to control, $P < 0.001$, Fig. 6.3H).

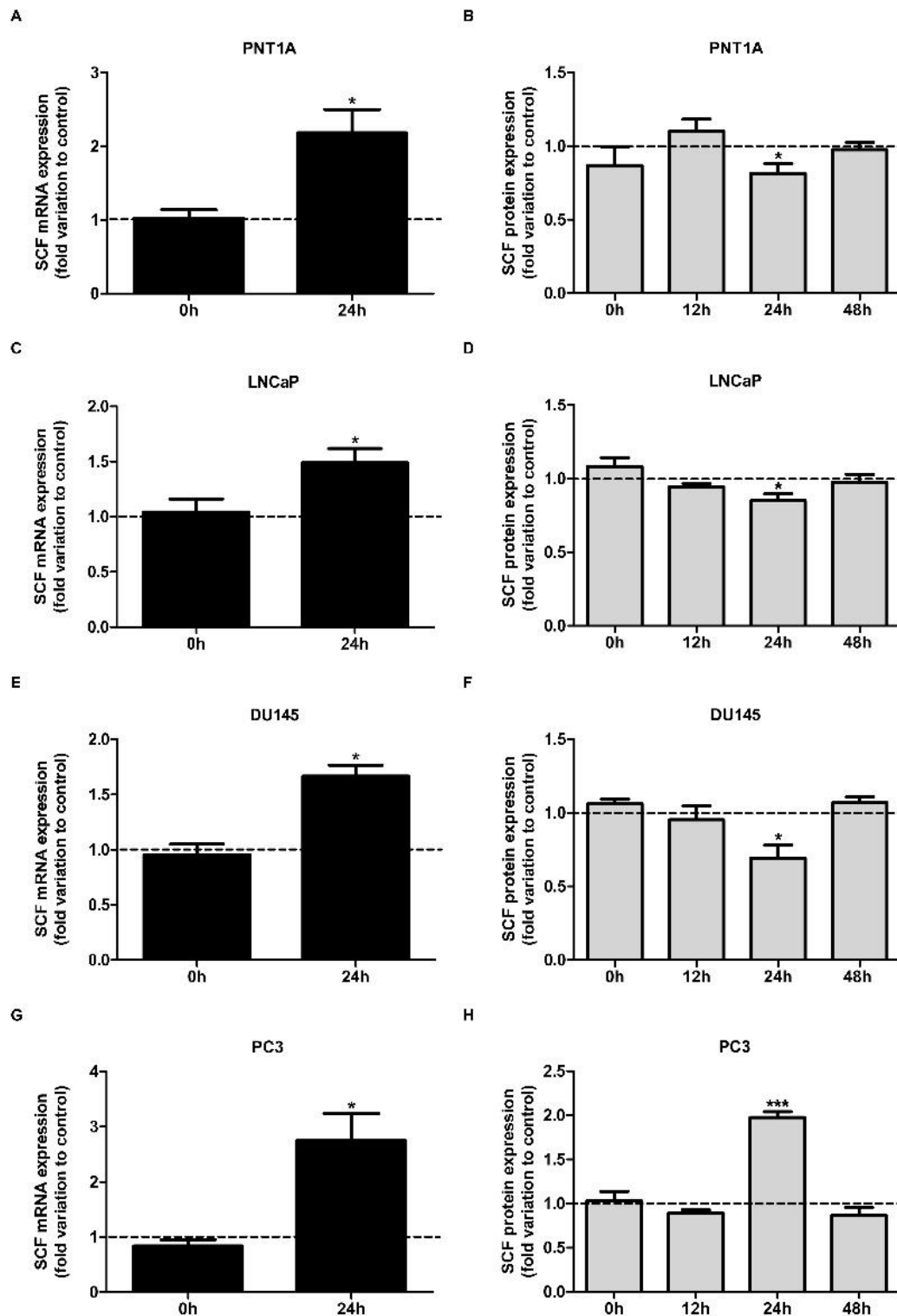
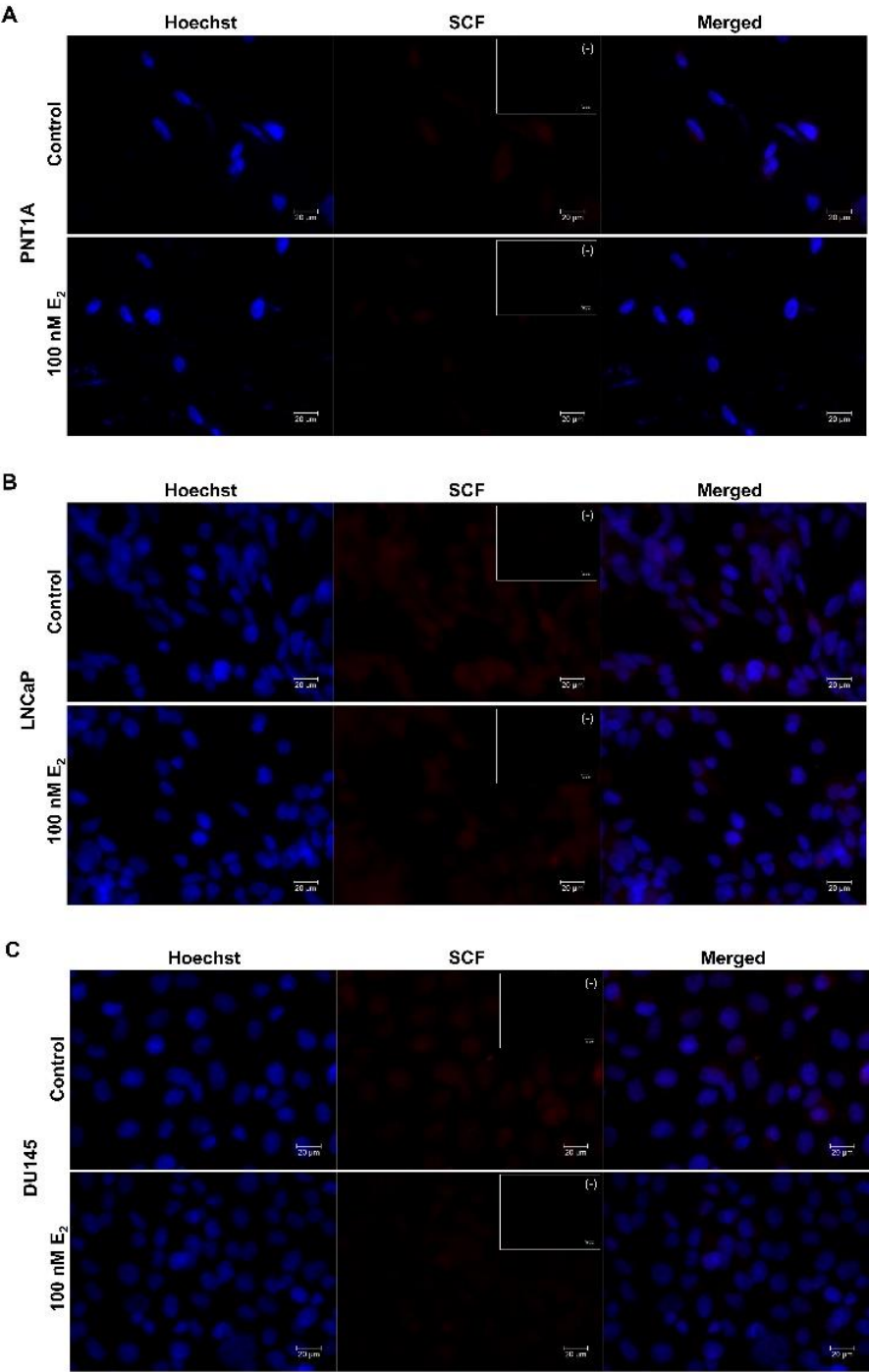


Figure 6.3. Effect of E₂ (100 nM) on the mRNA and protein expression of SCF in non-neoplastic (PNT1A) and neoplastic (LNCaP, DU145 and PC3) prostate cell lines. mRNA expression (**A, C, E and G**) was determined by qPCR after normalization with GAPDH and β 2M housekeeping genes. Protein expression (**B, D, F and H**) was determined by WB analysis after normalization with α -tubulin. Results are expressed as the fold variation relatively to control group (0 nM E₂, dashed line). Error bars indicate mean $\pm$ S.E.M (n $\geq$ 5). *P < 0.05, **P < 0.01.

No changes on SCF expression were observed for 12 or 48 h of stimulation with E₂. Immunofluorescent labeling of SCF in PNT1A, LNCaP, DU145 and PC3 cells stimulated with E₂ for 24 h (Fig. 6.4A, B, C, D, respectively) sustains the WB results.



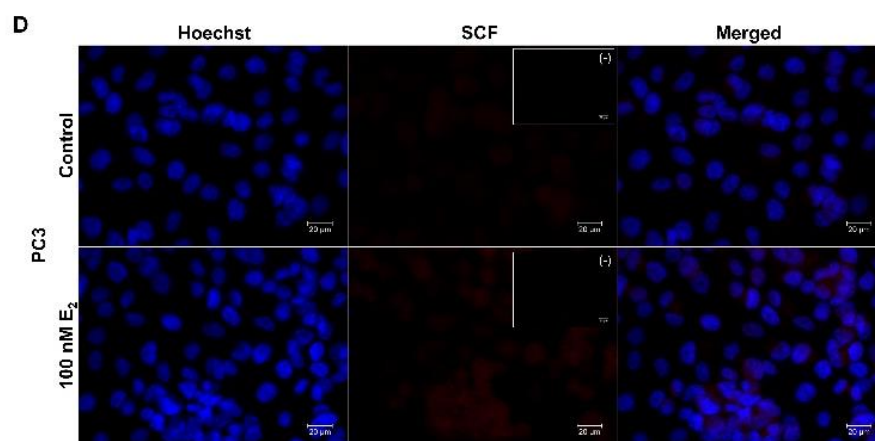


Figure 6.4. Representative images of SCF immunofluorescent labeling in non-neoplastic (PNT1A) and neoplastic (LNCaP, DU145 and PC3) human prostate cell lines treated with 100 nM E₂ for 24 h and controls. Images were obtained in the Axio Imager A1 microscope with an AxioCam MRc (Zeiss) under 630 x magnification. Nuclei are stained with Hoechst 33342 (blue) and SCF-positive staining is red. Negative controls for SCF obtained by omission of the primary antibody are provided as insert panels (-).

6.3.2. E₂ down-regulates the expression of SCF and c-KIT in rat prostate

In order to evaluate the effect of E₂ in regulating the expression of SCF and c-KIT *in vivo*, adult rats were daily injected with 250 µg/Kg of E₂ (or vehicle) for 5 days. E₂ treatment significantly increased the mRNA expression of c-KIT in rat prostate (2.53 ± 0.28 fold variation comparatively with the control, Fig. 6.5A). At protein level, estrogenic stimulation distinctly affected the expression of c-KIT isoforms. E₂ significantly up-regulated the expression of 50 kDa and 30 kDa isoforms of c-KIT (respectively, 1.17 ± 0.02 and 1.64 ± 0.09 fold variation relatively to control), whereas the expression of the full-length protein was decreased (0.89 ± 0.04 fold variation comparatively with the control group, $P < 0.05$, Fig. 6.5B). Results of dual-labeling fluorescent immunohistochemistry detecting c-KIT and cell membrane support the WB results showing increased expression of truncated cytoplasmic c-KIT in response to E₂ (Fig. 6.5D).

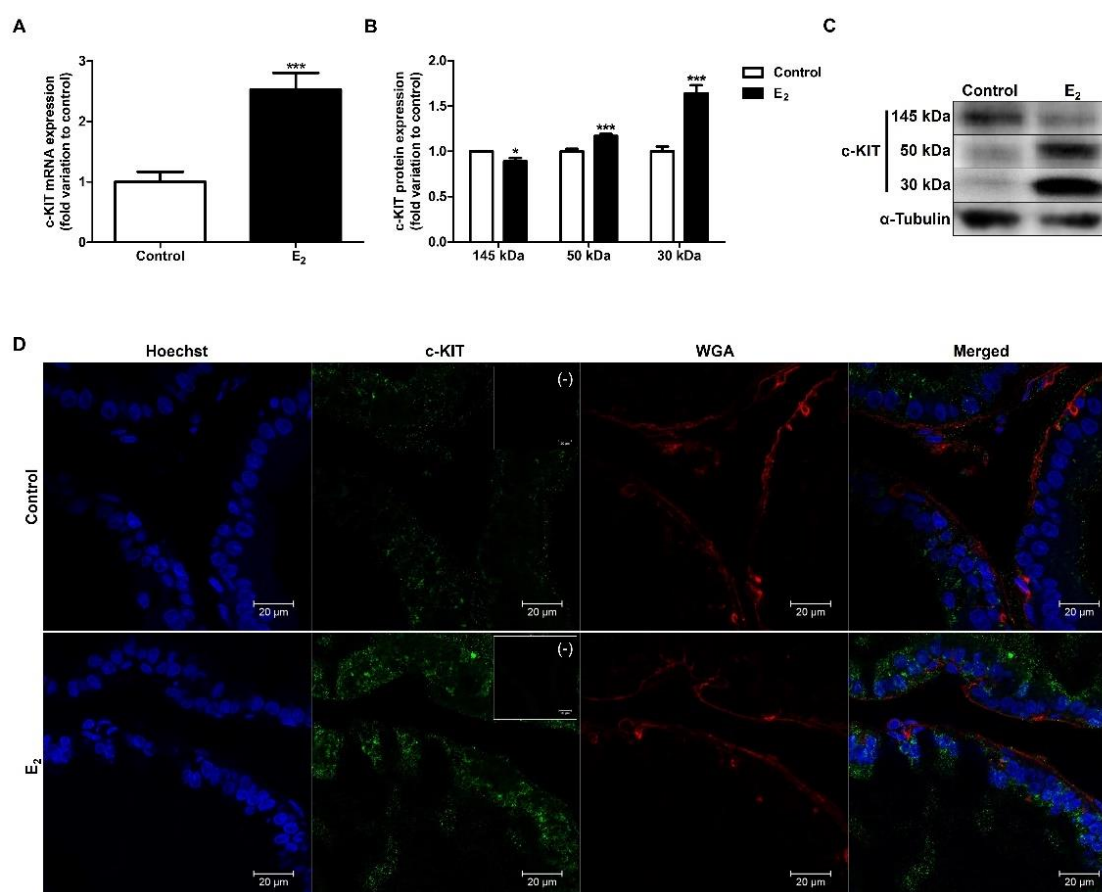


Figure 6.5. Effect of E₂ on the expression of c-KIT in rat prostate. (A) mRNA expression determined by qPCR after normalization with β -actin housekeeping gene. (B) Protein expression determined by WB analysis after normalization with α -tubulin. Results are expressed as fold variation relatively to the control untreated group. Error bars indicate mean $\pm$ S.E.M (n $\geq$ 5). *P < 0.05; ***P < 0.001. (C) Representative immunoblots are shown in panel. (D) Representative confocal microscopy images showing the co-localization of c-KIT with cell membrane in control and E₂-treated groups. Images were obtained in the Zeiss LSM 710 laser scanning confocal microscope under 630 x magnification. Nuclei are stained with Hoechst 33342 (blue), c-KIT-positive staining is green and cell membrane was stained with WGA (red). Negative controls for c-KIT obtained by omission of the primary antibody are provided as insert panels (-).

Considering SCF, its mRNA was up-regulated in the prostates of E₂-treated animals (3.70 ± 0.54 fold variation relatively to control, P < 0.001, Fig. 6.6A). Inversely, the SCF protein was down-regulated in response to E₂-treatment (0.78 ± 0.01 fold variation comparatively with the control group, P < 0.05, Fig. 6.6B). Although only one immunoreactive band was detected in WB (Fig. 6.6C), dual-labeling fluorescent immunohistochemistry allowed detecting both membrane and soluble forms of SCF (Fig. 6.6D) as evident by yellow color in merged images of SCF labeling (green) with cell membrane (red). In addition, the immunofluorescence results support the diminished expression of SCF protein in the E₂-treated group.

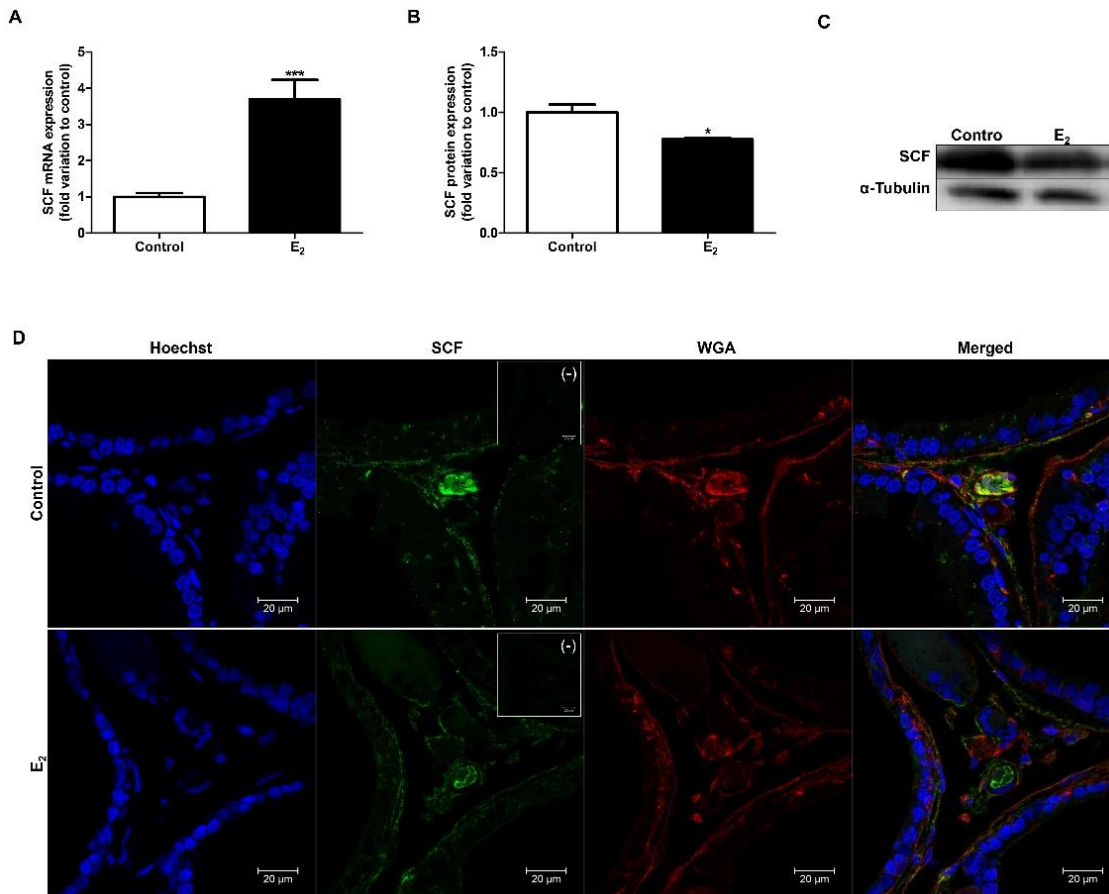


Figure 6.6. Effect of E₂ on the expression of SCF in rat prostate. (A) mRNA expression determined by qPCR after normalization with β -actin housekeeping gene. (B) Protein expression determined by WB analysis after normalization with α -tubulin. Results are expressed as fold variation relatively to the control untreated group. Error bars indicate mean $\pm$ S.E.M (n $\geq$ 5). *P < 0.05; ***P < 0.001. (C) Representative immunoblots are shown in panel. (D) Representative confocal microscopy images showing the co-localization of SCF with cell membrane in control and E₂-treated groups. Images were obtained in the Zeiss LSM 710 laser scanning confocal microscope under 630 x magnification. Nuclei are stained with Hoechst 33342 (blue), SCF-positive staining is green and cell membrane was stained with WGA (red). Negative controls for SCF obtained by omission of the primary antibody are provided as insert panels (-).

6.3.3. Down-regulated expression of SCF/c-KIT in response to E₂ is concomitant with decreased prostate weight and diminished proliferation

The activity of SCF/c-KIT system is highly related with the induction of cell proliferation [19]. Therefore, since E₂ diminished the expression of SCF and c-KIT receptor proteins in rat prostate, we decided to study the proliferation of prostate cells by comparing prostate weight and Ki proliferation index between control and E₂-treated groups. Although no changes were observed on animal weight between treated

and untreated animals (Fig. 6.7A), E₂-exposure significantly decreased prostate weight by almost an half comparatively with the control group (Fig. 6.7B).

Ki67 immunofluorescence was used to determine the cell proliferation index in the prostate of E₂-treated animals by determining the number of Ki67-positive cells relative to the total cell number (Fig. 6.7C and 6.7D). Ki67-proliferation index was reduced in rats treated with E₂ comparatively with the control group (0.50 ± 0.10 fold variation, $P < 0.05$, Fig. 6.7C).

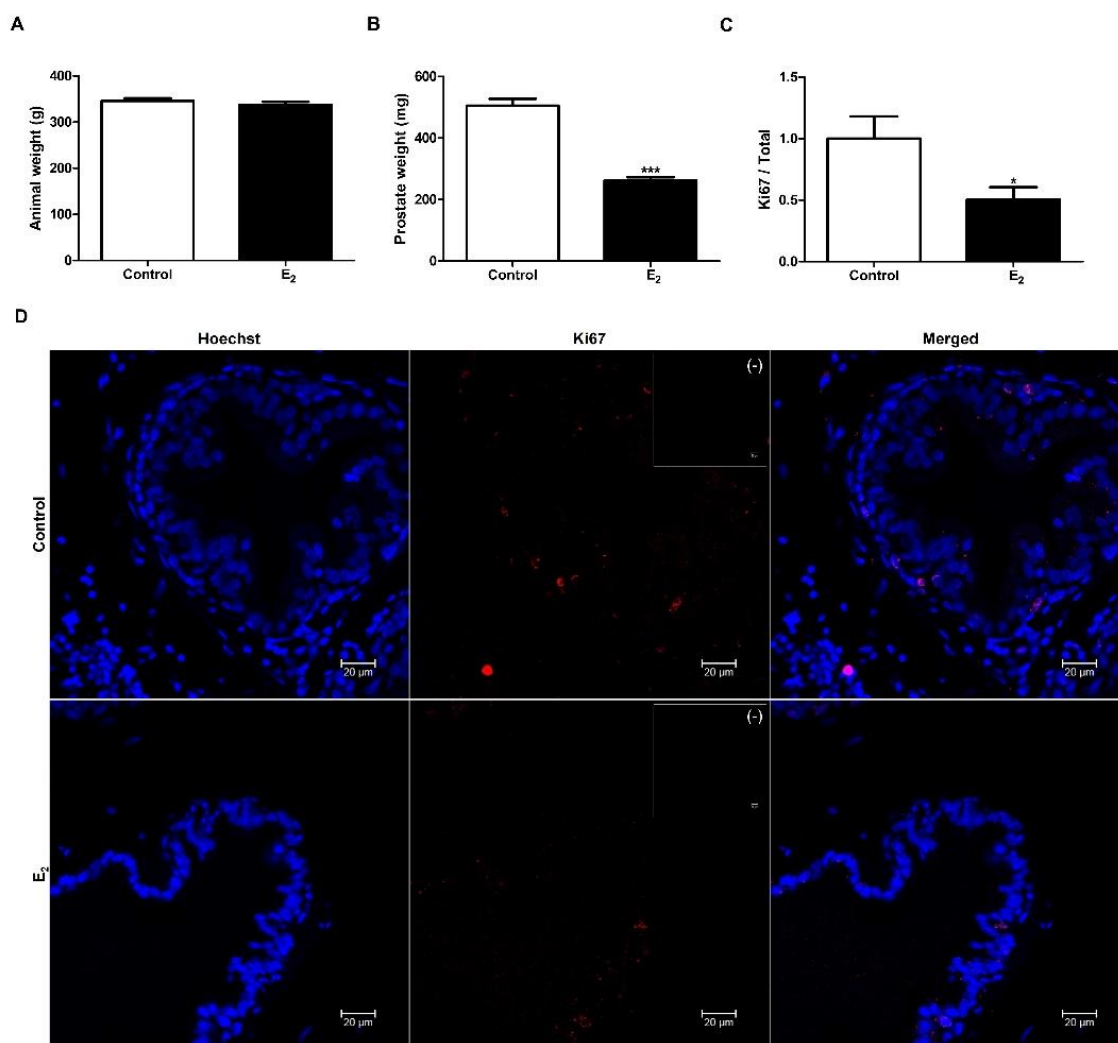
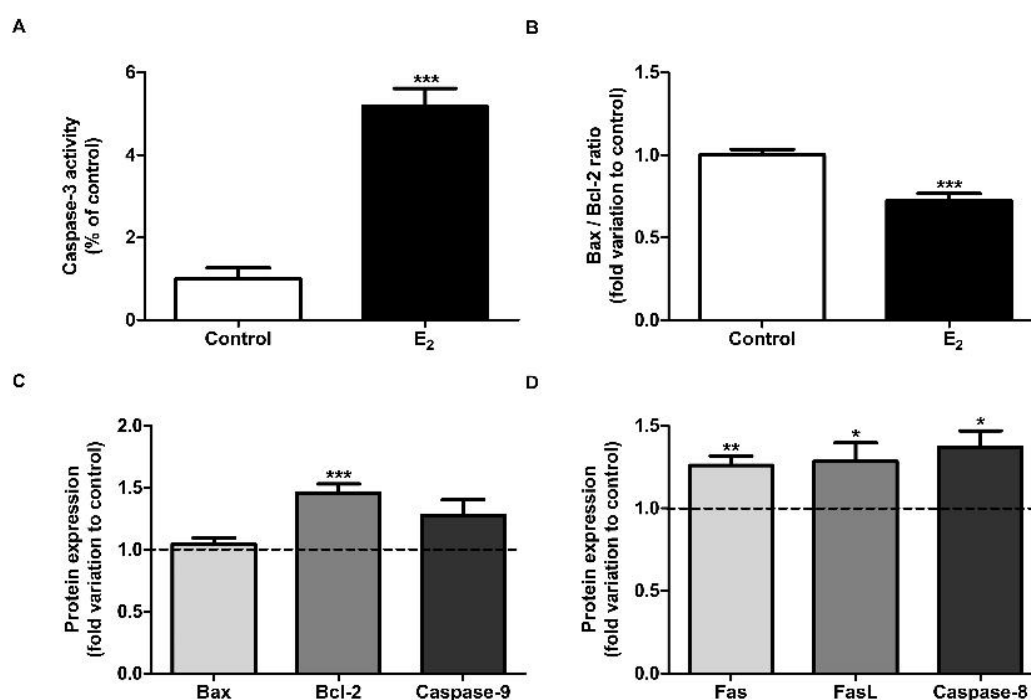


Figure 6.7. Effect of E₂ on animal weight (A), rat prostate weight (B) and cell proliferation. (C) Proliferation index determined by Ki67 immunofluorescence analysis. Percentage of Ki67-positive cells relative to the total cell number. Results are expressed as fold variation relatively to the control untreated group. Error bars indicate mean $\pm$ S.E.M ($n \geq 5$). *** $P < 0.001$ * $P < 0.05$. **(D)** Representative confocal microscopy images showing Ki67 labeling in control and E₂-treated groups. Images were obtained in the Zeiss LSM 710 laser scanning confocal microscope under 630 x magnification. Nuclei are stained with Hoechst 33342 (blue) and Ki67 positive staining is red. Negative controls for Ki67 obtained by omission of the primary antibody are provided as insert panels (-).

6.3.4. E₂-treatment induced apoptosis in rat prostate

The effect of E₂ on the expression and activity of apoptosis regulators in rat prostate was investigated. A huge increase in the activity of caspase-3 was observed in E₂-treated group relatively to the control group (5.18 ± 0.44 fold variation, $P < 0.001$, Fig. 6.8A).

The Bax and Bcl-2 proteins are, respectively, proapoptotic and antiapoptotic members of the Bcl-2 family of apoptosis regulators [38], and the Bax/Bcl-2 protein ratio is considered a powerful marker of apoptosis [39, 40]. Bax/Bcl-2 protein ratio was decreased in the prostates of E₂-treated group when compared with the control group (0.72 ± 0.04 fold variation, $P < 0.001$, Fig. 6.8B). This reduction seems to be due to an increase in Bcl-2 expression in treated rats (1.46 ± 0.07 fold variation comparatively with the control, $P < 0.001$, Fig. 6.8C), since no changes were observed on Bax expression (Fig. 6.8C). Also, caspase-9, did not showed altered expression between E₂-treated animals and the control group (Fig. 6.8C).



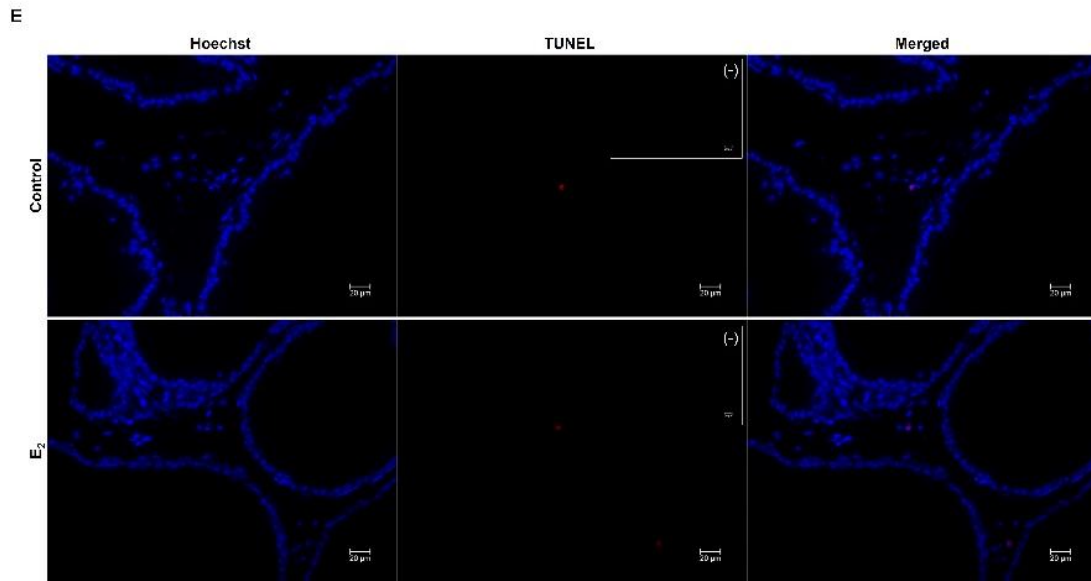


Figure 6.8. Apoptosis in the prostate of control and E₂-treated animals. (A) Caspase-3 activity. **(B)** Protein ratio of Bcl-2 family regulators of apoptosis, Bax and Bcl-2. **(C)** Expression of apoptosis regulators of the intrinsic pathway. **(D)** Expression of apoptosis regulators involved in the extrinsic pathway of apoptosis. Protein expression was determined by WB analysis after normalization with α -tubulin. Results are expressed as fold variation relatively to the control untreated group. Error bars indicate mean $\pm$ S.E.M (n $\geq$ 5). *P < 0.05, **P < 0.01, ***P < 0.001. (E) Representative fluorescence microscopy images showing TUNEL labeling in control and E₂-treated groups. Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany) under 630 x magnification. Nuclei are stained with Hoechst 33342 (blue) and TUNEL-positive staining is red. Negative controls are provided as insert panels (-).

The death factors Fas and FasL were up-regulated in the prostate of E₂-treated animals comparatively with the control group (respectively, 1.26 ± 0.06 (P < 0.01) and 1.28 ± 0.11 fold variation, (P < 0.05), Fig. 6.8D). Caspase-8 also showed to be significantly increased in the E₂-treated group comparatively with control (1.37 ± 0.10 fold variation, Fig. 6.8D). The presence of apoptotic cells in rat prostate was analyzed by detecting DNA fragmentation using the TUNEL-labeling assay. Representative photomicrographs showing increased number of TUNEL-positive cells in E₂-treated animals comparatively with controls are provided in Fig. 6.8E.

6.4. Discussion

In this study, we reported that estrogens are able to regulate the expression of the SCF/c-KIT system in human prostate cell lines and in rat prostate *in vivo*. The neoplastic prostate cell lines LNCaP, DU145 and PC3 mimicking different stages of PCa

and the non-neoplastic PNT1A cells were included to evaluate their responsiveness to E₂. Firstly, we confirmed that PNT1A, LNCaP, DU145 and PC3 cells express the c-KIT receptor and its ligand SCF because previous studies have produced somehow discordant results [24, 41].

c-KIT is a transmembrane glycoprotein with approximately 145-160 kDa in its full-length form [42]. Protein variants of c-KIT are originated by mechanisms of alternative splicing, differing in the presence or absence of the GNNK peptide [43]; and by the usage of an alternative promoter and transcription of a cryptic exon producing a truncated c-KIT protein (tr-KIT) with approximately 30-50 kDa [44-48]. Moreover, the proteolytic cleavage of c-KIT leads to its release from the cell membrane originating a soluble isoform [49, 50]. Herein, we showed that all cell lines (PNT1A, LNCaP, DU145 and PC3) present the full-length membrane-bound c-KIT protein, as well as the tr-KIT (30 kDa) variant (Fig. 6.1, 6.2). Furthermore, a 50 kDa c-KIT variant was also found, which may be generated by a covalent interaction of the 30 kDa tr-KIT protein with some other proteins present in prostate cells, as suggested by others [46]. Although the cytoplasmic tr-KIT has been described in PCa cases [51], its identification in these cell lines models of PCa is a novelty. Moreover, to the best of our knowledge this is the first report definitely characterizing the expression pattern of SCF/c-KIT system in PCa cells. Also, membrane and soluble isoforms of SCF were detected in PNT1A, LNCaP, DU145 and PC3 cells (Fig. 6.4).

It was verified that E₂ treatment modulated the levels of SCF and c-KIT in prostate cell lines both at mRNA and protein level, with a highly time-specific response. Interestingly, all changes in the expression of SCF and c-KIT were observed for 24 h of stimulation only (Fig. 6.1, 6.3). At 0, 12 or 48 h of stimulation no expression changes were detected. A down-regulated expression of c-KIT receptor protein was observed in all PCa cell lines, except PC3 cells, which displayed an up-regulated expression of c-KIT in response to E₂ (Fig. 6.1). No effect of E₂ was observed in the c-KIT protein levels in PNT1A cells. It is also remarkable that in DU145 cells the down-regulated expression of c-KIT occurred for all the variants detected, the 145 kDa full-length protein, the 50 kDa and the 30 kDa (tr-KIT), whereas no altered expression was found for the full-length c-KIT in the LNCaP cells in response to E₂. The function of tr-KIT in prostate cells is entirely unknown. However, despite not having tyrosine kinase activity, tr-KIT retains signal transduction capabilities, and it was shown to activate the phospholipase C- γ pathway in mice oocytes [52, 53]. Moreover, the c-KIT variants seem to be related to cell survival. For example, in the testis, the tr-KIT is associated with the DNA integrity of germ cells [46], and its expression has been shown to increase with the progression of PCa [51]. Thus, the increased expression of full-length and 30 kDa truncated c-KIT

variant observed in PC3 cells in response to E₂ may be an adaptive mechanism favoring survival and invasive features of this cancer cell model that mimics advanced stages of PCa.

Concerning the c-KIT ligand SCF, similar results were obtained. Hormonal stimulation down-regulated the SCF protein levels in all prostate cell lines tested except PC3 (Fig. 6.3). E₂ treatment significantly increased the SCF protein expression in PC3 cells (Fig. 6.3H), again showing a specific differential response in this cell line model.

Although E₂ treatment down-regulated the expression of SCF/c-KIT proteins in all cell lines except PC3, it cannot be excluded from the discussion the fact that the effects on mRNA expression do not always follow the results obtained at the protein level. Indeed, the increase in mRNA transcripts with non-correspondent increase in protein is widely described, and can be explained by innumerable reasons associated with the great complexity of gene expression regulatory mechanisms in eukaryotes, namely, mRNA retention in the nucleus, stability and half-life time of mRNA molecule, control of protein translation efficiency, post-translational modifications, and protein degradation [54]. Nevertheless, all the conclusions and discussion below were drawn exclusively from the protein differences. Moreover, the results of fluorescent immunocytochemistry were concordant with the WB analysis further confirming the obtained findings for both SCF and c-KIT.

LNCaP, DU145, and PC3 cells are largely known as cell line models of metastasized PCa representing, in this order, stages of increasing aggressiveness of the disease. Also, it has been established that these cells display a differential expression pattern of ERs. LNCaP and DU145 cells express the ER β but not ER α while PC3 cells express both classical ERs [55-57]. Thus, it is liable to assume that the specific behavior of PC3 cells concerning the up-regulated expression of SCF and c-KIT in response to E₂ may be the consequence of the presence of ER α . In the same way, the diminished expression of both SCF and c-KIT in LNCaP and DU145 cells is likely mediated by the ER β . In the last years, also it has been accepted that ER α and ER β have opposing roles in the control of prostate cell proliferation and apoptosis [58, 59]. Moreover, ER β is associated with an anti-tumorigenic role whereas the ER α seems to have pro-tumorigenic effects [6, 57, 60, 61].

The SCF/c-KIT system plays a crucial role in the control of cell proliferation, survival and invasion and has been shown to contribute to tumor onset, development and metastization [4, 5, 21, 22]. Therefore, in the light of the present findings it is expected that the stimulatory effects of ER α over PC3 cells proliferation depend on the augmented expression of SCF and c-KIT as showed here in response to E₂ treatment.

Contrastingly, as discussed above E_2 down-regulated the expression of SCF and c-KIT proteins in other PCa cell line models, as well as in the non-neoplastic PNT1A cells.

To confirm whether the *in vitro* results obtained in human prostate cell lines are translated *in vivo* and functionally relevant, adult male rats were daily injected with 250 $\mu\text{g } E_2/\text{Kg}$ for 5 days. The expression of the SCF/c-KIT system, as well as the proliferation and apoptosis in rat prostate was investigated. E_2 -treatment down-regulated the expression of the full-length c-KIT protein in rat prostate. However, the expression of 30 and 50 kDa truncated variants was up-regulated in the prostate of E_2 -treated animals (Fig. 6.5). This differential effect of E_2 in regulating c-KIT proteins did not occurred in prostate cell lines, and may be explained by the complexity of the *in vivo* systems, which include an intricacy of regulatory processes that there are absent in cell systems *in vitro*. It also suggests that the estrogenic regulation of SCF/c-KIT system in rat prostate may depend on the balance of $ER\alpha$ and $ER\beta$ expression.

Relatively to SCF, only one specific immunoreactive band was detected in WB, which was down-regulated after E_2 administration (Fig. 6.6B, C). Nevertheless, despite supporting the results of WB analysis, fluorescent immunohistochemistry confirmed the location of SCF both at the cell membrane and cytoplasm of rat prostate cells. In fact, the SCF is present at cell membrane as a noncovalent homodimer (mSCF) [62-64], but the proteolytic cleavage of an alternatively spliced variant of SCF may originates its soluble isoform (sSCF) [65-67]. The apparent discrepancy between immunohistochemistry and WB may be due to the high sensitivity of fluorescent labeling comparatively with detection method in WB technique.

Thus, our results demonstrated that E_2 -treatment down-regulated the expression of both SCF and c-KIT in rat prostate. Considering that the activity of SCF/c-KIT system is strongly related to the promotion of cell proliferation and inhibition of apoptosis [22], the diminished levels of c-KIT receptor and SCF in rat prostate in response to E_2 suggest the existence of modifications in proliferation and apoptosis.

The Ki67 is a recognized marker of cell proliferation, found in the nucleus of proliferating cells in all active phases of the cell division cycle but absent in non-proliferating cells [68]. Ki67 fluorescence immunohistochemistry was used to estimate the prostate cell proliferation index, which was significantly reduced in the prostate of E_2 -treated rats (Fig. 6.7C, D). The pronounced reduction observed in the prostate weight of E_2 -treated animals comparatively with the control group (Fig. 6.7A) further confirms the antiproliferative action of E_2 in rat prostate cells. In mice, similar results of prostate growth inhibitory effects were described upon administration of E_2 in the same range (200 and 300 $\mu\text{g}/\text{Kg}$) of that used in the present study. Contrastingly, lower

doses of estrogens enhanced the prostatic growth [15]. The duality of estrogenic effects depending on administration of normo- or supraphysiological doses was also discussed relatively to the proliferation and apoptosis of male germ cells [69]. For example, 10^{-10} M and 10^{-9} M of E_2 promoted germ cell survival *in vitro* [70] while 10^{-8} and 10^{-7} M doses induced the apoptotic death of germ cells [71, 72]. These findings raise the concern about the specific and accurate dosage of estrogens or estrogen-like compounds applied for PCa therapy and may explain some inconsistent results. Nevertheless, estrogens seem to have a protective role in PCa [16] and estrogen-based therapies have been used to treat PCa [17]. However, the effect of estrogens in PCa regression is thought to be mediated by their suppressive action on the hypothalamic-pituitary axis, thereby inhibiting testosterone synthesis [73]. Our results show that estrogens, and eventually similar compounds, may act directly on prostate cells with antitumor effects by their actions inhibiting cell cycle progression and also inducing apoptosis, as discussed below.

The apoptosis of rat prostate cells in response to E_2 stimulation and to the diminished levels of SCF/c-KIT system also was investigated. The apoptotic process may be triggered by two distinct but interconnected pathways: the intrinsic and the extrinsic pathways of apoptosis [74]. Nevertheless, independently of the activated pathway, cell-death signaling cascades converge at the activation of caspase-3 [38], and the measurement of caspase-3 enzymatic activity has been widely used as an indicator of apoptosis. A huge increase in the activity of caspase-3 was observed in the prostates of E_2 -treated group relatively to control (Fig. 6.8A). Also, the TUNEL assay showed increased DNA fragmentation in the prostates of animals exposed to E_2 (Fig. 6.8E). These results indicated that E_2 treatment, concomitantly with diminished expression of SCF and c-KIT, increased apoptosis in rat prostate cells, which also has been reported in other tissues of male reproductive tract [30].

To clarify the apoptotic pathway involved in the estrogenic regulation of apoptosis in rat prostate, the expression of several apoptosis related proteins was determined by WB analysis.

The Bax and Bcl-2 proteins, respectively, proapoptotic and antiapoptotic members of the Bcl-2 family of apoptosis regulators [38] are mainly involved in the activation of intrinsic pathway of apoptosis, although they can also be implicated in the extrinsic pathway [39]. The Bax/Bcl-2 protein ratio, considered a powerful marker of cell commitment to apoptosis [39, 40] was decreased in the prostate of rats under E_2 stimulation (Fig. 6.8B). This reduction was caused by the increased expression of Bcl-2 expression in treated rats since no changes were verified in Bax expression. In addition,

caspase-9, an initiator caspase involved in the intrinsic pathway of apoptosis, did not show altered expression with E₂ treatment (Fig. 6.8C).

The activation of the extrinsic pathway of apoptosis is triggered by death factors at the cell membrane, namely, by the receptor-ligand interaction between Fas and FasL [75]. Caspase-8 is a mediator of the extrinsic apoptosis pathway that interacts with death receptors [76] initiating apoptotic signaling cascade. Both Fas and FasL, and caspase-8 were up-regulated in the prostate of E₂-treated animals comparatively with the control group (Fig. 6.8D), which indicates that E₂ stimulates apoptosis in prostate cells through enhanced activity of the extrinsic pathway. Previous studies also have shown that estrogens trigger apoptosis mainly through the activation of extrinsic pathway [30, 72, 77, 78]. Moreover, attenuated activity of the SCF/c-KIT system is known to be linked with increased rates of apoptosis [22, 26, 71], which also contributes to the augmented apoptosis in the prostate of E₂-treated rats.

In conclusion, the present findings demonstrated that E₂ down-regulated the expression of the SCF/c-KIT system both in human PCa cell lines and in rat prostate *in vivo*. These results were underpinned by the antiproliferative and proapoptotic effects of E₂ and, thus, are supportive of the protective role of estrogens against PCa. Future approaches to disclosing the molecular mechanisms underlying the estrogenic effects in the prostate will be crucial to identify new targets for treatment and to establish the mode of action, safety and utility of estrogen-based therapies in PCa.

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Chapter 7

G protein-coupled estrogen receptor actions targeting the hallmarks of cancer in human prostate cells: from cell fate to metabolic reprogramming

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7. G protein-coupled estrogen receptor actions targeting the hallmarks of cancer in human prostate cells: from cell fate to metabolic reprogramming

Abstract

The G protein-coupled estrogen receptor (GPER), a membrane-associated estrogen receptor, has been pointed out as anti-tumorigenic in several types of cancer. However, its role in prostate cancer (PCa), the second most frequent cancer among men, remains not fully understood. This work investigated the role of GPER targeting the hallmarks of cancer in human prostate cells, namely, their actions in controlling cell proliferation, apoptosis, migration / invasion, metabolism and oxidative status of prostate cells. Tissue microarrays analysis by determining immunohistochemistry scores, confirmed the higher GPER immunoreactivity in human PCa cases compared to BPH, and its inverse correlation with the patients' PSA levels. Non-neoplastic (PNT1A) and neoplastic (LNCaP, DU145 and PC3) prostate cells were treated with the GPER specific agonist G1 (1 μ M) or dihydrotestosterone (DHT, 10 nM). G1 treatment modulated GPER subcellular location, which was found at the cell membrane, endoplasmic reticulum, and residually in the nucleus by GPER fluorescent immunocytochemistry in colocalization with wheat germ agglutinin, calnexin, and Hoescht. DHT-treatment reduced GPER protein levels non-neoplastic and androgen-sensitive PCa cell lines, without changing it in castrate-resistant PCa cells. The activation of GPER by G1 decreased PCa cells viability and proliferation, arresting cell cycle at G2/M phase, which was determined by the MTT assay, Ki-67 fluorescent immunocytochemistry and flow cytometry, respectively. Changes in phospho-c-Myc (p-cMyc), p21, p53, p-AKT and p-ERK were perceived in PCa-treated cells. Furthermore, G1-stimulated cells displayed augmented apoptosis as indicated by the TUNEL assay and measurement of caspase-3-like activity, with altered expression of apoptosis regulators of the intrinsic (Bax, Bcl-2 and caspase-9) and extrinsic (Fas-R, Fas-L and caspase-8) apoptotic pathways. GPER activation inhibited migration and invasion of PCa cells in transwell assays, and long-time exposure to G1 increased the expression of E-cadherin and cytokeratins 8 and 18, whereas reducing N-cadherin levels. Our results

first showed that G1 treatment also modulated the metabolic profile of PCa cells, changing glucose, amino-acids and lipids metabolism. Finally, GPER increased oxidative stress in PCa cells. Overall, it was shown that GPER activation covers a broad range of cancer hallmarks. G1 treatment decreased PCa cell viability and proliferation, arrested cell cycle, enhanced apoptosis, and controlled migration and invasion, with the altered expression of EMT markers. Moreover, PCa metabolism, including glucose, amino acids and lipid handling, and oxidative stress profile were altered in response to G1. These findings support the anti-cancer role of GPER and stimulate further research to ascertain its role in the regulation of metabolism and as a therapeutic target.

Keywords

Prostate cancer (PCa), G protein-coupled estrogen receptor (GPER), proliferation, apoptosis, migration and invasion, metabolism, oxidative stress

7.1. Introduction

Prostate cancer (PCa) is the second most frequent type of cancer among men, and its incidence continues to increase worldwide [1, 2]. Despite the continuous advances in PCa management, several unmet clinical needs exist, namely, in identifying effective treatment approaches to overcome PCa resistance to conventional androgen deprivation therapies [3, 4].

PCa is a hormone-dependent cancer highly regulated by androgens, which have a preponderant role in the onset and progression of the disease, being associated with augmented cell proliferation rates [5] and restricted apoptosis [6]. Also, it is widely accepted that PCa is sensitive to estrogens actions, with these hormones being used in treatment for decades and discontinued only because of the adverse side effects [7-12]. The G protein-coupled estrogen receptor (GPER), a membrane-bound ER, mediates the rapid, non-genomic effects of estrogens [13] by mobilizing intracellular calcium and activating the phosphatidylinositol 3-kinase (PI3K)/AKT, mitogen-activated protein kinase (MAPK), Src kinase and AmpC pathways [14].

A substantial amount of evidence in different cancer types has been describing the anti-tumorigenic effects of GPER [15-20]. Overall, it has been associated with suppression of proliferation, migration, invasion and epithelial-mesenchymal transition (EMT), cell cycle arrest and augmented apoptosis in several types of cancer

[16, 19, 21-25]. In PCa, some studies have reported the anti-cancer role of GPER, indicating that it reduces cell proliferation and tumor growth, arresting cell cycle and increasing apoptosis [26-28]. For this reason, the GPER-specific agonist, G1 (GPR30-specific compound 1) [29], a substituted dihydroquinoline, with a tetrahydro-3H-cyclopenta[c]quinoline core structure [29] was investigated considering its putative utility in PCa treatment. Indeed, it was shown that G1 inhibited the growth of castrate-resistant PCa cells but not of androgen-responsive, which supports GPER as a therapeutic target in castrate-resistant PCa (CRPC). However, other available studies have reported the pro-carcinogenic actions of GPER in prostate by inducing cell proliferation and tumor growth, restricting apoptosis and enhancing tumor progression [30-32]. Also, discrepancies exist concerning the expression pattern of GPER in the human prostate. Rago *et al.* [33] characterized GPER expression in benign and neoplastic tissues, which was inversely related to neoplastic cell differentiation [33]. Contrastingly, Lam *et al.* reported that GPER expression is higher in metastatic CRPC than in primary PCa [28]. These inconsistencies concerning GPER actions and expression strongly limit the perspective of targeting GPER in PCa therapy. Another bottleneck is that most studies have focused on the classical hallmarks of cancer (i.e. sustaining proliferative signaling and resisting cell death) not considering the new cancer hallmarks, such as metabolic reprogramming [34]. Furthermore, as GPER has been indicated as a metabolic regulator [35], it is of utmost importance to address cancer cell metabolism in response to GPER agonists.

The present study aimed to disclose the role of GPER in human prostate cells covering a broad range of cancer hallmarks. G1 effects in controlling PCa cell proliferation, apoptosis, migration, invasion, epithelial-mesenchymal transition (EMT), oxidative stress, and glucose, amino-acids and lipids metabolism were investigated. Also, the GPER expression pattern in non-neoplastic and neoplastic human prostate and its subcellular localization in human prostate cells were evaluated.

7.2. Material and methods

7.2.1. GPER immunohistochemistry and staining scores

Tissue microarrays (TMAs) containing 79 cases of benign prostatic hyperplasia (BPH, 34) and prostate adenocarcinoma (45), including non-neoplastic adjacent tissue and prostatic intraepithelial neoplasia (PIN) lesions were obtained from the archive of the Pathology Service of “Hospital Geral de Santo António”, Oporto. Samples were from

patients subjected to radical prostatectomy between 2010 and 2011 that did not receive preoperative chemotherapy or any other type of treatments.

TMA paraffin-blocks were cut into 2–4 μm sections, which were processed for immunohistochemical analysis. TMAs sections were deparaffinised in xylol and rehydrated through a series of graded ethanol solutions. Heat-induced antigen retrieval was performed in citrate buffer (10 mM, pH 6.0) for 20 min. Then, sections were permeabilized with 1 % Triton X-100 for 15 min at room temperature. The activity of endogenous peroxidases was blocked with 3 % hydrogen peroxyde for 10 min. Phosphate buffer saline (PBS) containing 1 % bovine serum albumin (PBA) and 0.3 M glycine was used to avoid unspecific staining. Sections were incubated overnight at 4 °C with rabbit anti-GPER (1:50, (K-19)-R: sc-48524-R; Santa Cruz Biotechnology (SCBT), Dallas, Texas, USA) primary antibody diluted in PBA, followed by 1 h incubation at room temperature with the goat anti-rabbit IgG biotinylated antibody (diluted 1:200 in PBA). Antibody binding was detected with the ExtrAvidin-Peroxidase reagent (1:20), min and 3,3-diaminobenzidine hydrochloride (Sigma-Aldrich, St. Louis, USA) as a chromogen. TMAs sections were counterstained with Mayer's haematoxylin (Bio-Optica, Milano, Italy), dehydrated, cleared and mounted with Entellan (Coverquick 2000, BDH Prolabo Chemicals, Fontenay-sous-Bois, France). Negative controls were performed by omission of the primary antibody.

The TMAs examination and immunoreactivity scoring were performed by an experienced pathologist. GPER immunoreactivity scores were determined based on the intensity of staining and classified as “1” (low intensity) or “2” (high intensity). GPER immunoreactivity was correlated with clinical and histopathological patient's data, namely, age, histological diagnosis, total PSA levels, percentage of free PSA, Gleason scores, tumour node and metastasis staging, and the presence of bone metastasis.

7.2.2. Cell lines and treatments

The human prostate non-neoplastic epithelial cell line PNT1A, and the human neoplastic cell lines LNCaP, DU145 and PC3 were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The neoplastic cell lines represent metastatic stages of PCa with different degrees of aggressiveness. LNCaP cells are derived from lymph nodules metastasis, are androgen-sensitive, and have low metastatic potential [36, 37]. The DU145 cell line are derived from brain metastasis and have moderate metastatic potential [38], whereas PC3 cells are originated from bone

metastasis and have high metastatic potential [39]. DU145 and PC3 cell lines are androgen-insensitive and represent the stage of CRPC.

PNT1A, LNCaP, DU145 and PC3 cells were maintained in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1 % penicillin/streptomycin (Sigma-Aldrich), at 37 °C in an atmosphere equilibrated with 5 % CO₂. At 60 % confluence, culture medium was replaced by phenol red-free RPMI 1640 medium (Sigma-Aldrich) containing 5 % charcoal-stripped FBS (CS-FBS) (Sigma-Aldrich), and cells were maintained for additional 24 h. Then, cells were exposed to a concentration range of GPER agonist G1 (0.001 µM – 100 µM, Tocris Bioscience, Madrid, Spain), or vehicle for 24 h. A long-time exposure to G1 (1 µM) was performed in the same conditions for 28 days.

Alternatively, PNT1A, LNCaP, DU145 and PC3 cells were also exposed to a normo-physiological concentration (10 nM) of 5α-dihydrotestosterone (DHT, Sigma-Aldrich) [40, 41] or vehicle for 24 and 48 h.

7.2.3. Protein extraction

Human prostate cells were homogenized in the appropriate volume of radioimmunoprecipitation assay buffer (RIPA) (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5 % Na-deoxycholate, 0.1 % SDS, 50 mM Tris, 1 mM EDTA, 1 % protease inhibitor cocktail and 10 % PMSF), and total proteins were recovered after a 14000 rpm centrifugation for 20 min at 4 °C. Protein concentration was determined by the BCA assay (Thermo Fisher, Rockford, USA).

7.2.4. Western blot (WB)

Total proteins were resolved by SDS-PAGE on 12.5 %, 10 %, 7.5 % or 7 % gels and electrotransferred to PVDF membranes (Bio-Rad, Hercules, USA). After blocking with 5% skimmed dried milk for 1 h, membranes were incubated overnight at 4 °C with the appropriate primary antibody (Table 7.1). Goat anti-rabbit IgG-HRP (1:40000, sc:2004; SCBT) or goat anti-mouse IgG-HRP (1:40000, sc:2005; SCBT) were used as secondary antibodies. A mouse anti-α-tubulin monoclonal antibody (1:10000, Sigma-Aldrich) was used for protein loading control in WB analyses of samples treated with G1, and mouse anti-β-actin monoclonal antibody (1:10000, Sigma-Aldrich) was used for samples treated with DHT.

Membranes were incubated with ECL substrate (Bio-Rad) for 5 min, and immuno-reactive proteins were visualized with the ChemiDoc™ MP Imaging System (Bio-Rad). Band densities were obtained using the Image Lab software (Bio-Rad) and normalized with the respective α -tubulin or β -actin band density.

Table 7.1. Primary antibodies and working dilutions used in the WB analysis.

Biological target/ process	Antibody	Host species	Dilution	Catalogue number	Supplier
Hormone receptors	Anti-GPER	Rabbit	1:500	(K-19)-R: sc-48524-R	SCBT
	Anti-GPER	Rabbit	1:250	ab 39742	Abcam, Cambridge, UK
Cell cycle	Anti-Stem Cell Factor (SCF)	Rabbit	1:250	H-189: sc-9132	SCBT
	Anti-c-KIT	Rabbit	1:250	C-19: sc-168	SCBT
	Anti-Phospho-c-Myc (Ser62) (E1J4K)	Rabbit	1:1000	#13748	Cell Signaling Technology, Danvers, MA, USA
	Anti-p53	Rabbit	1:1000	FL-393: sc-6243	SCBT
	Anti-p21	Rabbit	1:1000	C-19: sc-397	SCBT
	Anti-AKT	Rabbit	1:500	# 9272	Cell Signaling
Intracellular signaling	Anti-Phospho-AKT (Ser473)	Rabbit	1:500	# 9271	Cell Signaling
	Anti-p44/42 MAPK (Erk1/2)	Rabbit	1:1000	# 9102	Cell Signaling
	Anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Rabbit	1:500	# 9101	Cell Signaling
	Anti-Bax	Rabbit	1:1000	# 2772	Cell Signaling
Apoptosis	Anti-Bcl-2	Rabbit	1:1000	# 2876	Cell Signaling
	Anti-Caspase-9 p35	Rabbit	1:1000	H-170:sc-8355	SCBT
	Anti-Caspase-8 p18	Mouse	1:100	D-8: sc-5263	SCBT
	Anti-FAS	Rabbit	1:1000	A-20: sc-1023	SCBT

	Anti-FAS-L	Rabbit	1:1000	C-178: sc-6237	SCBT
	Anti-Glucose Transporter GLUT1	Rabbit	1:1000	CBL 242	Millipore, Darmstadt, Germany
	Anti-Glucose Transporter GLUT2	Rabbit	1:1000	H-67: sc-9117	SCBT
	Anti-Glucose Transporter GLUT3	Rabbit	1:1000	H-50: sc-30107	SCBT
	Anti-Phosphofructokinase 1 (PFK1)	Rabbit	1:1000	H-55: sc-67028	SCBT
	Anti-Lactate Dehydrogenase (LDH) [EP1566Y]	Rabbit	1:10000	ab 52488	Abcam
	Anti-Monocarboxylate Transporter 4 (MCT4)	Rabbit	1:1000	H-90: sc-50329	SCBT
	Anti-Glycogen Synthase 2 (GS2)	Mouse	1:200	G-8: sc-390391	SCBT
Metabolism	Anti-Alanine, Serine, Cysteine Transporter 2 (ASCT2) (V501)	Rabbit	1:500	# 5345	Cell Signaling
	Anti-Glutaminase (GLS)	Rabbit	1:1000	ab 93434	Abcam
	Anti-Glutamate Pyruvate Transaminase (GPT) / Alanine Aminotransferases (ALT)	Mouse	1:100	E-3: sc-374501	SCBT
	Anti- Cluster of Differentiation 36 (CD36)	Rabbit	1:400	ab 64014	Abcam
	Anti-Carnitine Palmitoyltransferase 1A (CPT1A) [8F6AE9]	Mouse	1:1000	ab 128568	Abcam
	Anti-Acetyl CoA Carboxylase (ACC)	Rabbit	1:1000	# 3662	Cell Signaling
	Anti-Fatty Acid Synthase (FASN) (C20G5)	Rabbit	1:1000	# 3180	Cell Signaling
	Anti-E-cadherin	Mouse	1:100	G-10: sc-8426	SCBT
EMT	Anti-N-cadherin	Mouse	1:500	13A9: sc-59987	SCBT
	Anti-Vimentin	Mouse	1:1000	V9: sc-6260	SCBT
	Anti-Cytokeratin-8	Rabbit	1:1000	ab 59400	Abcam
	Anti-Cytokeratin-18 [E431-1]	Rabbit	1:10000	ab 32118	Abcam
Housekeeping	Anti- α -Tubulin	Mouse	1:10000	T9026	Sigma-

genes				Aldrich
Anti- β -Actin	Mouse	1:10000	A1978	Sigma-Aldrich

7.2.5. Fluorescent immunocytochemistry

PNT1A, LNCaP, DU145 and PC3 cells were fixed with 4 % paraformaldehyde (PFA) and permeabilized with 1 % Triton X-100 for 5 min at room temperature. Unspecific staining was blocked by incubation with PBS containing 0.1 % Tween-20 (PBS-T) and 20 % FBS for 1 h. Then, cells were incubated with rabbit anti-Ki-67 (1:50, ab16667; Abcam), rabbit anti-GPER (1:50, ab39742; Abcam) or rabbit anti-calnexin (1:50, H-70: sc-11397; SCBT) primary antibodies for 1 h at room temperature. Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 546 goat anti-rabbit IgG were used as secondary antibodies (Invitrogen, Thermo Fisher). For dual-labelling, an additional step of 1 h of blockage was performed between the first staining for calnexin and the second staining with GPER. Specificity of the staining was assessed by the omission of the primary antibodies. Cell nuclei were stained with Hoechst 33342 (5 μ g/mL, Invitrogen, Thermo Fisher) for 10 min. Cell membrane was stained by incubation with wheat germ agglutinin (WGA, 5 μ g/ml, Invitrogen, Thermo Fisher) for 15 min before the permeabilization step. Lamellae were washed with PBS-T and mounted in Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Images were acquired using the Axio Imager A1 microscope (Carl Zeiss, Göttingen, Germany) or a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss).

7.2.6. Cell viability assay

PNT1A (10000 cells/well), LNCaP (15000 cells/well), DU145 (5000 cells/well) and PC3 (5000 cells/well) cells were seeded in 96-well plates and treated with G1 (0, 0.1, 1, 10 and 100 μ M) for 24 h. Cell viability was determined by the colorimetric MTT assay. After G1 treatment, thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich) was added to cell culture medium (0.5 mg/ml), and reaction occurred protected from light at 37 °C for 4 h. After incubation, the medium with MTT was removed and the formed formazan crystals were dissolved in 100 μ L of DMSO (Honeywell, Charlotte, USA). The absorbance of the resultant purple coloured solution was measured at 570

nm, using the xMark™ Spectrophotometer (Bio-Rad). The absorbance value is directly proportional to the number of viable cells in each experimental group.

7.2.7. Cell proliferation analysis

LNCaP, DU145 and PC3 cells counting was performed by nuclei staining with Hoechst 33342 (5 µg/mL) for 10 min. The number of cells in control and G1-treated groups (1 µM) was determined in a total of 10 400x magnification fields per slide. Images were acquired using the Axio Imager A1 microscope (Carl Zeiss).

The proliferation index was assessed by fluorescent immunocytochemistry analysis of the nuclear cell proliferation marker Ki-67 [42]. The percentage of Ki-67-positive cells out of the total number of Hoechst-stained nuclei was determined in 10, randomly selected, 400 x magnification fields in each lamella.

7.2.8. Flow cytometry

Propidium iodide (PI) staining and flow cytometry were used to evaluate cell cycle distribution and the occurrence of apoptotic nuclei. Briefly, LNCaP (1000000 cells / t-flask 25 cm²), DU145 (300000 cells / t-flask 25 cm²) and PC3 (200000 cells / t-flask 25 cm²) cells were treated with G1 as described (2.2). Twenty-four hours after stimulation, adherent viable cells were harvested by trypsin treatment and apoptotic cells collected in the medium. Cell suspension was kept on ice, pelleted, resuspended in 950 µL PBS and fixed with cold 70 % ethanol. Fixed cells were centrifuged, and ethanol solution was removed. For DNA extraction, 800 µL of PBS and 1 mL of DNA extraction buffer (0.2 M sodium phosphate, 0.1 % Triton X-100, pH 7.8) were added to the pellet of cells. After 5 min incubation, cells were centrifuged, the supernatant discarded, and the pellet resuspended in the staining solution (20 µg/mL PI and 0.2 mg/mL of RNase free DNase). A minimum of 20000 stained cells was acquired in a BD Biosciences FACSCalibur flow cytometer, using the 488-nm laser line. Debris and residual necrotic cells were eliminated by gating off the events with a lower diameter (Forward scatter; FSC) and reduced PI fluorescence (Fluorescence channel 3; FL3) than the hypodiploid apoptotic cells. Data analysis was performed with FlowJo (version 10.8.1) software.

7.2.9. Caspase-3 like activity assay

The caspase-3-like activity was determined spectrophotometrically by detecting the presence of the yellow product p-nitro-aniline (pNA), upon cleavage of caspase-3 substrate (Ac-DEVD-pNA). In a 96-well plate, 5 μ L of total protein extracts from PNT1A, LNCaP, DU145 and PC3 cells were mixed with 85 μ L of assay buffer (20mM HEPES, pH 7.4, 2mM EDTA, 0.1 % CHAPS, 5mM DTT) and 200 μ M of Ac-DEVD-pNA (Sigma-Aldrich). The reaction was left to occur overnight at 37 °C and the yellow colour obtained by the release of pNA (Sigma-Aldrich) was measured at 405 nm using the xMark™ Spectrophotometer (Bio-Rad). The amount of generated pNA was calculated by extrapolation with a standard curve. All measurements were normalized to the total μ g of protein in each sample.

7.2.10. Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL)

TUNEL analysis was performed using the *In Situ* Cell Death Detection Kit, TMR red (Roche, Basel, Switzerland) following the manufacturer's instructions. Briefly, cells were washed with PBS, fixed with 4 % paraformaldehyde (PFA), and permeabilized with 0.1 % Triton X-100 in 0.1 % sodium citrate for 2 min in ice. Afterwards, cells were incubated in a humidified atmosphere with TUNEL reaction mixture (enzyme solution equilibrated in label solution), for 1 h at 37 °C, protected from the light. Then, fixed cells were washed with PBS and cell nuclei stained with Hoechst 33342 (5 μ g/mL) for 10 min. Cells were then washed and mounted in Dako fluorescent mounting medium (Dako). Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss). The apoptosis index was determined by the percentage of TUNEL-positive cells out of the total number of Hoechst-stained nuclei in 10, randomly selected, 400 x magnification fields in each lamella.

7.2.11. Transwell migration and invasion assays

Migration and invasion assays were performed in 24-well plates, using, respectively, uncoated (SPL Life Sciences, Gyeonggi-do, Korea) or Matrigel-coated (Corning, New York, USA) chambers with 8 μ m pore-size membranes. Briefly, one day before the assays, cell culture medium was replaced by phenol red-free RPMI 1640

medium containing 5 % CS-FBS. Cell suspensions were prepared in serum free medium and $1-3 \times 10^5$ cells were placed in the upper transwell chamber. Culture medium with 10 % FBS (750 μ L) was added to the lower chamber as the chemoattractant.

Cells were maintained in an incubator at 37 °C with 5% CO₂ for 24 h. After that, cells in the top chamber were removed, using a cotton bud moistened in serum free medium. Migrated and invaded cells at the bottom of membranes were fixed with 4 % PFA for 10 min, and the nuclei stained with Hoechst 33342 (5 μ g/mL). The number of migrated and invaded cells was counted in 5, randomly selected, 400x magnification fields using the Axio Imager A1 microscope (Carl Zeiss). Representative images of migrated or invaded cells were acquired using the Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss).

7.2.12. Quantification of glucose and lactate

The concentration of glucose and lactate in the culture medium of untreated and G1-treated cells was assessed by means of spectrophotometric analysis using commercial kits (Spinreact, Girona, Spain) following the manufacturers' instructions. For glucose and lactate quantification, 1 μ L of cell culture medium of each sample was placed in a 96-well plate and mixed with 100 μ L of kit reaction buffer. The plate was incubated at 37 °C for 10 min, and the absorbance measured at 505 nm (xMark™ Spectrophotometer, Bio-Rad). Glucose consumption and lactate production after 24 h of treatment were calculated relatively to the initial concentration of these metabolites at 0 h of stimulation.

7.2.13. Lactate dehydrogenase (LDH) enzymatic activity assay

The enzymatic activity of LDH was measured using a commercial kit (Spinreact), following the manufacturers' instructions. LDH catalyses the reduction of pyruvate to lactate with the oxidation of NADH to NAD⁺, so, the activity of LDH in the sample is proportional to the rate of decrease in NADH concentration, which was measured spectrophotometrically at 340 nm using the xMark™ Spectrophotometer (Bio-Rad). 1 μ L of cells protein extract was added to 150 μ L of a kit reagent in a 96-well plate and incubated for 1 min at 37 °C. The initial absorbance was acquired, followed by subsequent readings every minute for 3 min. LDH activity was calculated by

normalizing values to the total protein concentration in each sample (U/L/ μ g of protein).

7.2.14. Glutathione peroxidase (GPX) activity assay

GPX activity was determined using a commercial kit (703102; Cayman Chemical, Michigan, USA) according to the manufacturer's protocol. Enzymatic activity was measured indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione, produced upon reduction of hydroperoxide by GPX, is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP⁺, catalysed by glutathione reductase, is accompanied by a decrease in the absorbance at 340 nm (A₃₄₀), which is directly proportional to the GPX activity if the GPX activity is rate limiting. In brief, 10 μ L of protein extract were pipetted into a 96-well microplate (containing 25 μ L of assay buffer, 25 μ L of co-substrate mixture and 25 μ L of NADPH) and the decrease in the A₃₄₀ was read at 1 min intervals for 5 min in the xMark™ Spectrophotometer (Bio-Rad). The rate of Δ A₃₄₀ per minute was used to calculate GPX activity. Results were expressed as nmol/min/mL/ μ g total protein.

7.2.15. Superoxide dismutase (SOD) activity assay

SOD activity was assessed using the commercial kit SOD Assay Kit (19160-1KT-F; Sigma-Aldrich) following the manufacturer's instructions. Briefly, this kit uses the highly water-soluble tetrazolium salt, WST-1 that produces a water-soluble formazan dye upon reduction with the superoxide anion. The rate of formazan reduction is linearly related with the activity of xanthine oxidase and inhibited by SOD. Thus, the percentage of reaction inhibition rate indicates the amount of SOD activity, which was monitored by measurement of absorbance at 450 nm in the xMark™ Spectrophotometer (Bio-Rad). The amount of protein extract used was 5 μ L into a 96-well microplate (containing 100 μ L of WST working solution plus 5 μ L of enzyme working solution). The reduction reaction occurred at 37 °C for 20 min. Results were expressed as a proportion of activity (percentage of inhibition) relative to the control group.

7.2.16. Total oxidant status (TOS) analysis

TOS was determined using a commercial kit (RLo024, Rel Assay Diagnostics, Gaziantep, Turkey). The principle of the assay is that oxidants present in the sample oxidize the ferrous ion-chelator complex to ferric ion. The ferric ion produces a coloured complex with chromogen in an acidic medium. The colour intensity, measured spectrophotometrically, is associated with the total amount of oxidant molecules present in the sample. Briefly, 7.5 μL of sample or standard (H_2O_2) were pipetted into a 96-well microplate. Then, sample (or standard) and buffer solution were mixed and the initial absorbance at 530 nm was read after 30 s in the xMark™ Spectrophotometer (Bio-Rad). The reaction was incubated for 5 min at 37 °C and the final absorbance was read at 530 nm. Results were expressed in $\mu\text{mol H}_2\text{O}_2 \text{ Equiv./L}$.

7.2.17. Statistical analysis

In the case of GPER expression in TMAs, statistical analysis was performed using IBM SPSS (v23.0 IBM Corp, Armonk, USA). Pearson chi-square and Fisher exact tests were used to determine the correlation between GPER immunoreactivity and individual variables of clinical-pathological data.

For the *in vitro* analysis, statistical significance of differences between experimental groups was evaluated by unpaired T-test with the appropriate correction or one-way ANOVA, followed by the adequate post-test, using GraphPad Prism v6.01 (GraphPad Software, Inc., La Jolla, USA).

$P < 0.05$ was considered statistically significant. All experimental data are shown as mean $\pm$ standard error of the mean (SEM).

7.3. Results

7.3.1. GPER immunoreactivity is higher in human PCa and correlated with total PSA levels

As a basis before exploring GPER actions modulating the behaviour of PCa cells *in vitro*, we evaluated the receptor expression pattern in human PCa cases. TMAs were used and GPER immunoreactivity scores divided into low (score 1) or high (score 2), based on the intensity of staining. GPER expression levels were higher in PCa

comparatively to the BPH cases ($P < 0.001$, Table 7.2). Though, no significant differences were found between the GPER immunoreactivity in BPH and PIN ($P = 0.271$) or PIN and adenocarcinoma ($P = 0.412$). In fact, 40% of the adenocarcinoma specimens showed high GPER immunoreactivity, contrasting with only 22.22% of PIN lesions that showed high immunoreactivity, and all the cases of BPH that displayed low immunoreactivity for GPER (Table 7.2).

Table 7.2. Association of GPER expression with clinical and histopathological data in benign prostate hyperplasia and prostate cancer cases

Clinical and histopathological data	GPER immunoreactivity		<i>p</i> value ^a
	Low (score 1)	High (score 2)	
Age			
< 55	77.78% (7/9)	22.22% (2/9)	0.618
55-65	71.43% (25/35)	28.57% (10/35)	
> 65	81.40% (35/43)	18.60% (8/43)	
Diagnosis			
BPH	100.00% (34/34)	0.00% (0/34)	0.000244
PIN	77.78% (7/9)	22.22% (2/9)	
Adenocarcinoma	60.00% (27/45)	40.00% (18/45)	
Total PSA (ng/ml)			
≤ 4	20.00% (1/5)	80.00% (4/5)	0.047
> 4	73.68% (14/19)	26.32% (5/19)	
Free PSA (%)			
≤ 10	44.44% (4/9)	55.56% (5/9)	0.637
> 10	66.67% (6/9)	33.33% (3/9)	
Gleason			
< 7	66.67% (8/12)	33.33% (4/12)	0.735
≥ 7	57.58% (19/33)	42.42% (14/33)	
TNM			
T2	65.00% (13/20)	35.00% (7/20)	1.000
T3	57.14% (4/7)	42.85% (3/7)	
Bone metastasis			
Absent	68.75% (11/16)	31.25% (5/16)	1.000
Present	50.00% (1/2)	50.00% (1/2)	

^aPearson Chi-square correlation; TNM - tumour, node and metastasis (*TNM*) staging

Representative images of immunohistochemical analysis showing GPER high immunoreactivity in BPH, PIN lesions and adenocarcinoma of prostate are shown in Fig. 7.1. BPH sections displayed the normal glandular tissue, with glands structure surrounded by stroma. GPER immunoreactivity in BPH was observed in both epithelial

and stroma cells (Fig. 7.1). GPER immunoreactivity was maintained in the stroma compartment and luminal epithelial cells of prostate specimens with PIN (Fig. 7.1). In PCa adenocarcinoma, a different cell arrangement of tumor cells can be observed, with a strong immunoreactivity for GPER (Fig. 7.1).

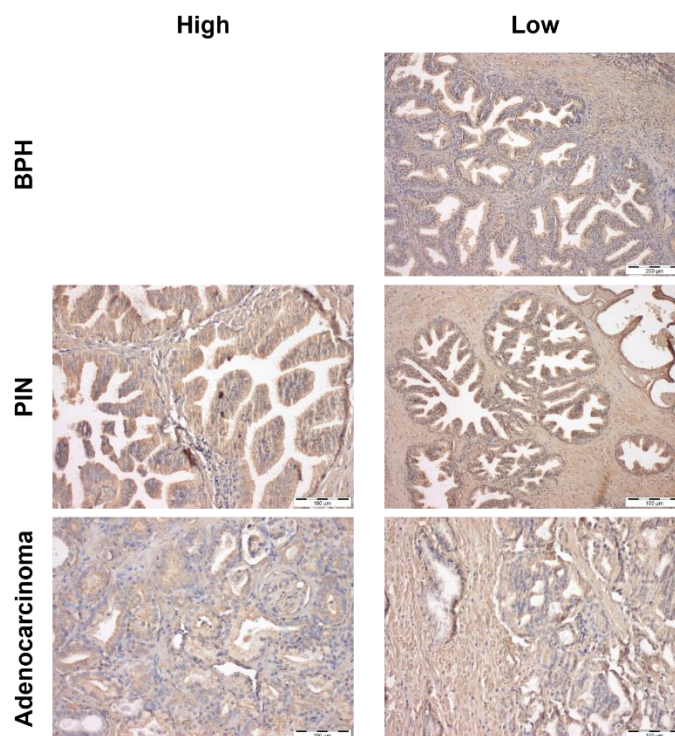


Figure 6.1. Representative images of GPER high vs. low immunoreactivity in human benign prostate hyperplasia (BPH), prostatic intraepithelial (PIN) lesions and prostate adenocarcinoma. Haematoxylin-eosin counterstaining magnification 40X. Scale bar 200 μ m for BPH and 100 μ m for PIN and adenocarcinoma.

The correlation of GPER immunoreactivity with clinical and histopathological data (Table 7.2), such as age, total prostatic-specific antigen (PSA), percentage of free PSA, Gleason score, pathologic stage and the presence of bone metastasis, was determined. GPER immunoreactivity was associated with the total PSA levels ($P < 0.05$, Table 7.2). 80 % of cases with 4 or less ng/ml PSA showed high GPER immunoreactivity, contrastingly with only 26.32 % of the cases with PSA levels higher than 4 ng/ml (Table 7.2).

7.3.2. GPER expression pattern and subcellular localization in human prostate cells

After confirming GPER expression in PCa human samples, and before proceeding with the *in vitro* studies to investigate the GPER role modulating PCa cells behaviour, we aimed to characterize the receptor expression in the prostate cell line models being used. Non-neoplastic (PNT1A), and neoplastic (LNCaP, DU145 and PC3) cell lines, representing different stages of PCa were used. LNCaP cells are known to be sensitive to androgens, whereas DU145 and PC3 cells are CRPC cells, mimicking more aggressive stages of disease. LNCaP cells displayed the highest expression of GPER protein. GPER expression is significantly higher in LNCaP cells compared with both non-neoplastic PNT1A, and neoplastic DU145 and PC3 cells (Fig. 7.2A and 7.2B). GPER expression in DU145 and PC3 cells resembled that of PNT1A cells (Fig. 7.2A and 7.2B). Immunofluorescence cytochemistry confirmed GPER expression in PNT1A, LNCaP, DU145 and PC3 cells (Fig. 7.2C), with the green fluorescence intensity indicating the higher expression levels in LNCaP cells.

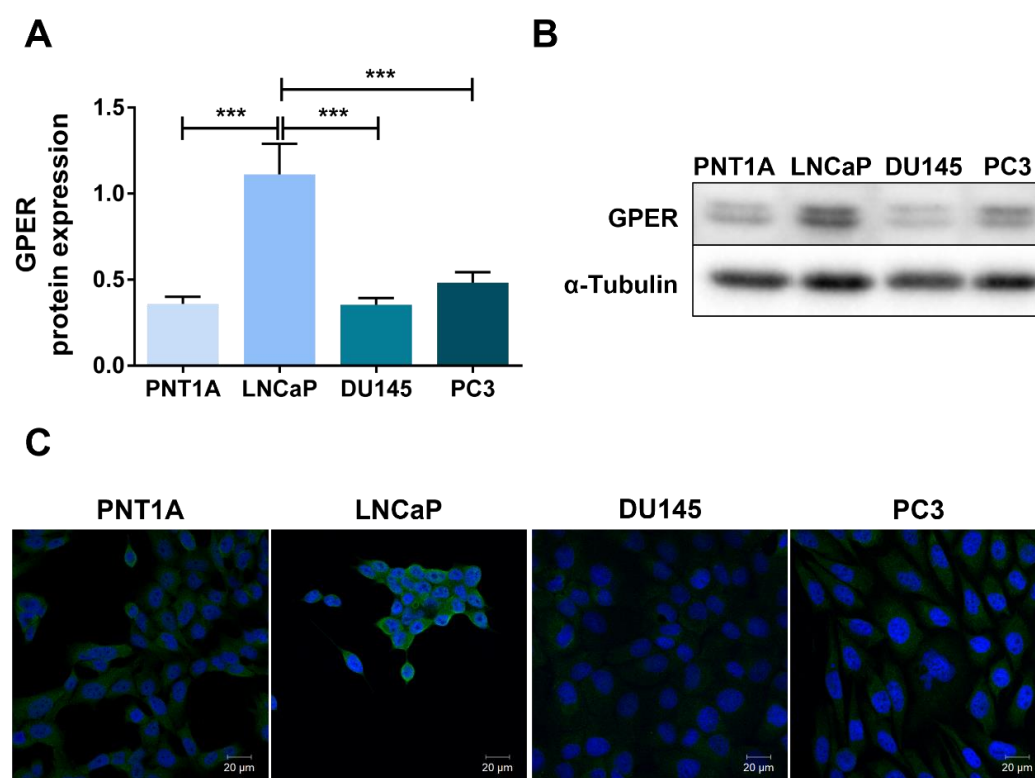


Figure 7.2. GPER expression in non-neoplastic PNT1A and neoplastic LNCaP, DU145 and PC3 human prostate cells. (A) Protein expression determined by WB analysis after normalization with α -tubulin. Error bars indicate mean $\pm$ S.E.M. *** $P < 0.001$; (B) Representative immunoblots; (C) Representative images of GPER subcellular localization obtained with a Zeiss LSM 710 laser scanning confocal microscope under 400 X magnification. Nucleus are stained blue and GPER green.

GPER has been reported to be localized in the endoplasmic reticulum and cell nucleus in different cell types [43, 44]. This prompted us to investigate the GPER subcellular distribution in human prostate cells using fluorescent immunocytochemistry to determine its co-localization with WGA, a specific marker of cell membrane; calnexin, an endoplasmic reticulum marker and Hoechst, a nuclei staining dye. Immunofluorescence cytochemistry confirmed that GPER was mainly localized in the endoplasmic reticulum in all prostate cells (average Pearson's coefficient of 0.429, 0.355, 0.430 and 0.357 for PNT1A, LNCaP, DU145 and PC3 cells, respectively, Fig. 7.3A and 7.3C). GPER also was present at the cell membrane, but in less extension (average Pearson's coefficient of 0.165, 0.119, 0.092 and 0.123 for PNT1A, LNCaP, DU145 and PC3 cells, respectively, Fig. 7.3A and 7.3D). Residual GPER staining was observed in the nuclei of non-neoplastic and neoplastic cells (Fig. 7.3C and 7.3D).

It has been described that GPER subcellular location can be modified by estrogen ligands binding and receptor activation. Therefore, we tested whether G1 would affect GPER distribution within cell compartments. G1-treatment increased GPER localization at cell membrane in PNT1A and DU145 (1.53 ± 0.11 and 1.69 ± 0.04 fold-change relative to control, respectively), but no changes were observed in LNCaP and PC3 cells (Fig. 7.3B and 7.3C). G1 also increased GPER localization at the endoplasmic reticulum in PC3 cells (1.38 ± 0.04 fold-change relative to control, Fig. 7.3B and 7.3D). However, G1-treated PNT1A cells displayed decreased location of GPER at the endoplasmic reticulum (0.81 ± 0.04 fold-change relative to control, Fig. 7.3B and 7.3D). G1-treatment did not change the endoplasmic reticulum localization of GPER in LNCaP and DU145 cells (Fig. 7.3B and 7.3D). No apparent alteration was observed in GPER staining in the nuclei in response to G1 treatment in any cell line (Fig. 7.3C).

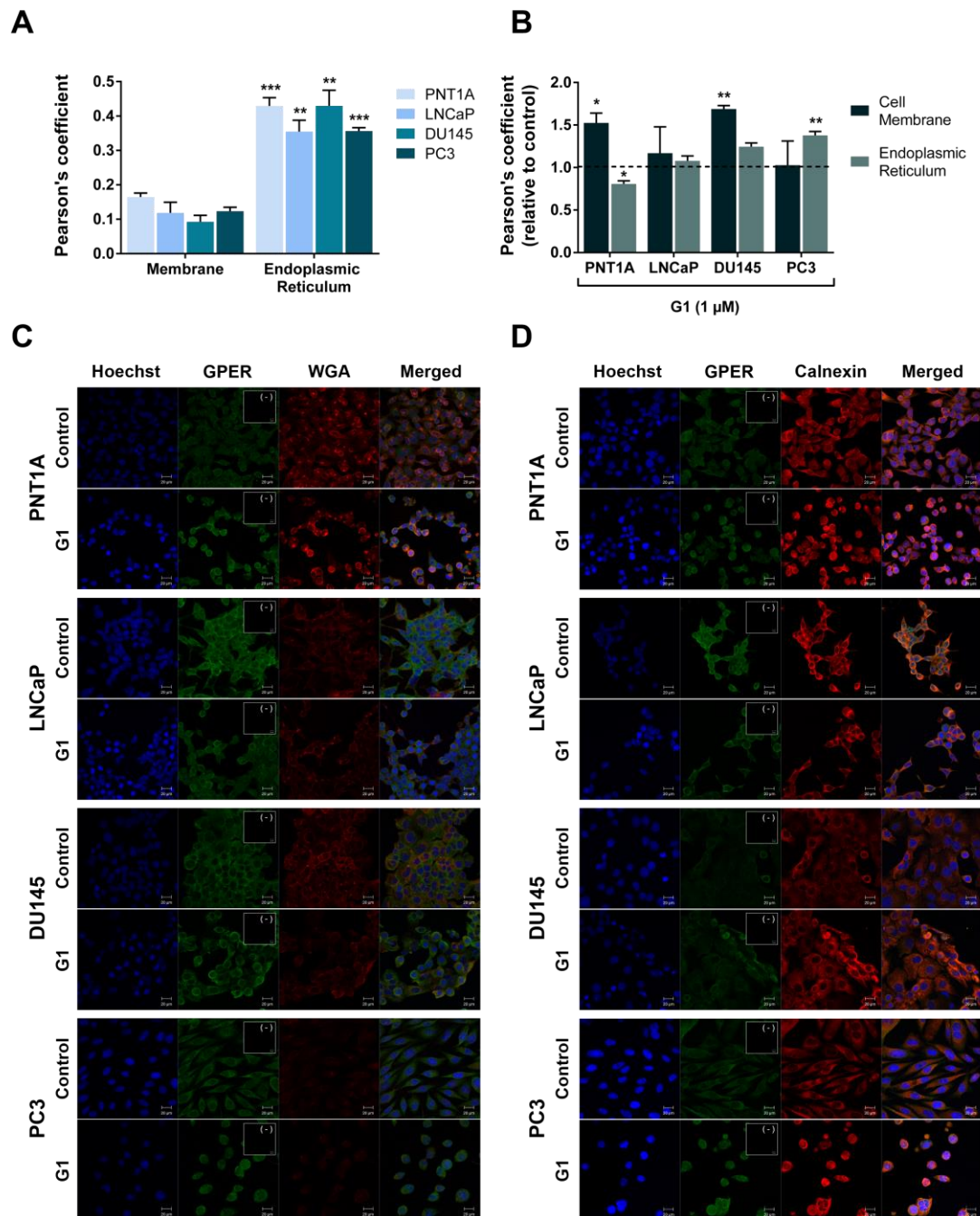


Figure 7.3. GPER subcellular localization in non-neoplastic PNT1A and neoplastic LNCaP, DU145 and PC3 human prostate cells. (A) Pearson's correlation coefficient for the GPER co-localization with cell membrane and endoplasmic reticulum. (B) Pearson's correlation coefficient for the GPER co-localization with cell membrane and endoplasmic reticulum after GPER activation by G1 (1 μ M). Results are expressed as fold-change relative to the control untreated group (0 μ M G1, dashed line). Error bars indicate mean $\pm$ S.E.M. * P < 0.05 ** P < 0.01. (C, D) Representative confocal microscopy images showing the co-localization of GPER (green) with cell membrane (WGA in red), endoplasmic reticulum (calnexin in red) or nucleus (Hoechst in blue) in control and G1-treated (1 μ M) cells. Images were obtained in the Zeiss LSM 710 laser scanning confocal microscope under 400 X magnification. Negative controls obtained by omission of the primary antibody or WGA are provided as insert panels (-).

7.3.3. Androgens modulated GPER protein expression levels in prostate cells

The differential expression of GPER in CRPC and androgen-sensitive LNCaP cells suggests it could be a target of regulation by androgens. Therefore, non-neoplastic PNT1A and neoplastic LNCaP, DU145 and PC3 cells were treated with DHT 10 nM for 24 and 48 h, and GPER protein expression analysed by WB. DHT-treatment diminished GPER protein levels in PNT1A (0.64 ± 0.08 fold-change relative to control, 24 h) and LNCaP (0.78 ± 0.05 variation to control, 48 h) cells, though no changes were observed in the DU145 and PC3 cells (Fig. 7.4).

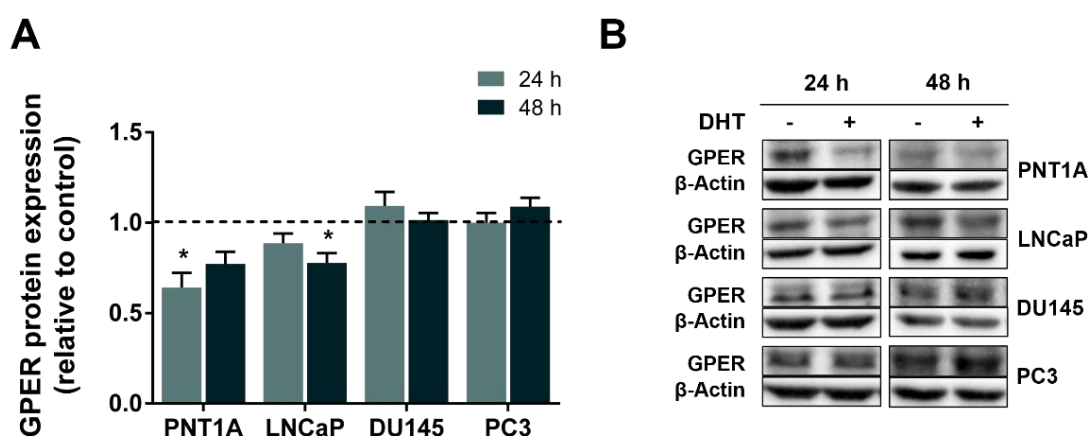


Figure 7.4. Effect of androgens in regulating GPER expression in LNCaP, DU145 and PC3 human PCa cells. (A) GPER protein expression after treatment with DHT (10 nM) for 24 or 48 h. Protein expression was determined by WB analysis after normalization with β -actin. Results are expressed as fold-change relative to the control untreated group (0 nM DHT, dashed line). Error bars indicate mean $\pm$ S.E.M. * $P < 0.05$. (B) Representative immunoblots.

7.3.4. G1-treatment decreased the viability and the proliferative behavior of PCa cells

To start evaluating the role of GPER in modulating prostate cells fate, PNT1A, LNCaP, DU145 and PC3 cells were exposed to a concentration range of GPER specific agonist G1 for 24 h, and cell viability analysed (Fig. 7.5). The highest G1 concentration used (100 μ M) diminished the viability of all PCa cells by approximately 22, 66 and 81 % relative to control in LNCaP, PC3 and DU145, respectively (Fig. 7.5). In DU145 cells, for concentrations higher than 0,1 μ M, G1 effects were concentration-dependent (Fig. 7.5).

100 μ M G1 also diminished the viability of non-neoplastic PNT1A cells, though lower concentrations (0.1 and 1 μ M) have significantly increased viability when compared to the control group (approximately 29 and 23 % increase, respectively, Fig. 7.5).

1 μ M G1 was the concentration selected for use in the subsequent experiments, as it reduced DU145 and PC3 cells viability by 50 %, and is a concentration reported to have biological effects in several cancer cell line models [45, 46].

In parallel with decreasing cell viability, G1-exposure significantly decreased the number of PCa cells (Fig. 7.6A). Accordingly, the proliferation index, obtained by immunocytochemistry analysis of the Ki67 nuclear proliferation marker, was significantly reduced in LNCaP, DU145 and PC3 G1-treated cells (0.88 ± 0.01 , 0.76 ± 0.05 and 0.86 ± 0.02 fold-change relative to control, Fig. 7.6B and 7.6C). Representative images of immunofluorescence staining (Fig. 7.6C) suggest higher Ki67 staining in some LNCaP, DU145 and PC3 cells after G1-treatment (Fig. 7.6C).

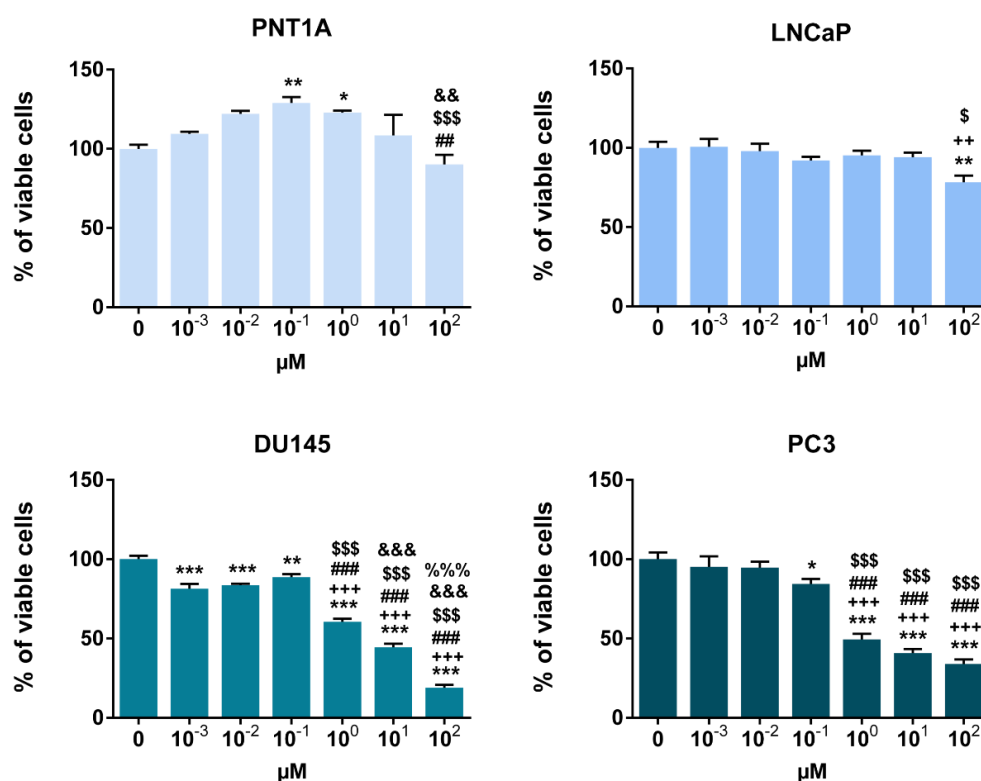
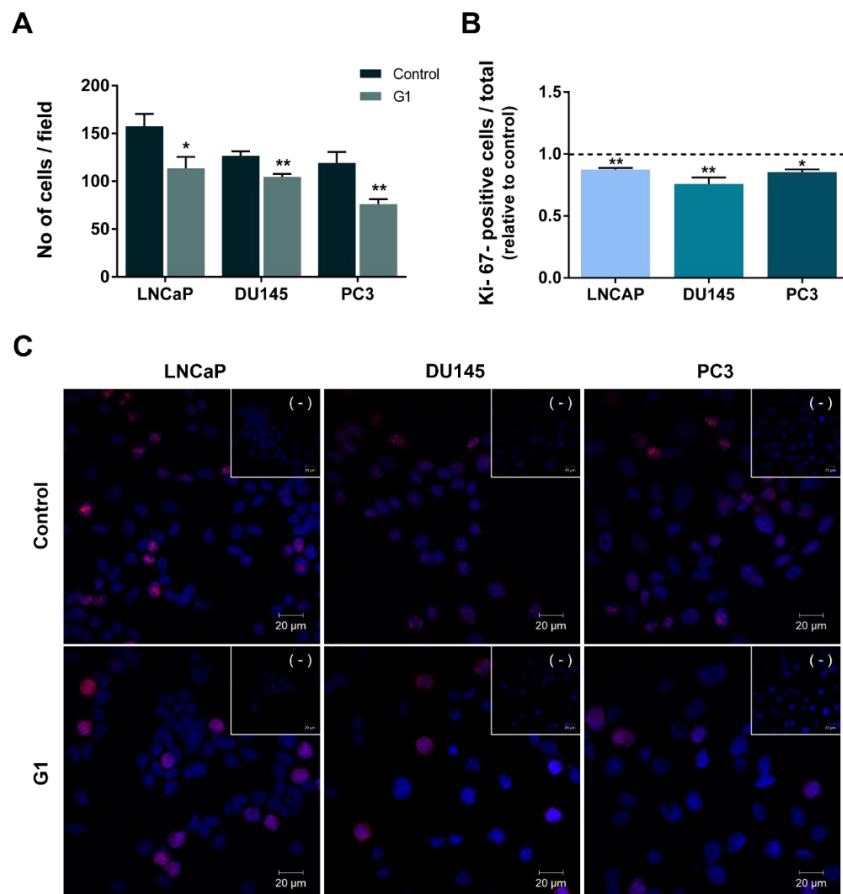


Figure 7.5. Viability of non-neoplastic PNT1A and neoplastic, LNCaP, DU145 and PC3 human prostate cells in response to G1. Cells were treated with a range of G1 concentrations (0 μ M – 100 μ M) for 24 h. Results are expressed as % of control (0 μ M G1). Error bars indicate mean $\pm$ S.E.M. * P < 0.05 ** P < 0.01 *** P < 0.001 relative to 0 μ M; ++ P < 0.01 +++ P < 0.001 relative to 0.001 μ M; ## P < 0.01 ### P < 0.001 relative to 0.01 μ M; \$ P < 0.05 \$\$\$ P < 0.001 relative to 0.1 μ M; &&& P < 0.001 relative to 1 μ M; %%% P < 0.001 relative to 10 μ M.

Flow cytometry was used to analyse cell cycle distribution in control and G1-treated cells. G1 stimulation significantly increased the percentage of PCa cells in the G2/M-phase population; 23 %, 17 % and 9 % in LNCaP, DU145 and PC3, respectively (Fig. 7.6D).

G1 effects influencing the proliferative behaviour of PCa cells were accompanied by the altered expression of oncogenes and regulators of cell cycle and proliferation. We analysed the expression of the proliferation driver and oncogene receptor tyrosine kinase c-KIT and its ligand, SCF (Fig. 7.7A). The c-KIT receptor was decreased in DU145 and LNCaP cells in response to G1-treatment (0.88 ± 0.02 and 0.75 ± 0.08 fold-change relative to control, respectively, Fig. 7.7A) with no alterations observed in SCF expression. In PC3 cells, G1-treatment increased the expression of SCF in (1.29 ± 0.08 fold-change relative to control, Fig. 7.7A), with no alteration on c-KIT expression levels. Regarding other cell cycle regulators and oncogenes, we focused on p21 and p-cMyc as its relevance in PCa has been demonstrated [47]. Exposure to GPER agonist markedly increased p-cMyc expression in PCa cells (2.35 ± 0.15 , 4.95 ± 0.94 , and 13.38 ± 1.12 fold-change relative to control in DU145, LNCaP and PC3, respectively, Fig. 7.7B). None of the cell lines displayed altered expression of the cell cycle inhibitor p21 protein in response to G1 treatment (Fig. 7.7B).



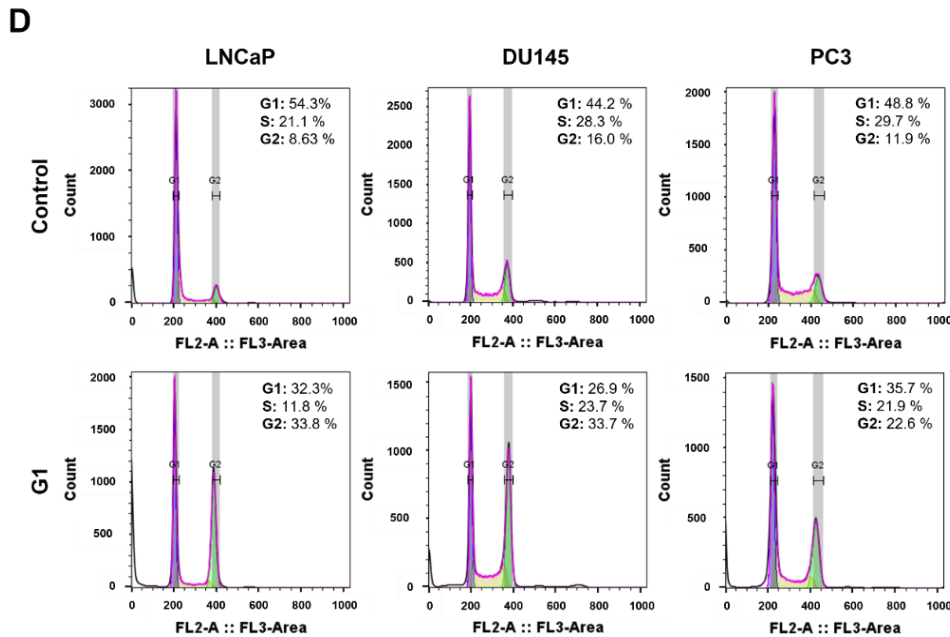


Figure 7.6. Effect of G1 on LNCaP, DU145 and PC3 human PCa cell fate. Cells were treated with 1 μ M G1 for 24 hours. (A) Cell number determined through counting Hoechst-stained nuclei. (B) Percentage of Ki67-positive cells relative to the total cell number (Hoechst stained nuclei). A total of 10 400x magnification fields per slide was assessed. Results are expressed as fold-change relative to the control untreated group (0 μ M G1, dashed line). Error bars indicate mean $\pm$ S.E.M. * P < 0.05 ** P < 0.01. (C) Representative confocal microscopy images showing Ki67 labelling in control and G1-treated cells. Images were obtained in the Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany) under 400 X magnification. Nuclei are stained with Hoechst 33342 (blue) and Ki67 positive staining is red. Negative controls obtained by omission of the primary antibody are included as insert panels. (D) Representative flow cytometry histograms and respective percentage of cells. The number of events were counted according to the DNA content, by propidium iodide (PI) staining. Cell cycle analysis was processed in FlowJo.

Concerning the intracellular signalling pathways that would be involved in cell response to G1, we evaluated the AKT and MAPK / ERK pathways (Fig. 7.7C). Upon exposure to G1, LNCaP cells displayed increased expression of p-AKT (2.62 ± 0.45 fold-change relative to control, Fig. 7.7C), though p-MAPK was diminished (0.76 ± 0.02 fold-change relative to control, Fig. 7.7C). p-AKT and p-MAPK expression levels were not significantly altered in DU145 and PC3 cells in response to G1 (Fig. 7.7C and 7.7D).

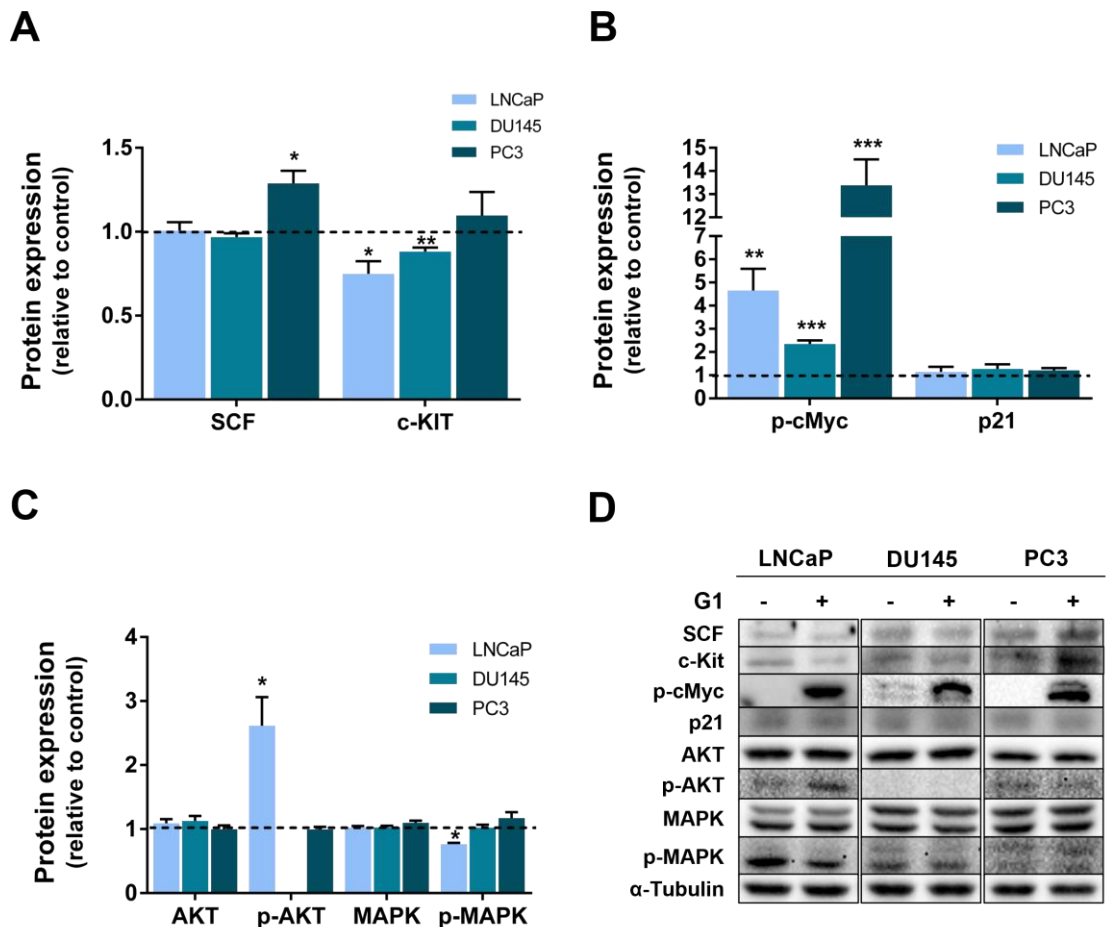


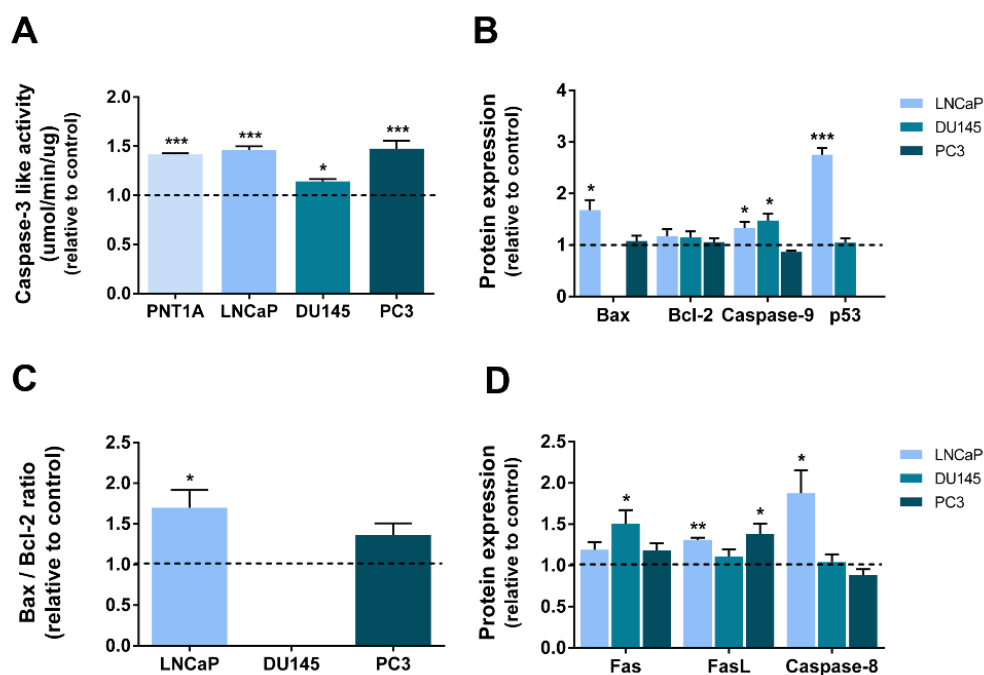
Figure 7.7. Effect of G1 on the expression of cell cycle regulators and intracellular signaling players in LNCaP, DU145 and PC3 human PCa cells. Cells were treated with 1 μ M G1 for 24 hours. Protein expression of (A) SCF ligand and receptor tyrosine kinase c-KIT, (B) p-cMyc and p21, and (C) AKT, p-AKT, MAPK and p-MAPK determined by WB analysis after normalization with α -tubulin. Results are expressed as fold-change relative to the control untreated group (0 μ M G1, dashed line). Error bars indicate mean $\pm$ S.E.M. * P < 0.05; ** P < 0.001 *** P < 0.01. (D) Representative immunoblots.

7.3.5. GPER activation induced apoptosis of PCa cells

Caspase-3 like activity was determined in PNT1A, LNCaP, DU145 and PC3 G1-treated cells as an indication measure of apoptosis. All cell lines showed augmented caspase-3 like activity in response to GPER activation by G1 (1.42 ± 0.01 , 1.46 ± 0.04 , 1.14 ± 0.02 , and 1.47 ± 0.08 fold-change relative to control in PNT1A, LNCaP, DU145 and PC3 cells, respectively, Fig. 7.8A). Also, altered expression of regulators of both the intrinsic and extrinsic pathways of apoptosis was observed in response to G1 treatment. The expression of the mitochondrial pro-apoptotic protein Bax was increased in LNCaP-treated cells (1.68 ± 0.19 fold-change relative to control, Fig. 7.8B), with no change perceived on the Bcl-2 (anti-apoptotic) levels (Fig. 7.8B). Accordingly, Bax/Bcl-

2 ratio was significantly increased in LNCaP-treated cells compared with the control group (1.7 fold-increase, Fig. 7.8C). Bax and Bcl-2 expression and Bax/Bcl-2 ratio remained unchanged in response to G1 treatment in DU145 (not expressing Bax) and PC3 cells (Fig. 7.8B and 7.8C). Expression levels of the initiator caspase-9 were increased in LNCaP and DU145 cells upon exposure to G1 (1.33 ± 0.12 and 1.48 ± 0.13 fold change to control, Fig. 7.8B). p53 expression was increased after G1 treatment in LNCaP cells only (2.75 ± 0.13 fold-change relative to control, respectively, Fig. 7.8B). No change was perceived in DU145, and PC3 cells are known to be p53 negative [48]. G1 also enhanced the expression of the initiator caspase of the extrinsic pathway of apoptosis; caspase-8 in LNCaP cells (1.88 ± 0.28 fold-change relative to control, Fig. 7.8D). Stimulation with the GPER agonist augmented the expression of Fas receptor in DU145 cells (1.51 ± 0.17 fold-change relative to control) and FasL ligand in LNCaP (1.31 ± 0.02 fold change relative to control) and PC3 cells (1.39 ± 0.12 fold-change relative to control, Fig. 7.8D).

Results of the TUNEL assay (Fig. 7.8F and 7.8G) showed that DNA fragmentation was significantly enhanced in G1-treated cells compared with the respective controls (7.7 ± 0.07 , 1.60 ± 0.12 and 3.89 ± 0.59 fold-change in LNCaP, DU145 and PC3 cells, respectively, Fig. 7.8F). Moreover, flow cytometry results confirmed that the sub-G1 populations of LNCaP, DU145 and PC3 cells were augmented by G1 treatment in, approximately, 11 %, 9 % and 8 %, respectively (Fig. 7.6D).



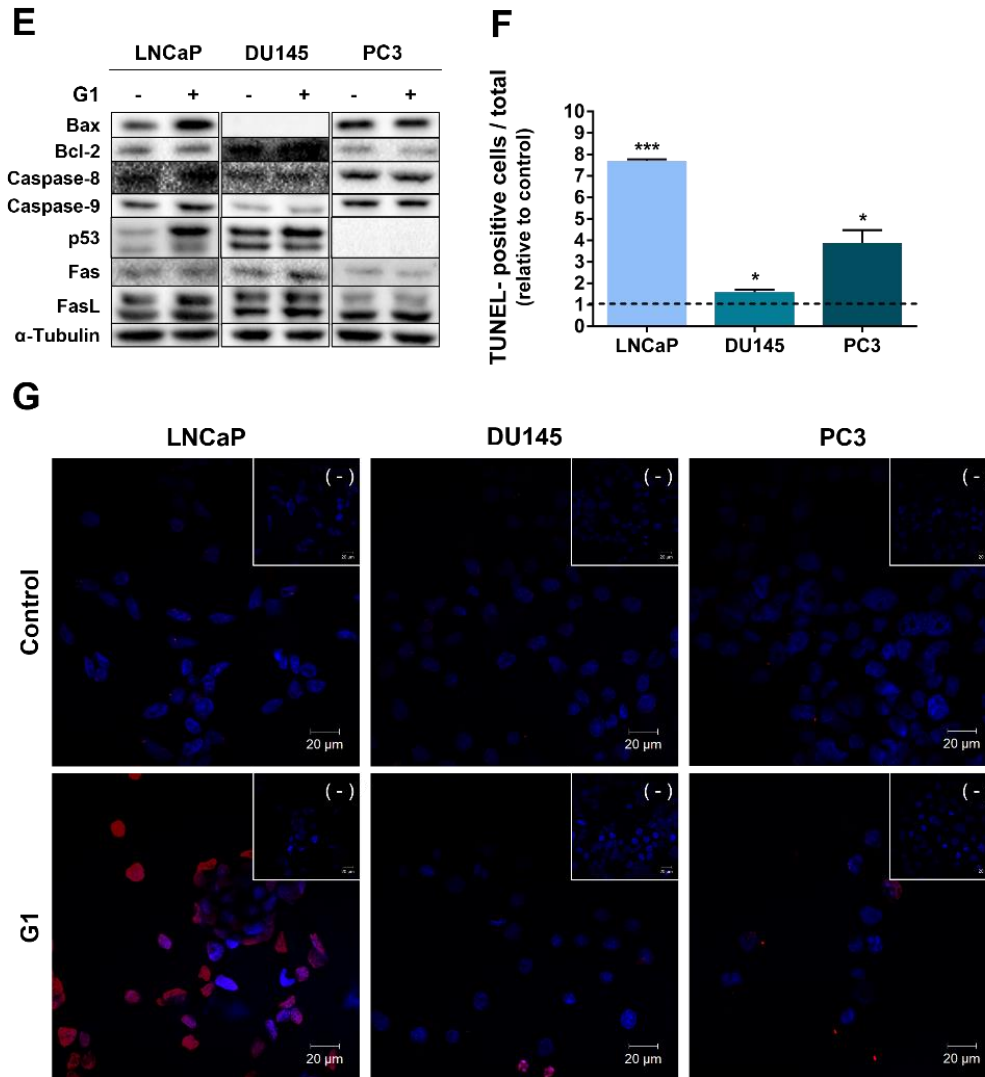


Figure 7.8. Effect of G1 in LNCaP, DU145 and PC3 human Pca cells apoptosis. Cells were treated with 1 μ M G1 for 24 hours. (A) Caspase-3 like activity determined spectrophotometrically. (B-D) Protein expression of apoptosis regulators of the (B, C) intrinsic and (D) extrinsic pathways of apoptosis determined by WB analysis after normalization with α -tubulin. (E) Representative immunoblots. (F) Percentage of TUNEL-positive cells relative to the total cell number (Hoechst stained nuclei). A total of 10 400 X magnification fields per slide was assessed. Results (A-D, F) are expressed as fold-change relative to the control untreated group (0 μ M G1, dashed line). Error bars indicate mean $\pm$ S.E.M. * P < 0.05 ** P < 0.01 *** P < 0.001. (G) Representative fluorescence microscopy images showing TUNEL labelling in control and G1-treated cells. Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany) under 400 X magnification. Nuclei are stained with Hoechst 33342 (blue) and TUNEL positive staining is red. Negative controls for TUNEL were performed following the manufacturer's instructions.

7.3.6. Migration and invasion of PCa cells were shaped by GPER

G1-treatment significantly reduced DU145 cells migration and invasion in about 30 and 42 % relative to control untreated cells (Fig. 7.9A and 7.9B). In contrast, G1-treated PC3 cells showed increased invasion in about 19 % compared with control, though no statistically significant differences were observed on migration (Fig. 7.9A and 7.9B). LNCaP cells migration and invasion ability was not changed by G1 (Fig. 7.9A and 7.9B).

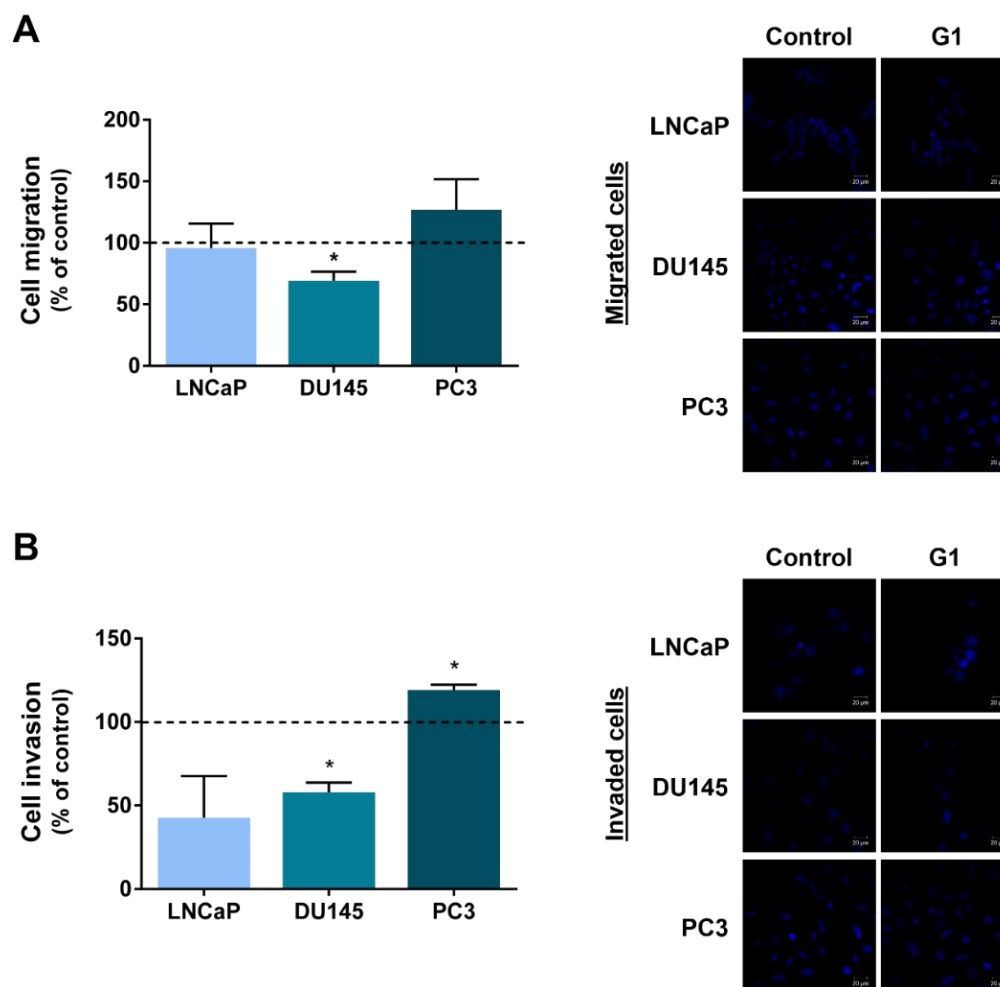


Figure 7.9. Effect of G1 in LNCaP, DU145 and PC3 human PCa cells migration and invasion.

(A) Cell migration and (B) Cell invasion determined by trans-wells assays in uncoated and Matrigel-coated chambers, respectively. The upper chambers contained serum free medium and cells in the presence or absence of G1 (1 μM). FBS-complete medium in the lower chambers was used as chemoattractant. Cells were stained and the number of migrated or invaded cells per 400 X magnification field (5 fields were assessed for each experimental condition) after 24 hours was counted. Results are expressed as % of the control untreated group (0 μM G1, dashed line). Error bars indicate mean ± S.E.M. * $P < 0.05$. Right panels show representative fluorescence microscopy images of migrated and invaded cells in control and G1-treated (1 μM) cells. Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany) under 400 X magnification. Nuclei are stained with Hoechst 33342.

7.3.7. GPER activation modulated the expression of epithelial and mesenchymal markers in PCa cells

CRPC cell lines DU145 and PC3 were long-term exposed to 1 μ M G1 and the expression of epithelial and mesenchymal markers was evaluated at different time points, after 1, 7, 14 and 28 days of treatment. The long exposure to G1 increased E-cadherin expression since the first day in both DU145 and PC3 cells (Fig. 7.10A and 7.10B), being this alteration more evident at the beginning of stimulus in PC3 cells (Fig. 7.10B). A huge augment was verified in the cytokeratin 8 expression by DU145 cells exposed to G1 (Fig. 7.10A), and a less evident increase was verified until 2 weeks of PC3 cells treatment with G1 (Fig. 7.10B). In these cells, after 14 days of exposure, the increase in cytokeratin 8 expression was lost (Fig. 7.10B). Cytokeratin 18 also showed to be highly upregulated by G1 stimulation in DU145 cells, but no differences were found in PC3 cell in any time point (Fig. 7.10A and 7.10B). Regarding N-cadherin, despite its initial increased in response to G1, the treatment did not cause alterations in its expression in DU145 cells after 7 days until the end of stimulus (Fig. 7.10A). Similarly to DU145, in PC3 cells, N-cadherin expression was increased after 24 h of treatment and remains higher than in the control group after 7 days, but after 14 days its expression was reduced comparatively to control (Fig. 7.10B). Moreover, vimentin expression also was increased in DU145 exposed cells, comparatively to the control at 1 and 7 days of stimulation, but after 14 days no differences were observed and after 28 days its expression tends to decrease with treatment (Fig. 7.10A). In the case of PC3 cells, the vimentin expression highly increased with G1 treatment over the time (Fig. 7.10B). No data is presented for PC3 cells after 28 days of treatment because at this time we did not collect sufficient protein to proceed the analysis since the number of cells was substantially reduced in the treated group. Additionally, the cell morphology changed with the long G1 stimulation as showed in Fig. 7.10C.

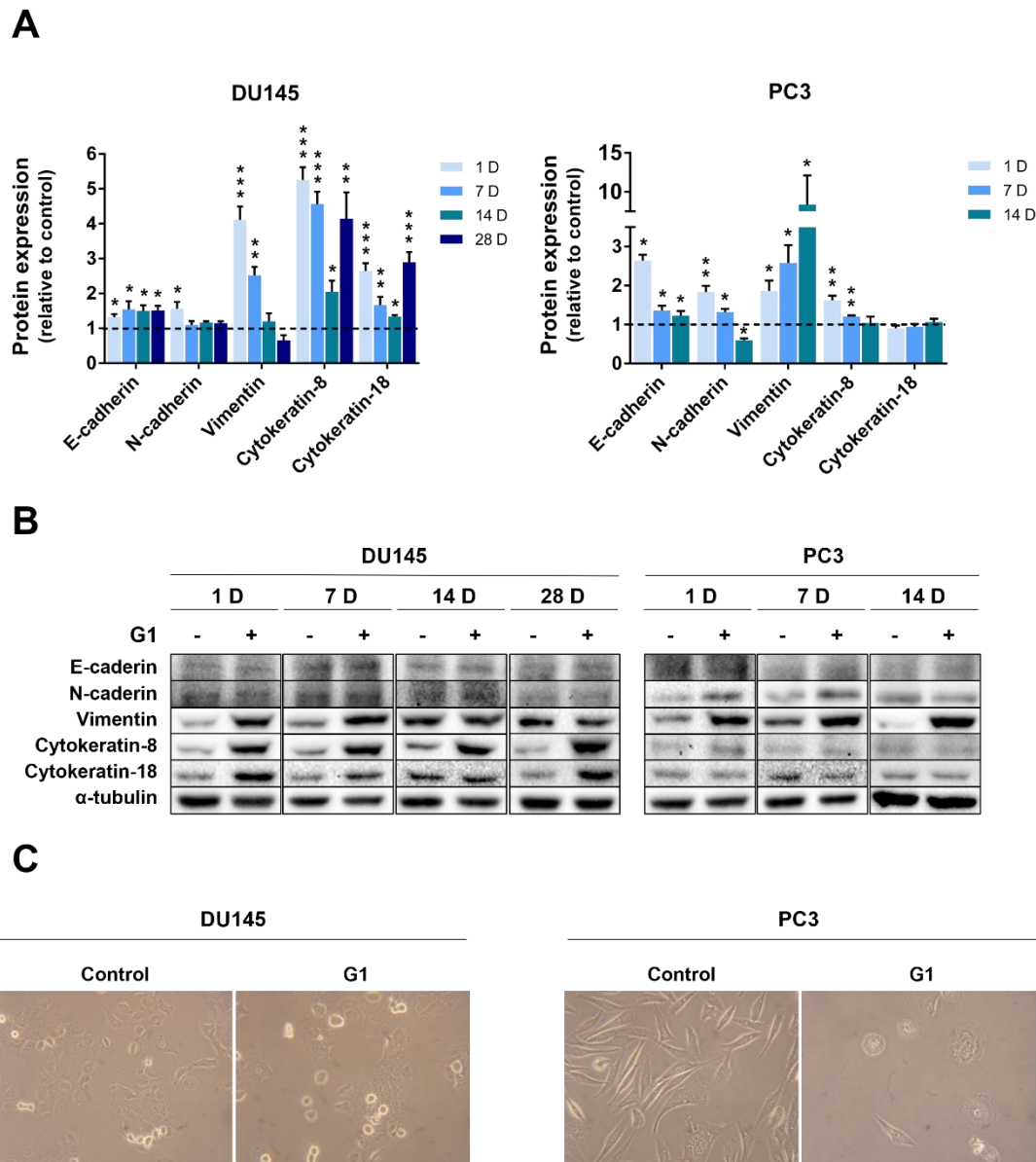


Figure 7.10. G1 effects in modulating the expression of epithelial and mesenchymal markers in DU145 and PC3 human Pca cells in long-term culture. Cells were treated with 1 μ M G1 for 1 to 28 days (1-28 D). (A) Protein expression determined by WB analysis after normalization with α -tubulin. Results are expressed as fold-change relative to the control untreated group (0 μ M G1, dashed line). Error bars indicate mean $\pm$ S.E.M. * P < 0.05 ** P < 0.01 *** P < 0.001. (B) Representative immunoblots. (C) Representative images of DU145 and PC3 cells morphology after 28 D stimulation with G1 compared with control. Images were obtained in optic microscope under 200 X magnification.

7.3.8. GPER modulated the metabolic profile of PCa cells

The effect of G1-treatment on glucose, amino acids and lipid metabolism was evaluated (Fig. 7.11A). Stimulation with G1 significantly augmented glucose consumption in PCa cells (1.93 ± 0.12 , 1.76 ± 0.07 and 1.31 ± 0.06 fold-change relative

to control in LNCaP, DU145 and PC3 cells, respectively, Fig. 7.11B). The increase in glucose consumption was accompanied by an increase in lactate production in CRPC treated cells (2.04 ± 0.11 and 1.51 ± 0.09 fold-change relative to control, respectively for DU145 and PC3 cells), whereas a reduction was observed in LNCaP cells (0.79 ± 0.03 fold-change relative to control, Fig. 7.11C). LDH is the enzyme responsible for the conversion of pyruvate, derived from glycolysis, into lactate [49] that is then exported to the extracellular space by the MCT4 isoform of MCTs family [49, 50] (Fig. 7.11A). G1-treated LNCaP cells, displayed reduced LDH activity compared to the control group (0.60 ± 0.03 fold-change), but no significant differences were observed for the CRPC cell lines (Fig. 7.11D). G1 treatment increased the expression of LDH and MCT4 in PC3 cells (1.46 ± 0.16 and 1.53 ± 0.09 fold-change to control, respectively), but had no effect on the expression of these proteins in LNCaP and DU145 cells (Fig. 7.11E).

Enhanced glucose consumption in LNCaP and PC3 cells was underpinned by the altered expression of GLUTs (Fig. 7.11E). GLUT1 and GLUT3 expression was increased with GPER activation in LNCaP cells (1.30 ± 0.05 and 1.23 ± 0.08 fold-change relative to control), whereas PC3 cells displayed augmented protein levels of GLUT1 (1.42 ± 0.10 fold-change relative to control, Fig. 7.11E).

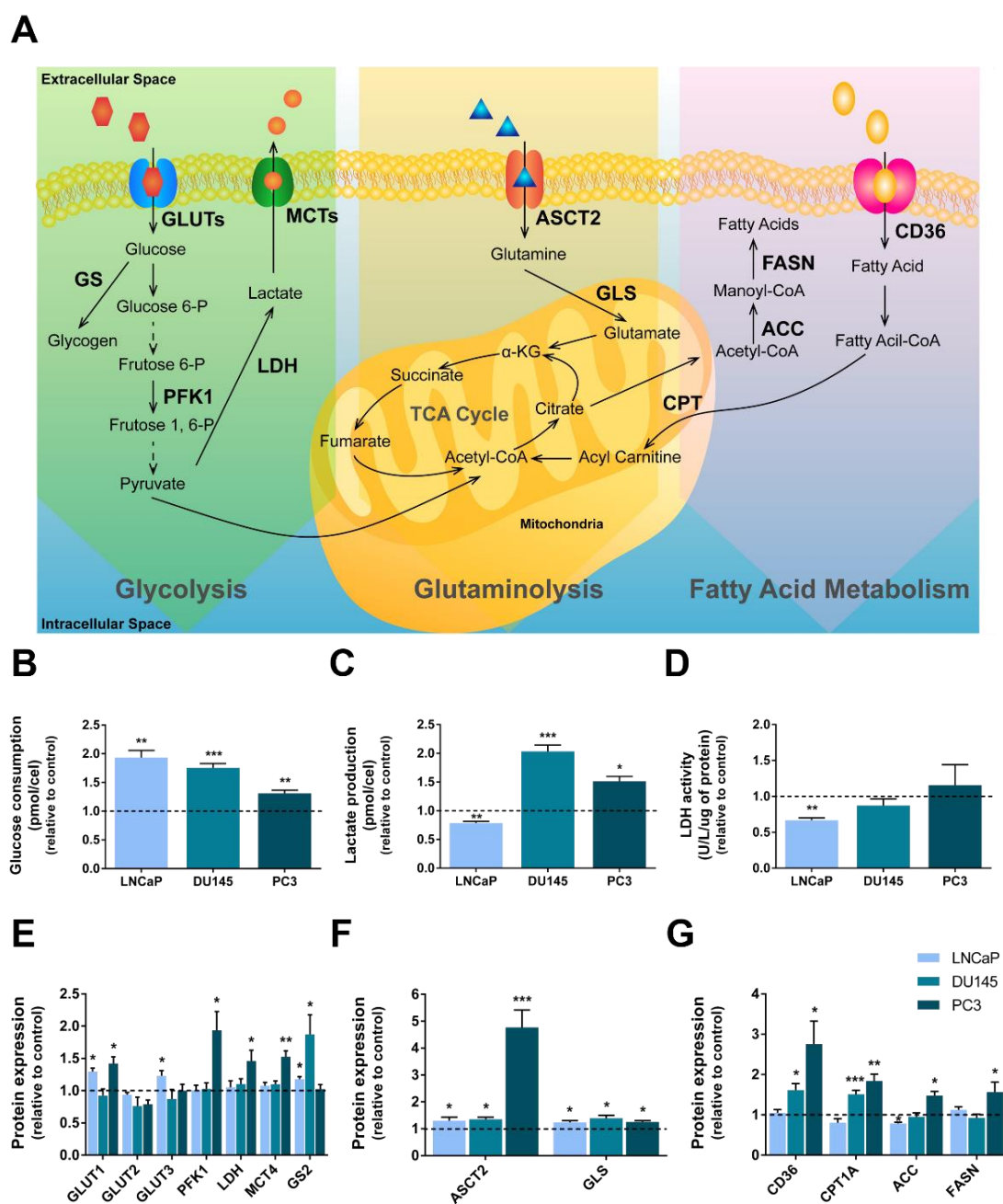
PFK1 the enzyme that phosphorylates fructose-6-phosphate (F-6-P) into fructose 1,6-biphosphate (F-1,6-P) catalyzes a limiting step of glycolysis [51], determining the rate of glycolytic flux (Fig. 7.11A). PFK1 expression was augmented by G1 in PC3 cells (1.94 ± 0.29 fold-change to control), but no differences were observed in the other cell lines (Fig. 7.11E).

If glucose doesn't enter the glycolytic cascade, it can be converted into glycogen through the enzyme glycogen synthase (GS2, Fig. 7.11A) [52]. Herein, both LNCaP- and DU145-treated cells displayed augmented expression of GS isoform 2 (GS2) (1.18 ± 0.04 and 1.87 ± 0.30 fold-change relative to control, respectively, Fig. 7.11E).

Regarding amino acids metabolism, we focused on glutamine handling (Fig. 7.11A). Glutamine enters the cell by the glutamine transporter ASCT2, being converted into glutamate by GLS [53] (Fig. 7.11A). Both ASCT2 and GLS expression was augmented in the G1-treated cells compared with the respective control groups (LNCaP: 1.39 ± 0.12 and 1.23 ± 0.07 fold-change, respectively; DU145: 1.36 ± 0.07 and 1.39 ± 0.10 fold-change, respectively; PC3: 4.77 ± 0.65 and 1.25 ± 0.05 fold-change, respectively, Fig. 7.11F).

GPER activation also modulated the expression of several targets of lipid metabolism, namely, CD36, CPT1A, ACC and FASN (Fig. 7.11A). Treatment with G1 increased the expression of CD36 in the CRPC cell lines DU145 and PC3 (almost 2 and 3-fold change relative to control, respectively) (Fig. 7.11G). Also, the expression of

CPT1A that is required for the transport of FA to the mitochondria [54] (Fig. 7.11A), was enhanced by G1 treatment in these cells lines (1.51 ± 0.10 and 1.84 ± 0.17 fold-change relative to control in DU145 and PC3 cells, respectively, Fig. 7.11G). ACC and FASN are lipogenic enzymes involved in *de novo* lipid synthesis [55-57] (Fig. 7.11A). PC3 cells showed increased expression of both ACC and FASN in response to G1 (1.48 ± 0.10 and 1.57 ± 0.24 fold-change relative to control; Fig. 7.11G). In the case of LNCaP cells, and considering the main targets of lipid metabolism, only ACC expression was changed upon G1 administration (an approximate reduction of 20% relative to control was observed, Fig. 7.11G).



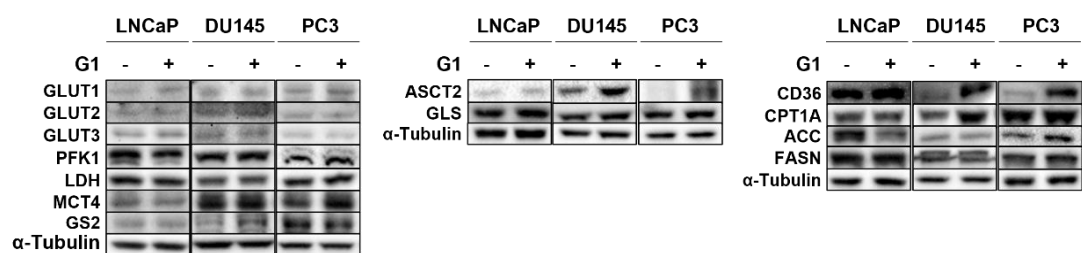


Figure 7.11 G1 actions in modulating the metabolic profile of LNCaP, DU145 and PC3 human Pca cells. Cells were treated with 1 μ M G1 for 24 hours. (A) Schematic representation of glycolysis, glutaminolysis and fatty acid metabolism. Glucose enters the cell by the activity of GLUTs, being metabolized through glycolytic enzymes, namely, PFK1. Pyruvate, the end product of glycolysis can be driven to mitochondria or converted into lactate by LDH. MCT4 at cell membrane exports lactate to the extracellular space. Alternatively, glucose can be converted into glycogen through GS. Glutamine is incorporated into the cell by the membrane transporter ASCT2 and is converted to glutamate by the mitochondrial GLS. CD36 transporter is responsible for the uptake of fatty acids that can be incorporated to mitochondria by CPT1A undergoing β -oxidation. ACC and FASN are the target players in fatty acid *de novo* synthesis from citrate. (B) Glucose consumption and (C) Lactate production determined by spectrophotometric measurement of glucose and lactate content in the extracellular medium. (D) LDH activity determined spectrophotometrically. (E-G) Protein expression of target players in (E) glycolytic metabolism (F) glutamine metabolism, and (G) lipid handling determined by WB analysis after normalization with α -tubulin. Results are expressed as fold-change relative to the control untreated group (0 μ M G1, dashed line). Error bars indicate mean $\pm$ S.E.M. * P < 0.05 ** P < 0.01 *** P < 0.001. Representative immunoblots are shown as bottom panels.

7.3.9. Oxidative stress was a target of GPER in PCa cells

The effect of G1 in modulating the oxidant status of PCa cells was evaluated. G1-stimulated PCa cells displayed an increase in the TOS relative to control (4.27 ± 0.96 , 1.59 ± 0.13 and 1.70 ± 0.08 fold-change in LNCaP, DU145 and PC3 cells, respectively; Fig. 7.12A). SOD inhibition activity was slightly augmented by G1 treatment in LNCaP (3.8 %; Fig. 12B), and slightly reduced in DU145 and PC3 cells (2.7 % and 2.5 %, respectively; Fig. 7.12B). Furthermore, G1 stimulation reduced GPx activity in LNCaP cells (0.82 ± 0.01 fold-change relative to control; Fig. 7.12C), whereas increasing it in DU145 and PC3 cells (1.44 ± 0.05 and 1.58 ± 0.05 fold-change relative to control; Fig. 7.12C).

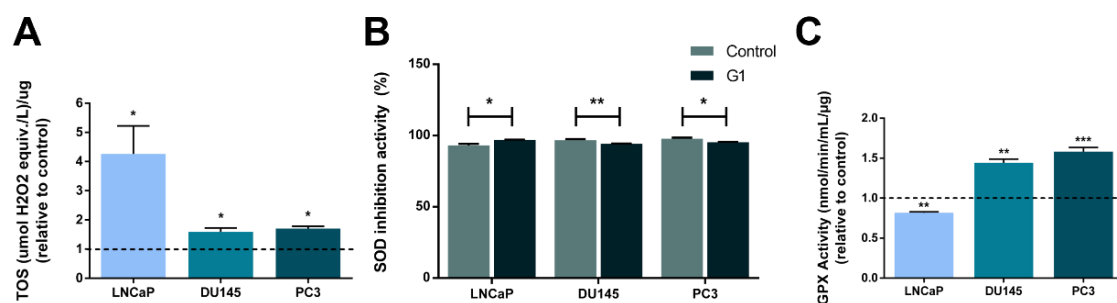


Figure 7.12. Effect of G1 in modulating the oxidant and antioxidant status of LNCaP, DU145 and PC3 human PCa cells. Cells were treated with 1 μ M G1 for 24 hours. (A) Total oxidant status, (B) SOD activity determined spectrophotometrically (C) GPX activity determined spectrophotometrically. Results are expressed as fold-change relative to the control untreated group (0 μ M G1, dashed line). Error bars indicate mean $\pm$ S.E.M. *P < 0.05 **P < 0.01 ***P < 0.001.

7.4. Discussion

This study demonstrated the broad role of GPER in modulating PCa cell proliferation, apoptosis, migration, invasion, EMT transition, energy metabolism and oxidative stress.

First, we characterized the expression of GPER in human prostate tissues using TMAs. Some previous studies have described the GPER expression in benign and neoplastic human prostate, though producing discrepant results [26, 31-33]. Herein, we showed that GPER expression was augmented in human PCa samples compared with BPH and inversely correlated with the total PSA levels. This is the first report correlating GPER expression with PSA levels in PCa specimens. This inverse correlation raises curiosity about the potential role of GPER as a predictive biomarker of biochemical recurrence after radical prostatectomy, together with PSA.

Histological analysis confirmed GPER expression in epithelial and stroma cells, both in BPH and PIN prostate specimens, following the previously reported studies [26, 31-33, 58], and further supporting the existence of estrogen signaling mediated by GPER in the prostate stromal and epithelial compartments.

To ascertain the usefulness of PNT1A, LNCaP, DU145 and PC3 cell line models to investigate GPER actions, we characterized the receptor expression in these cells. All prostate cell lines under study expressed GPER, with a higher expression detected in LNCaP cells compared with the non-neoplastic PNT1A, which resembles the TMAs results. Moreover, GPER expression was decreased in CRPC cells compared with the androgen-sensitive LNCaP. The reduced levels of GPER in CRPC cell lines follow the findings reported in human PCa cases, indicating decreased GPER expression with the

aggressiveness of disease and progression from moderately to poorly differentiated PCa [33].

The differential expression found in androgen-sensitive and CRPC cell lines led us to investigate whether GPER protein levels are affected by DHT, the biologically active androgen in the prostate, produced locally from the reduction of testosterone by the 5 α -reductase enzyme [59]. Our results showed that DHT-treatment (10 nM) down-regulated GPER protein expression in PNT1A (24 h) and LNCaP (48 h) cells but not in the CRPC cell lines DU145 and PC3. Although not evaluating protein levels, and using a different time frame and hormone concentrations, Lam *et al.* [28] also observed that DHT treatment (0.1 and 1 nM for 4 days) reduced the GPR30 mRNA levels in LNCaP cells, with the effect being abolished in the presence of the androgen receptor antagonist bicalutamide, or by siRNA silencing of the receptor. Overall, these data allow establishing GPER as a target of DHT for a wide range of concentrations, from infra- (0.1 and 1 nM) to normo-physiological (10 nM) [40, 41], and demonstrated that at physiological concentrations, the DHT effects are perceived even if the exposure time is short.

GPER is known as a cell membrane receptor, but its presence in the endoplasmic reticulum, the Golgi apparatus, and also in the nucleus has been demonstrated [33, 60-67]. Accordingly, we found that GPER was localized at the cell membrane, endoplasmic reticulum and residually in the nucleus of PCa cells, with fluorescence intensity of the co-localization with the endoplasmic reticulum being largely higher (~ 2-5 fold) than that with the cell membrane. No other reports exist quantifying the subcellular distribution of GPER in PCa cells. However, co-localization studies in MDA-MB231 breast cancer cells and HEC50 endometrial cancer cells failed to detect the GPER at the cell membrane, though they have described its association with the endoplasmic reticulum [68].

The results herein also showed that treatment with the GPER agonist G1 altered the GPER location at the cell membrane and endoplasmic reticulum of prostate cells, whereas it did not affect the residual nuclear staining observed. The selective GPER agonist increased the receptor localization at the cell membrane in the non-neoplastic PNT1A and neoplastic DU145 cells. This is the first report describing the role of G1 influencing the localization of GPER at cell membrane, which has been shown to modulate intracellular signaling with the activation of heterotrimeric G proteins, increase of cAMP concentration and mobilization of intracellular calcium [13, 69-71].

GPER location at the endoplasmic reticulum has been a matter of controversy. It has been linked to the receptor synthesis and endoplasmic reticulum - Golgi intracellular traffic before its translocation to the cell membrane (93, 94) or the

constitutive retrograde transport from the plasma membrane to the endosomal compartment (104-106). However, some authors reported that GPER at the reticulum is signaling-active mediating cell responses by mobilizing intracellular calcium [71, 72]. G1 reduced the association of GPER to the endoplasmic reticulum in PNT1A cells, whereas increasing it in PC3. Our findings indicate that G1 modulates GPER subcellular localization, which seems to be a complex mechanism depending on the cell type and physiopathological status.

Considering the effects of G1/GPER in regulating prostate cell behaviour, we started by evaluating cell viability. G1-treatment increased the viability of non-neoplastic PNT1A prostate cells, except for the highest G1 concentration, which decreased cell viability. This duality of effects on cell viability depending on high or low G1 concentrations follows the described in prostate cell lines and tissues using E₂ or other estrogenic compounds [73, 74]. The activation of GPER widely decreased the viability of CRPC cells, which were more sensitive to G1 treatment than LNCaP cells. LNCaP, DU145 and PC3 cells also displayed reduced cell number and diminished Ki-67 cell proliferation index in response to G1, which is in line with another previous study demonstrating that G1 administration inhibited the growth of PCa cell line models [26]. These findings support targeting GPER to counteract PCa growth and are further supported by a panoply of studies in other cancer types showing the anti-proliferative actions of GPER [16, 19, 21, 75-78].

Despite the effect of G1 in diminishing the number of Ki-67 positive cells, the expression of this proliferation marker seemed to be higher in the nuclei of treated cells. Cell cycle analysis allowed to confirm that G1 treatment caused PCa cell cycle arrest in G2/M phase, which follows a previous study reporting that the GPER agonist G1 induced a persistent cell-cycle arrest at the G2/M phase in LNCaP and PC3 cells [26]. Also, it may explain the apparent high Ki-67 nuclear expression, as it varies depending on the cell cycle phase, being increased in proliferating cells in mitosis, as well as in cells in the G2 phase [79, 80].

G1 effects suppressing the viability of CRPC cells were underpinned by the altered expression of cell cycle and cell survival regulators. The tyrosine kinase receptor c-KIT and its specific ligand, the stem cell factor (SCF), have an important role in the control of cell proliferation, survival and apoptosis, and have been shown to be involved in prostate carcinogenesis [81-84]. Moreover, we have previously reported that E₂ regulates the expression of the SCF/c-KIT system, diminishing the expression of c-KIT both *in vitro* and *in vivo* with an impact on prostate cell proliferation and apoptosis [85]. Therefore, we decided to investigate the effect of G1 on SCF and c-KIT expression. SCF was upregulated in PC3 cells when treated with G1, but no changes were observed

in LNCaP and DU145 cells. Nevertheless, G1-treatment downregulated full-length c-KIT expression in LNCaP and CRPC DU145 cells, concomitantly with the observed diminished cell proliferation. Although G1 also decreased the proliferation of PC3 cells, no changes were found in the expression of the full-length c-KIT.

The c-Myc is a well-known oncogene with a crucial role in cell cycle progression and cell proliferation [86]. The phosphorylation of c-Myc regulates its stability since it is a required step for ubiquitin-mediated degradation [87-89]. p-cMyc expression was highly increased in response to G1 stimulation in all neoplastic cell lines, in line with the reduced proliferation. Repression of p21 expression is one of the mechanisms by which c-Myc promotes cell proliferation and cell cycle progression [90-92]. However, no changes were detected in p21 expression in PCa cells treated with the GPER agonist, which suggests the involvement of another molecular target downstream p-cMyc.

GPER-mediated responses in the control of cell proliferation and cancer growth have been described in various cell types, involving several molecular targets and signaling pathways, namely, the phosphatidylinositol 3-kinase (PI3K), the serine/threonine protein kinase AKT and the mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase (ERK)1/2 [14, 19, 76, 93-97]. Herein, despite the reduced proliferative ability of PCa cells treated with G1, no changes were observed in the expression of AKT and MAPK. The increased expression of p-AKT in the androgen-sensitive LNCaP cells would suggest the activation of this pathway and enhanced survival. However, no stimulatory effect by G1 was observed in LNCaP cell growth. There is no definitive explanation for this fact at the moment, which is likely related to the observed down-regulated expression of p-MAPK. Similar findings were obtained with the selective estrogen receptor modulator raloxifene. Modulation of GPER activity by raloxifen increased p-AKT expression and reduced p-ERK suppressing LNCaP cells viability [98].

G1 treatment induced apoptosis in PNT1A, LNCaP, DU145 and PC3 cells, as indicated by the activity-like of the executioner caspase-3, an endpoint of apoptotic signaling at the crossroad of intrinsic and extrinsic pathways [99]. These results were confirmed by the increase of DNA fragmentation detected in LNCaP, DU145 and PC3 G1-stimulated cells.

To shed light on the apoptotic pathways being activated by G1 we analyzed the expression of several apoptosis-related proteins of both the intrinsic and extrinsic pathways [100]. G1-treatment augmented the expression of proapoptotic Bax protein and caspase-9 in LNCaP and DU145 cells, which indicates the activation of the intrinsic pathway. Bax and Bcl-2 proteins are target players in the activation of the intrinsic pathway of apoptosis, with the augmented Bax/Bcl-2 protein ratio recognized as a

powerful marker of cell apoptosis [101, 102]. Bax/Bcl-2 ratio could not be determined in DU145, as these cells do not express the Bax protein, which was also reported by others [103-105]. In G1-treated LNCaP cells, the enhanced apoptotic rate also is supported by the increased expression of the tumour suppressor p53, which is widely known to control the intrinsic pathway [106]. p53 acts as a transcription factor controlling the expression of several apoptotic regulators, namely, inducing Bax and repressing Bcl-2 transcription, thus establishing the increased proapoptotic Bax/Bcl-2 protein ratio [107]. In addition, p53 promotes the transcription of Bcl-2 antagonists [108] and the cytoplasmic p53 binds to proapoptotic Bcl-2-family proteins, antagonizing Bcl-2 function or leading to permeabilization of mitochondria and release of cytochrome C [108]. Altogether, results suggest that G1 induces apoptosis of LNCaP and DU145 cells, likely by activating the intrinsic pathway. Indeed, GPER's proapoptotic actions have been widely reported in distinct types of cancer, including prostate, breast, ovarian and Leydig cells tumors [21, 22, 26, 109, 110] and mainly related to the mitochondrial pathway and altered expression or activity of Bcl-2 protein family members [99]. However, no changes were detected in the expression of regulators of the intrinsic pathway of apoptosis in PC3 cells, which can likely be explained by the lack of p53 expression [48]. Although no alterations were observed in caspase-8, a trigger of the extrinsic pathway of apoptosis, increased expression of FasL was detected in PC3 cells, suggesting that this would be involved in GPER-induced apoptosis. It is also predictable that this pathway is activated in LNCaP and DU145 cells, in line with the increased expression of caspase -8 and FasL, and Fas receptor, respectively.

The pro-apoptotic effects of estrogens have been frequently associated with the activation of the extrinsic pathway of apoptosis [111-113], and other studies also have reported enhanced apoptosis of androgen-sensitive LNCaP and CRPC PC3 cells upon GPER activation [26]. However, our results first indicate that the GPER agonist G1 can activate apoptotic signaling by both the intrinsic and extrinsic pathways.

Our results showed that besides modulating the proliferative and apoptotic behavior of PCa cells, GPER could also influence other cancer hallmarks, namely, migration and invasion. G1 treatment decreased migration and invasion of DU145 cells. Studies analyzing the effects of G1 in modulating the migration and invasion of PCa cells are scarce, but a previous report also found inhibited migration and invasion of DU145 cells after GPER activation, which was supported by reduced formation of filopodia and stress fibers [27]. However, G1 did not affect migration and invasion of LNCaP cells and enhanced the invasion capabilities of PC3 cells. There is no definitive

explanation for this fact at this stage, but PC3 cells represent an aggressive stage of CRPC with high invasive capability compared with other PCa cell models [114].

The acquisition of invasive properties by tumor cells is strictly related to EMT, which comprises loss of polarity, disruption of cell-cell adhesion, and migratory ability [115]; cellular changes coordinated by alterations in the gene expression pattern of epithelial and mesenchymal markers [116]. Loss of cytokeratin 8 and 18 expression also is associated with EMT, metastasis and chemoresistance [117]. Herein, we hypothesize that G1 can modulate EMT in PCa cells. In addition to the well evident changes on cell morphology with the long-term stimulation with G1, marked alterations on the expression of epithelial and mesenchymal markers were observed. In DU145 cells, GPER activation by G1 increased the expression of cell-cell adhesion protein E-cadherin, as well as the epithelial cellular intermediate filament proteins, cytokeratin 8 and 18, throughout all the experimental time-frame. Concerning mesenchymal markers, both N-cadherin and vimentin expression increased in response to G1 for short-term exposure times but decreased to basal control levels in the long-term. These results suggest that the continuous exposure to G1, by increasing E-cadherin expression over N-cadherin and increasing cytokeratin 8 and 18, may enhance the epithelial profile of DU145 cells and revert the EMT process. In line with our findings, studies in breast and pancreas cancer cells reported that GPER prevented the transition from the epithelial to the mesenchymal phenotype [118, 119].

Information on GPER actions in the EMT process in PCa is scarce. Nevertheless, a study showed that GPER activation reduced the expression of vimentin and increased E-cadherin expression in PC3 cells [119]. Herein, the results obtained indicated that despite the increased E-cadherin expression and reduced N-cadherin levels with the long-term (14 days) exposure to G1, vimentin expression was highly increased in PC3 cells. The different response patterns seen in the invasiveness capacity of DU145 and PC3 cells in response to G1 may be underlined by the observed differences in the expression of EMT markers.

Reprogramming of energy metabolism and its role as a hallmark of cancer has been a topical cancer research subject in recent years [120, 121]. Furthermore, new accumulating evidence has been pointing out GPER as a metabolic regulator, namely, in the maintenance of glucose homeostasis [35]. The *GPER* knockout mice displayed hyperglycemia and glucose intolerance [122], demonstrating a relevant GPER action in glycolytic metabolism. Considering the importance of glucose and metabolic reprogramming in shaping cancer cell fate [123], we decided to investigate the influence of GPER in PCa cell metabolism.

G1 treatment modulated the metabolic profile of androgen-sensitive LNCaP cells and CRPC cells. Enhanced glucose consumption was observed in all cell lines, which was supported by the augmented expression of GLUT1 (LNCaP and PC3) and GLUT3 (LNCaP), the GLUTs isoforms mostly associated with cancer [124]. Despite the increased glucose consumption, GLUT1 and GLUT3 expression remained unchanged in G1-treated DU145 cells, indicating that other GLUT isoform may be involved. A likely candidate would be GLUT12, which has been detected in PCa cases and suggested as one of the main glucose suppliers of glycolytic metabolism [125-127]. These novel findings linking GPER with the regulation of glycolytic metabolism in PCa cells are supported by the recently reported ability of GPER to modulate GLUTs expression in other cell types [128-130].

After entering the cells, glucose follows the glycolytic pathway, being converted into pyruvate, the end product of glycolysis [51]. PFK1 catalyzes a limiting step of glycolysis, the conversion of fructose 6-phosphate into fructose 1,6-bisphosphate, and has been considered to indicate the rate of glycolytic flux [51]. GPER activation increased PFK1 expression in PC3 cells, which were already described as being more glycolytic than LNCaP [131]. Indeed, in G1-treated PC3 cells, the increased glucose consumption was accompanied by augmented lactate production, supported by the augmented expression levels of LDH, the enzyme responsible for the conversion of pyruvate into lactate [49], and MCT4, the transporter responsible for lactate export to the extracellular space [49, 50]. In DU145, the increased lactate production was not followed by changes in the expression of PFK, LDH or MCT4, suggesting that lactate could be being produced from sources other than glucose [132, 133]. Moreover, the expression of GS2, the enzyme that converts glucose into glycogen [52], was increased in G1-treated DU145 cells suggesting that glucose is being routed for storage.

In the less-glycolytic LNCaP cells [131], despite the increased glucose consumption in response to G1 stimulation, lactate production was reduced, in accordance with the diminished LDH activity and the unaltered expression of LDH and MCT4. Similarly, to what was found in DU145, G1 also increased the expression of GS2 in LNCaP cells. The implications of putative glucose storage for PCa cell fate have not been investigated, but previous studies reported that glycogen accumulation is associated with androgen-responsiveness [134].

Interestingly, GPER activation could also modulate glutamine metabolism, as G1 increased the expression of ASCT2 and GLS, the membrane glutamine transporter and the enzyme that converts glutamine into glutamate, respectively. The role of glutaminolysis has been implicated in the onset and progression of PCa, [135] and our previous study showed that restriction on glutamine decreased cell viability and the

inhibition of glutamine metabolism by inhibiting GLS activity increased apoptosis and reduced PCa cells migration [136]. Further studies are needed to clarify the relevance of G1/GPER modulation of glutaminolysis in PCa proliferative activity.

Lipids are another energy source relevant for sustaining the high proliferative rates of cancer cells [137], and several players involved in lipid metabolism have been shown to be upregulated in PCa cells, namely, CD36, FASN and CPT1A [138-143]. Although no studies have been directed to investigate GPER as a metabolic regulator in PCa cells, its influence over lipid metabolism in other cell types has been suggested. For example, GPER activation was able to increase the expression of low-density lipoprotein (LDL) receptor and to induce the uptake of LDL in liver cells (176, 177). Additionally, GPER seems to promote adipogenesis, leading to obesity in female mice (178). Our results showed that GPER activation in PCa cells up-regulated the expression of ACC and FASN, lipogenic enzymes involved in *de novo* lipid synthesis [55-57]. Furthermore, in CRPC cell lines, G1 treatment enhanced the expression of the fatty acid transporter CD36 and CPT1A, the enzyme required for the transport of fatty acids to the mitochondria for β -oxidation [54]. Overall, GPER activation in PCa cells seems to drive lipid utilization by favouring its uptake and β -oxidation.

Fatty acid β -oxidation has been reported as an important bioenergetic pathway supporting PCa cell proliferation and tumour growth [141]. However, the acceleration of energy metabolism by GPER, i.e. glucose, glutamine and lipid usage, was not underpinned by increased cell viability and proliferation. On the contrary, G1 treatment suppressed cell proliferation and increased apoptosis, which indicates that other processes superimpose metabolic plasticity driving the alterations in PCa cell fate. Nevertheless, this is the first report of GPER modulation of PCa cell metabolism, opening new perspectives to explain changes in tumour cell behaviour under oestrogens actions.

Oxidative stress caused by a disruption of the balance between the production of reactive oxygen species (ROS) and the efficiency of antioxidant defences [144] can damage lipids, nucleic acids, and proteins, disrupting normal cell function [145]. Indeed, moderate levels of oxidative stress have been pointed out as a common feature associated with the onset of PCa [146, 147]. However, increased oxidative stress above some threshold can trigger programmed cell death. It has been demonstrated the reduced viability of PCa cell and the anti-proliferative and pro-apoptotic effects in response to augmented levels of ROS [148]. Herein, GPER activation increased TOS in PCa cells, indicating an augment of ROS, which can sustain the pro-apoptotic effect of G1 observed. Other studies also reported the involvement of estrogens and their receptors in the regulation of oxidative stress [149]. Enzymes of the antioxidant defence

system, such as SOD and GPx, which chelate superoxide, preventing and repairing ROS-induced damage, have been shown to be a target of estradiol and G1 [150-152], and significantly reduced in PCa [153-156]. SOD catalyzes the dismutation of superoxide anion radical into hydrogen peroxide (H_2O_2), which is then transformed into water and oxygen by GPx [157, 158]. We found a slight increase in SOD activity and a reduction of GPx activity in the androgen-sensitive LNCaP cells, suggesting that increased TOS in LNCaP cells may occur because GPx don't acts in the H_2O_2 resultant from SOD activity. On the other hand, CRPC cells presented reduced SOD activity and augmented activity of GPx, suggesting that TOS increased in these cells is motivated by the reduction of SOD activity. Our findings are supported by the reports in lung cancer cells showing that G1 increased oxidant levels and the activity of antioxidant enzymes SOD and GPx [150].

As mentioned, the augmented oxidative stress can drive PCa cell apoptosis, which we cannot exclude being related with the enhanced metabolic activity. It is well known that the metabolic processes lead to the formation of oxidant species, changing cell oxidant status [159]. Moreover, it has been shown that high glucose induces oxidative stress and causes activation of several proteins involved in apoptotic cell death, including members of the caspase and Bcl-2 families [160].

In sum, the present results confirmed the higher GPER expression in PCa cases and its inverse correlation with PSA levels. GPER protein levels also were higher in neoplastic cells. Nevertheless, CRPC cells displayed reduced expression of GPER compared to the androgen-responsive cell line, with GPER protein levels being modulated by androgens. G1-treatment showed the multiplicity of benefic effects driven by GPER in PCa, including diminished cell viability and proliferation, arrested cell cycle and increased apoptosis. GPER was also related to reduced migration and invasion, as well as with EMT attenuation, depending on the cell line. Moreover, GPER activation increased energetic metabolism and oxidative stress in the PCa cells. To the best of our knowledge, this is the first report compiling GPER effects over a broad range of cancer hallmarks, including metabolic reprogramming. Overall, the obtained findings support the anti-cancer role of GPER and its feature as an appealing target for PCa management. However, our results highlight that should not be ignored the metabolic alterations driven by GPER activation when considering GPER as a target for treatment in PCa. Further investigation would be needed to better understand the role of GPER as a metabolic regulator in PCa and the underpinning mechanisms depending on the cell type, stage and aggressiveness of disease.

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Chapter 8

Diosgenin effects in suppressing proliferation and promoting apoptosis of human prostate cancer cells in an interplay with the G protein-coupled estrogen receptor

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8. Diosgenin effects in suppressing proliferation and promoting apoptosis of human prostate cancer cells in an interplay with the G protein-coupled oestrogen receptor

Abstract

Diosgenin is a phytosteroid sapogenin, which has been reported to have antitumoral activity in several types of cancer. A reduced number of studies have investigated diosgenin effects in prostate cancer (PCa) and its mechanisms of action remain to be disclosed. Nevertheless, it has been shown that some phytoestrogens exert their actions by activating the G protein-coupled estrogen receptor (GPER), a membrane-bound receptor suggested to have anti-tumorigenic effects in PCa. The present study evaluated the effect of diosgenin in the control of proliferation, apoptosis and glycolytic metabolism of PCa cells and its possible relationship with the GPER. Non-neoplastic (PNT1A) and neoplastic (LNCaP, DU145 and PC3) prostate cells were stimulated with diosgenin alone or in combination with the GPER agonist G1. Diosgenin reduced the viability of PCa cells (IC₅₀: 14.02 - 66.10 μ M) as indicated by the MTT assay. Moreover, castrate-resistant PCa (CRPC) cells were more sensitive than LNCaP to diosgenin treatment. Treatment with diosgenin increased prostate cells apoptosis, mainly through the extrinsic pathway, confirmed by increased caspase-3 activity, underpinned by altered expression apoptosis regulators, evaluated by Western blot. The phytosteroid also changed the glucose metabolism of PCa cells. Furthermore, the effects of diosgenin in viability, apoptosis and metabolism of prostate cells were enhanced by co-treatment with G1, suggesting the involvement of GPER in interplay with diosgenin. It was confirmed that diosgenin augmented GPER expression in prostate cell lines. Moreover, the knockdown of *GPER* gene showed to suppress the effect of diosgenin in PC3 apoptosis, suggesting that GPER is required for diosgenin-induced apoptosis in these cells. Altogether, the results obtained, suggest that diosgenin can be a promising agent to be tested in PCa therapy alone or in combination with G1.

Keywords

Diosgenin, GPER, prostate cancer, proliferation, apoptosis, metabolism

8.1. Introduction

Prostate cancer (PCa) is a hormone-dependent cancer highly dependent on steroid hormones actions [1-9]. Huggins and Hodges [10] were the first to demonstrate the hormone-sensitivity of PCa, namely, to androgens and estrogens, i.e. being stimulated by androgenic activity and inhibited by suppression of androgens levels or estrogens administration, which was the basis for using hormone therapy in PCa. The synthetic estrogen diethylstilboestrol (DES) was used for years as an effective therapy for delaying the progression of metastatic PCa [11-16]. However, the associated adverse effects, namely, at the cardiovascular level, lead to the discontinuation of estrogenic therapies [12-14]. However, the panoply of the beneficial roles of estrogens to control PCa [7-9, 17-19] and the lack of treatment options for advanced castrate-resistant PCa, resulted in efforts to find safe approaches for estrogens use, and their re-introduction in PCa therapy has been discussed [20]. The lower incidence of PCa in Eastern countries associated with a plant-based diet and higher consumption of phytoestrogens [17-19] stimulated research exploring the use of different classes of these compounds in PCa treatment [21]. Diosgenin, (25R)-5-Spirosten-3 β -ol, is a phytosteroid sapogenin and a biologically active molecule found in some species of *Dioscorea*, *Costus*, *Smilax*, and *Trigonella*, in particular in tubers and seeds [22-25]. This sapogenin has a structure similar to cholesterol and other steroids, and for this reason, has been used in the pharmaceutical industry for the synthesis of synthetic hormones and corticosteroids for application as anti-inflammatory, androgenic and contraceptive drugs (623, 624). Among a diversity of beneficial effects over different biological processes, the anti-cancer properties of diosgenin have been reported in several types of cancer [26, 27]. Also in PCa, diosgenin actions reducing tumor development and progression [28-30] have been described. However, scientific gaps exist considering the effect of diosgenin over the new cancer hallmarks as for example, the metabolic reprogramming, and its mechanism of action. It was reported that diosgenin could act by activating the classical nuclear estrogen receptor [31-33], but nothing is known about its interaction with the membrane-bound G protein-coupled estrogen receptor (GPER), which has been implicated as anti-tumorigenic in some types of cancer,

namely, in PCa [34-49]. Furthermore, other phytoestrogens, were shown to activate GPER [50-57]. This study aimed to evaluate the role of diosgenin in modulating PCa cell fate and glycolytic metabolism. Moreover, the possible relationship between diosgenin and GPER in PCa cells was evaluated.

8.2. Material and methods

8.2.1. Cell culture and treatments

Human prostate cell lines PNT1A, LNCaP, DU145 and PC3 were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). PNT1A is a non-neoplastic epithelial cell line, and LNCaP, DU145 and PC3 are neoplastic cell lines representing androgen-sensitive (LNCaP) and castrate-resistant (DU145 and PC3) metastatic stages of PCa [58-61].

PNT1A, LNCaP, DU145 and PC3 cells were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich) at 37 °C in an atmosphere equilibrated with 5% CO₂. At 60% confluence, culture medium was replaced by phenol red-free RPMI 1640 medium (Sigma-Aldrich) containing 5% charcoal-stripped FBS (CS-FBS) (Sigma-Aldrich), and cells maintained for additional 24 h. Then, cells were treated with diosgenin (Sigma-Aldrich) alone or in combination with the GPER agonist G1 (Tocris Bioscience, Madrid, Spain), or vehicle for 24 h.

8.2.2. MTT assay

PNT1A (10000 cells/well), LNCaP (15000 cells/well), DU145 (5000 cells/well) and PC3 (5000 cells/well) cells were seeded in 96-well plates and treated with diosgenin (0, 0.001, 0.01, 0.1, 1, 10 and 100 µM) for 24 h. Cell viability was determined by the colorimetric MTT. After diosgenin and /or G1 treatment, thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich) was added to cell culture medium (0,5 mg/ml), and reaction occurred protected from light at 37°C for 4 h. After incubation, the medium with MTT was removed, and the formed formazan crystals were dissolved in 100 µL DMSO (Honeywell, Charlotte, USA). The absorbance of the resultant purple coloured solution was measured at 570 nm using the xMark™ Microplate Absorbance

Spectrophotometer (Bio-Rad Hercules, USA). The absorbance value is directly proportional to the number of viable cells in each experimental group.

8.2.3. Cell number analysis

PNT1A, LNCaP, DU145 and PC3 cell nuclei were stained with Hoechst 33342 (5 µg/mL, Invitrogen, Thermo Fisher) for 10 min. Lamellae with control and diosgenin-treated (20 µM) cells were visualized in a 400x / 630x magnification. Images were acquired using the Axio Imager A1 microscope (Carl Zeiss).

8.2.4. Protein extraction

Human prostatic cells were homogenized in the appropriate volume of radioimmunoprecipitation assay buffer (RIPA) (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris, 1 mM EDTA) supplemented with 1% protease inhibitors cocktail and 10% PMSF, kept on ice for 20 min with occasional mixing, and total proteins recovered after a 14000 rpm centrifugation for 20 min at 4 °C. Protein concentration was determined using the BCA protein assay (Thermo Fisher, Rockford, USA), according to the manufacturer's instructions.

8.2.5. Western blot (WB)

Total proteins (25 µg) were resolved by SDS-PAGE on 12.5% gels and electrotransferred to PVDF membranes (Bio-Rad). After blocking with 5% skimmed dried milk for 1 h, membranes were incubated overnight at 4 °C with the appropriate primary antibodies: rabbit anti-GPER (1:250, ab 39742; Abcam, Cambridge, UK); rabbit anti-phospho-c-Myc (1:1000, #13748, Cell Signalling Technology, Danvers, MA, USA); rabbit anti-p53 (1:1000, FL-393: sc-6243, Santa Cruz Biotechnology, Dallas, Texas, USA); rabbit anti-p21 (1:1000, C-19: sc-397, Santa Cruz Biotechnology); rabbit anti-Bax (1:1000, # 2772, Cell Signalling Technology); rabbit anti-Bcl-2 (1:1000, # 2876, Cell Signalling Technology); rabbit anti-caspase-9 p35 (1:1000, H-170:sc-8355, Santa Cruz Biotechnology); mouse anti-caspase-8 p18 (1:100, D-8: sc-5263, Santa Cruz Biotechnology); rabbit anti-FAS (1:1000, A-20: sc-1023, Santa Cruz Biotechnology); rabbit anti-FAS-L (1:1000, C-178: sc-6237, Santa Cruz Biotechnology); rabbit anti-

Glucose Transporter 1 (GLUT1) (1:1000, CBL 242, Millipore, Darmstadt, Germany); rabbit anti- GLUT2 (1:1000, H-67: sc-9117, Santa Cruz Biotechnology); rabbit anti- GLUT3 (1:1000, H-50: sc-30107, Santa Cruz Biotechnology); rabbit anti-phosphofructokinase 1 (PFK1) (1:1000, H-55: sc-67028, Santa Cruz Biotechnology); rabbit anti-lactate dehydrogenase (LDH) [EP1566Y] (1:10000, ab 52488, Abcam); rabbit anti-monocarboxylate transporter 4 (MCT4) (1:1000, H-90: sc-50329, Santa Cruz Biotechnology); mouse anti-glycogen synthase 2 (GS2) (1:200, G-8: sc-390391, Santa Cruz Biotechnology).

Goat anti-rabbit IgG-HRP (1:40000, sc:2004; Santa Cruz Biotechnology) or goat anti-mouse IgG-HRP (1:40000, sc:2005; Santa Cruz Biotechnology) were used as secondary antibodies. A mouse anti- α -tubulin monoclonal antibody (1:10000, Sigma-Aldrich) or a mouse anti- β -actin monoclonal antibody (1:10000, Sigma-Aldrich) were used for protein loading control in WB analyses.

Membranes were incubated with ECL substrate (Bio-Rad) for 5 min, and immuno-reactive proteins were visualized with the ChemiDoc™ MP Imaging System (Bio-Rad). Band densities were quantified using the Image Lab software (Bio-Rad) and normalized with the respective α -tubulin or β -actin band density.

8.2.6. Caspase-3-like activity assay

The caspase-3-like activity was determined spectrophotometrically by detecting the presence of the yellow product p-nitro-aniline (pNA), upon cleavage of caspase-3 substrate (Ac-DEVD-pNA). In a 96-well plate, 5 μ L of total protein extracts from PNT1A, LNCaP, DU145 and PC3 cells, or PC3 si-RNA transfected cells were incubated overnight at 37 °C with 85 μ L of assay buffer (20mM HEPES, pH 7.4, 2mM EDTA, 0.1% CHAPS, 5mM DTT) and 200 μ M of Ac-DEVD-pNA (Sigma-Aldrich). The yellow colour obtained by the release of pNA (Sigma-Aldrich) was measured at 405 nm using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). The amount of generated pNA was quantified by extrapolation with a standard curve. All measurements were normalized to the total amount (μ g) of protein in each sample.

8.2.7. GPER gene knockdown

PC3 (10000 cells/well) were seeded in 6-well plates containing 2 mL/well of RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS)

(Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich). After 24 h at 37 °C in an atmosphere equilibrated with 5% CO₂, cell culture medium was replaced by phenol red and antibiotics-free medium containing 5% of charcoal stripped FBS (CS-FBS). PC3 cells, at 50% confluence, were transfected with 40 nM of a small interfering RNA (siRNA) targeting GPER (GPER1, Silencer Select, s6053, Ambion, Carlsbad, CA, USA), or scramble siRNA (Negative Control, Silencer Select, 4390846, Ambion) for 24 h. For this purpose, the appropriated quantity of si-scramble and si-GPER (per well) was diluted in 150 µL of Opti-MEM® (mix A), and simultaneously, 9 µL of Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) were diluted in 150 µL of Opti-MEM® (mix B). Mix A and B were incubated for 5 min at room temperature (RT), and then were combined, allowing the formation of siRNA:Lipofectamine RNAiMAX complexes for additional 20 min at RT. Then, the siRNA:Lipofectamine RNAiMAX complexes were added to each well and cells were incubated at 37 °C in a humid atmosphere equilibrated with 5 % CO₂ for additional 24 h. *GPER* gene knockdown at 24 h was evaluated by WB analysis.

8.2.8. Quantification of glucose and lactate

The concentration of glucose and lactate in the culture medium of control and diosgenin and / or G1-treated PNT1A, LNCaP, DU145 and PC3 cells was determined by means of spectrophotometric analysis using commercial kits (Spinreact, Girona, Spain) following the manufacturers' instructions. For glucose and lactate quantification, 1 µL of cell culture medium of each sample was placed in a 96-well plate and mixed with 100 µL of kit reaction buffer. The plate was incubated at 37°C for 10 min, and the absorbance measured at 505 nm (xMark™ Spectrophotometer, Bio-Rad). Glucose consumption and lactate production after 24 h of treatment were calculated relatively to the initial concentration of these metabolites at 0 h of stimulation. All measurements were normalized to the cell number in each sample.

8.2.9. Lactate dehydrogenase enzymatic activity assay

The enzymatic activity of LDH was measured using a commercial kit (Spinreact), following the manufacturers' instructions. 1 µL of cells protein extract was added to 150 µL of a kit reagent in a 96-well plate and incubated for 1 min at 37°C. The initial absorbance was acquired, followed by subsequent readings every min for 3 min.

LDH catalyses the reduction of pyruvate to lactate with the oxidation of NADH to NAD⁺. Thus, the activity of LDH in the sample is proportional to the rate of decrease in NADH concentration, which was measured spectrophotometrically at 340 nm using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). LDH activity was calculated by normalizing values to the total protein concentration in each sample (U/L/μg of protein).

8.2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism v6.01 (GraphPad Software, Inc., La Jolla, USA). The statistical significance of differences between experimental groups was evaluated by unpaired T-test with the appropriate correction or one-way ANOVA, followed by the adequate post-test. $P < 0.05$ was considered statistically significant. All experimental data are shown as mean ± standard error of the mean (S.E.M).

8.3. Results

8.3.1. Diosgenin treatment reduced the viability of PCa cells

As a starting point to evaluate the effect of diosgenin in human prostate cells, we started by exposing PNT1A, LNCaP, DU145 and PC3 cells to a concentration range of diosgenin, from 0.001 to 100 μM, for 24 h, and cell viability was analysed (Fig. 8.1). The highest diosgenin concentration (100 μM) diminished the viability of all prostate cell line models (Fig. 8.1A). Moreover, in DU145 and PC3 cells, for concentrations higher than 0.1 μM, diosgenin effects seem to be concentration-dependent (Fig. 8.1A).

The non-neoplastic PNT1A cells showed to be less sensitive to diosgenin, with an IC₅₀ of 66.10 μM (Fig. 8.1A). The IC₅₀ concentration in LNCaP cells was around 56.12 μM. In contrast, CRPC cells, DU145 and PC3, started to respond to diosgenin at lower concentrations, displaying IC₅₀ values of 23.21 and 14.02 μM, respectively (Fig. 8.1A).

20 μM diosgenin was the concentration selected to be used in the subsequent experiments, as it showed to reduce DU145 and PC3 cell viability by 50 %, and it represents an intermediate value considering the observed IC₅₀ in all the prostate cell

lines. The viability of PNT1A, LNCaP, DU145 and PC3 cells in response to 20 μM diosgenin was tested. It was confirmed that 20 μM diosgenin had no effect on the viability of PNT1A cells (Fig. 8.1B). Nevertheless, 20 μM diosgenin significantly diminished PCa cells' viability compared to the respective control groups (by approximately 30 % in LNCaP cells, and 60 % in DU145 and PC3, Fig. 8.1B). Representative images of Hoechst-stained nuclei (Fig. 8.1C) support the reduction in PCa cell' viability and cell number upon exposure to 20 μM diosgenin, which is visible compared with the unaltered cell number/viability in PNT1A cells (Fig. 8.1C).

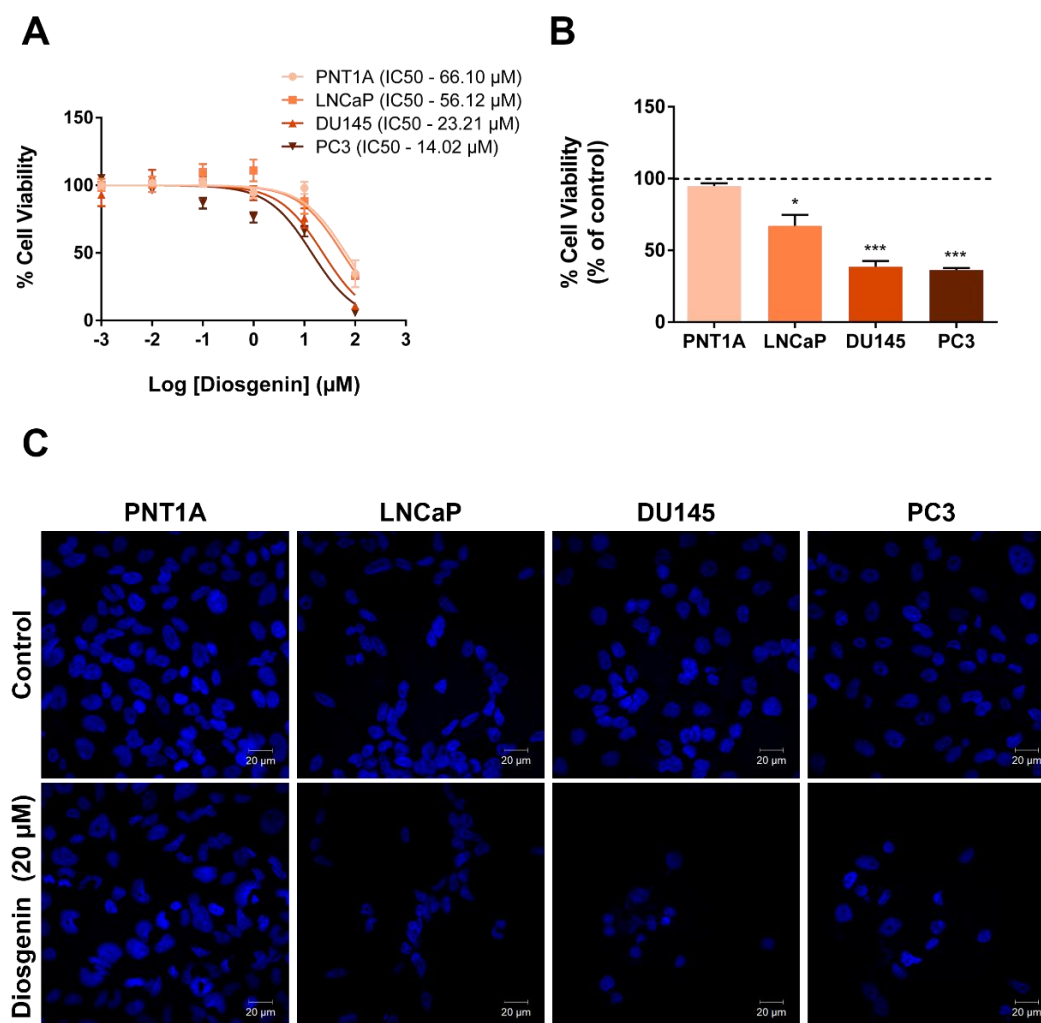


Figure 7.1. Effect of diosgenin on the viability of non-neoplastic (PNT1A) and neoplastic (LNCaP, DU145 and PC3) human prostate cells. (A) Diosgenin concentration-response curve and IC₅₀ determined by MTT analysis of cell viability with a concentration range from 0.001 to 100 μM for 24 h. The IC₅₀ value was obtained by the logarithmic transformation of the concentrations. (B) Cell viability after treatment with 20 μM Diosgenin for 24 h. For each cell line, results are expressed as % of control (0 μM Diosgenin, dashed line). Error bars indicate mean S.E.M. * $P < 0.05$, *** $P < 0.001$. (C) Representative fluorescence microscopy images of Hoechst 33342 stained cell nuclei in control and diosgenin-treated cells. Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany) under 400 X magnification.

8.3.2. Diosgenin effects in suppressing the viability of PCa cells are increased in the presence of the GPER agonist G1

PNT1A, LNCaP, DU145 and PC3 cells were treated with 20 μ M diosgenin and 1 μ M GPER agonist G1, alone or in combination. Diosgenin did not influence PNT1A cell viability while suppressing the viability of LNCaP, DU145 and PC3 cells by approximately 28, 49 and 43%, respectively (Fig. 8.2). G1 also reduced LNCaP, DU145 and PC3 cells viability, respectively, by 18, 64 and 32%, approximately (Fig. 8.2). However, it increased the viability of PNT1A cells (~31%). The combined treatment of diosgenin plus G1 had a highly pronounced effect diminishing PCa cells viability, which was reduced by 83, 95 and 76% in LNCaP, DU145 and PC3 cells, respectively). Diosgenin did not change G1 effect increasing PNT1A cells viability (Fig. 8.2).

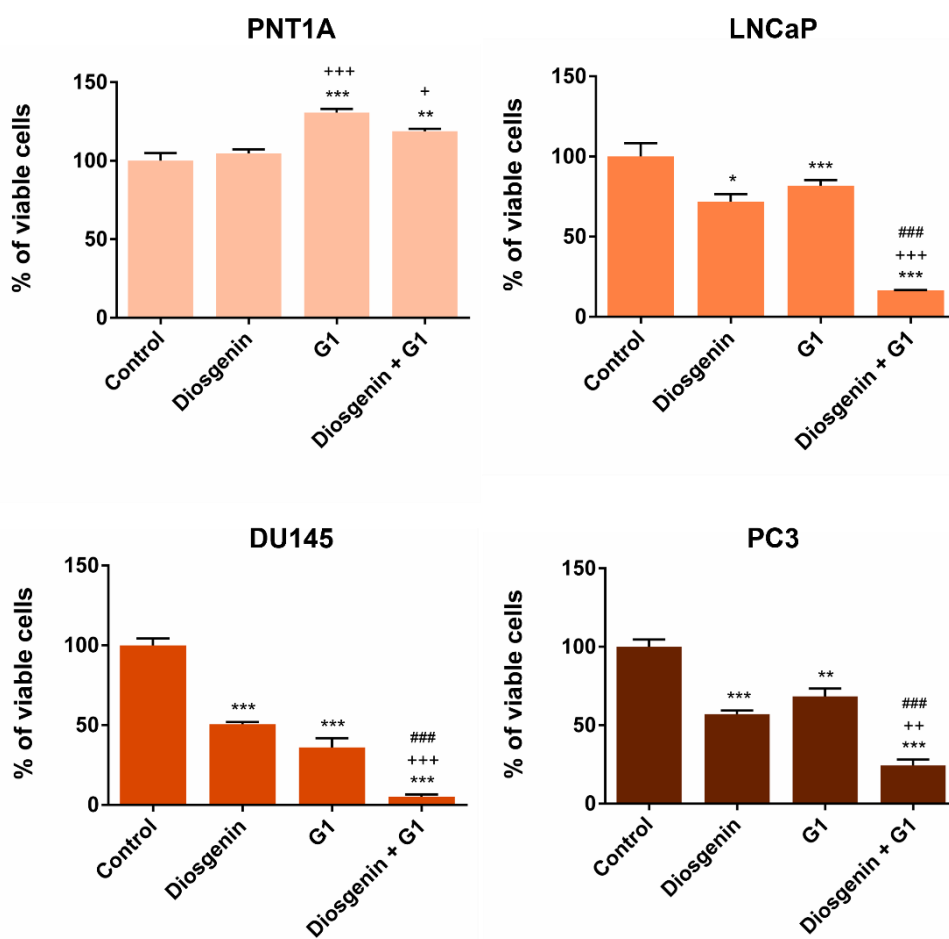
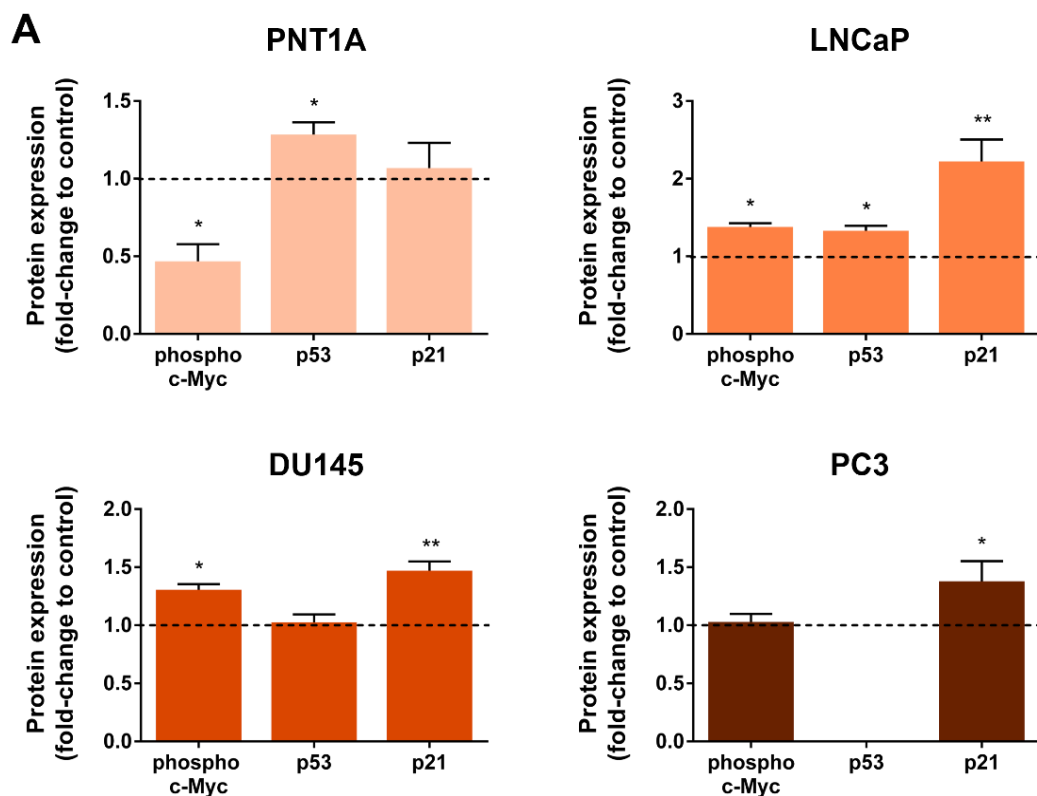


Figure 8.2. Viability of non-neoplastic (PNT1A) and neoplastic (LNCaP, DU145 and PC3) human prostate cells in response to diosgenin and GPER agonist G1. Cells were treated with 20 μ M diosgenin alone or in combination with 1 μ M G1 for 24 hours and viability determined by the MTT assay. Results are expressed as % of control. Error bars indicate mean $\pm$ S.E.M. * P <0.05 ** P <0.01 *** P <0.001 compared to control; + P <0.05 ++ P <0.01 +++ P <0.001 compared to Diosgenin; # P < 0.05 ### P < 0.001 compared to G1; \$\$ P < 0.01 \$\$\$ P < 0.001 compared to Diosgenin + G1.

Considering the effects of diosgenin in suppressing PCa cells viability, we evaluated its influence on the expression of cell cycle regulators, namely, phospho c-Myc, p53 and p21 (Fig. 8.3), as they are essential targets in PCa [62]. Diosgenin reduced the expression of phospho-c-Myc (0.47 ± 0.11 fold-change relative to control) in PNT1A cells (Fig. 8.3A), though increasing it in PCa cells. Exposure to diosgenin increased phospho-c-Myc expression in LNCaP and DU145 cells, respectively, by 1.38 ± 0.05 and 1.31 ± 0.05 fold-change relative to control (Fig. 8.3A). No changes were observed in phospho-c-Myc in PC3-treated cells (Fig. 8.3A).

PNT1A and LNCaP showed increased p53 expression after treatment with diosgenin (1.28 ± 0.08 and 1.33 ± 0.07 fold-change relative to control, respectively, Fig. 8.3A). We did not detect p53 in PC3 cells, as these cells are known to be p53 negative [63]. Concerning p21, diosgenin increased its expression in all PCa cell lines (2.22 ± 0.28 ; 1.47 ± 0.08 and 1.38 ± 0.17 fold-change relative to control, in LNCaP, DU145 and PC3, respectively, Fig. 8.3A). G1 did not change p21 expression in PNT1A cells (Fig. 8.3A).



B

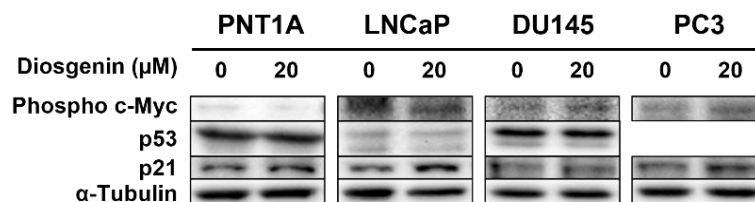
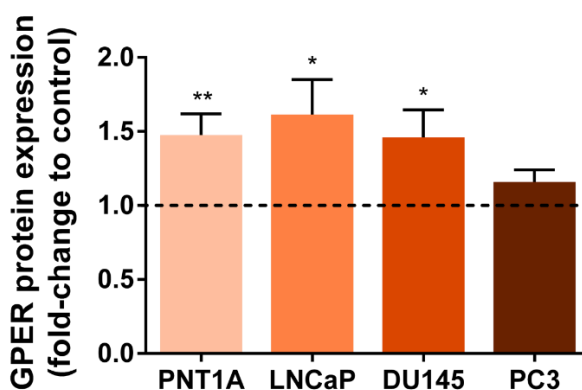


Figure 8.3. Effect of diosgenin on the expression of cell cycle regulators in non-neoplastic (PNT1A) and neoplastic (LNCaP, DU145 and PC3) human prostate cells. Cells were treated with 20 μM diosgenin for 24 h. (A) Protein expression of phospho c-Myc, p53 and p21 determined by WB analysis after normalization with α-tubulin. Results are expressed as fold-change relative to control untreated group (0 μM Diosgenin, dashed line). Error bars indicate mean ± S.E.M. **P* < 0.05; ***P* < 0.01. (B) Representative immunoblots are shown in the bottom panel.

8.3.3. Diosgenin increased GPER expression in non-neoplastic and neoplastic human prostate cells

Previously published data indicated that some phytoestrogens showed to reduce PCa growth by modulating GPER expression. Therefore, we decided to investigate if diosgenin regulates GPER expression in prostate cells. Non-neoplastic PNT1A and neoplastic LNCaP, DU145 and PC3 cells were treated with 20 μM diosgenin for 24 h, and GPER protein expression was analysed by WB. Diosgenin treatment increased GPER expression in both non-neoplastic PNT1A (1.48 ± 0.14 fold-change relative to control) and neoplastic LNCaP and DU145 cells (1.61 ± 0.24 and 1.46 ± 0.19 fold-change relative to control, respectively) (Fig. 8.4). No changes were observed in GPER expression in PC3 cells after diosgenin stimulation (Fig.8. 4).

A



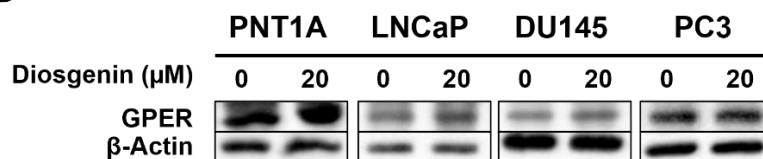
B

Figure 8.4. Effect of diosgenin in regulating GP_{ER} expression in non-neoplastic (PNT1A) and neoplastic (LNCaP, DU145 and PC3) human prostate cells. Cells were treated with 20 μM diosgenin for 24 h. (A) GP_{ER} protein expression determined by WB analysis after normalized with β-actin. Results are expressed as fold-change relative to the control untreated group (0 μM diosgenin, dashed line). Error bars indicate mean ± S.E.M. **P* < 0.05 ***P* < 0.01. (B) Representative immunoblots.

8.3.4. Diosgenin-induced apoptosis of prostate cells is enhanced by G1 and suppressed by GP_{ER} knockdown

To evaluate the role of diosgenin in prostate cell apoptosis, caspase-3-like activity was assessed in PNT1A, LNCaP, DU145 and PC3 cells after treatment with diosgenin or/and G1. Upon exposure to diosgenin, caspase-3-like activity augmented in PNT1A, LNCaP and PC3 cells by 1.42 ± 0.05 , 1.42 ± 0.08 and 1.54 ± 0.11 fold-change relative to control (Fig. 8.5A), respectively. Also, G1 increased caspase-3-like activity in all cell lines except DU145 (1.42 ± 0.01 , 1.38 ± 0.03 , and 1.63 ± 0.03 fold-change relative to control in PNT1A, LNCaP and PC3 cells, respectively, Fig. 8.5A).

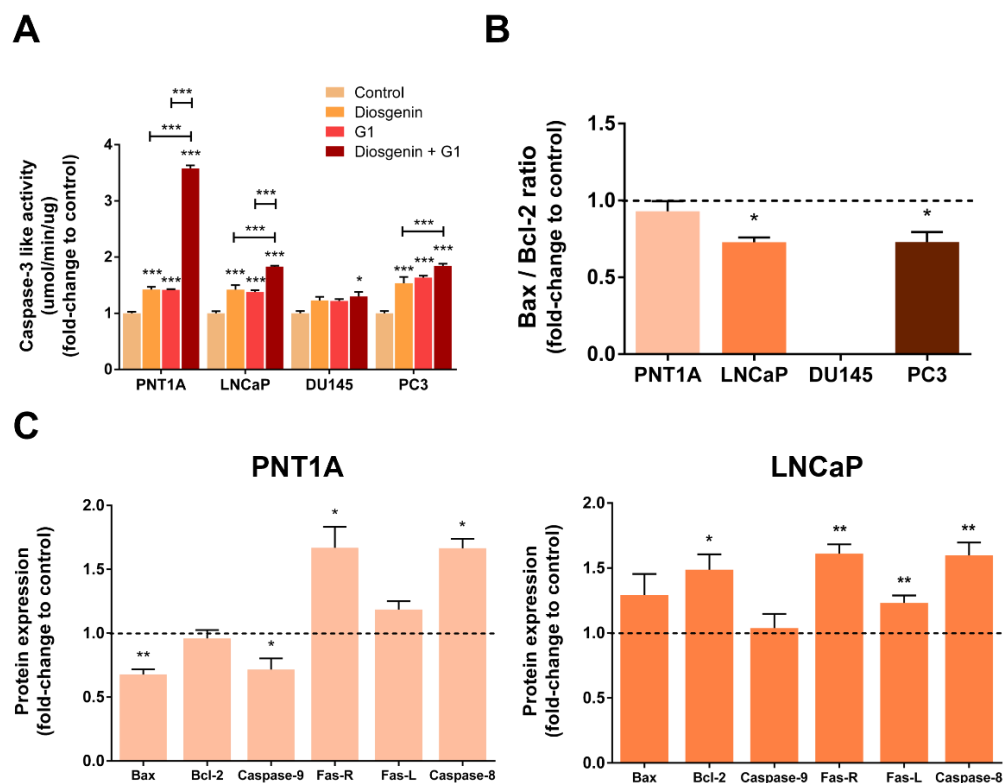
Diosgenin effects were accentuated in combination with G1. Caspase-3-like activity was significantly enhanced in PNT1A (3.58 ± 0.05 fold-change relative to control), LNCaP (1.83 ± 0.02 fold-change relative to control), and PC3 (1.84 ± 0.04 fold-change relative to control) cells compared to the effect of diosgenin alone (Fig. 8.5A). Furthermore, contrarily to the use of compounds alone, the combination of diosgenin plus G1 increased caspase-3-like activity in DU145 cells (1.30 ± 0.08 fold-change relative to control, Fig. 8.5A).

The expression of regulators of both the intrinsic and extrinsic pathways of apoptosis was also evaluated (Fig. 8.5E). Diosgenin reduced the Bax/Bcl-2 ratio in LNCaP and PC3 cells (0.73 ± 0.03 and 0.73 ± 0.06 fold-change relative to control, Fig. 8.5B). In PNT1A cells, despite the reduced expression of the pro-apoptotic protein Bax in response to diosgenin (0.68 ± 0.04 fold-change relative to control, Fig. 8.5C and D), the Bax/Bcl-2 ratio remained unchanged (Fig. 8.5B). Concerning PCa cells, diosgenin increased Bax expression, although without statistical significance in LNCaP cells (Fig. 8.5C and D).

The expression levels of the Bcl-2 (anti-apoptotic) increased after diosgenin treatment (Fig. 8.5C and D) in LNCaP and DU145 cells (1.49 ± 0.12 and 1.50 ± 0.07 fold-change relative to control, respectively, Fig. 8.5C and D), with no effects being perceived in PNT1A and PC3.

Levels of the initiator caspase-9 upon exposure to diosgenin were increased in DU145 cells only (1.28 ± 0.05 and 1.26 ± 0.05 fold change to control, Fig. 8.5C and D). Contrarily, diosgenin significantly reduced caspase-9 expression in PN1A cells (0.72 ± 0.09 fold-change relative to control, Fig. 8.5C and D).

Regarding the extrinsic pathway of apoptosis (Fig. 8.5E), treatment with diosgenin increased Fas receptor expression in the non-neoplastic cells PNT1A (1.67 ± 0.17 fold change to control, Fig. 8.5C and D), and in LNCaP and PC3 cells (1.61 ± 0.07 and 1.26 ± 0.06 fold-change relative to control, respectively, Fig. 8.5C and D). Additionally, diosgenin induced Fas-L expression in PCa cells (1.23 ± 0.06 ; 1.45 ± 0.10 and 1.37 ± 0.06 fold-change relative to control, respectively for LNCaP, DU145 and PC3, Fig. 8.5C and D). The expression of the caspase-8 was increased in response to diosgenin stimulation in PNT1A, LNCaP and DU145 cells (1.67 ± 0.07 ; 1.60 ± 0.10 and 1.48 ± 0.10 fold-change relative to control, respectively, Fig. 8.5C and D).



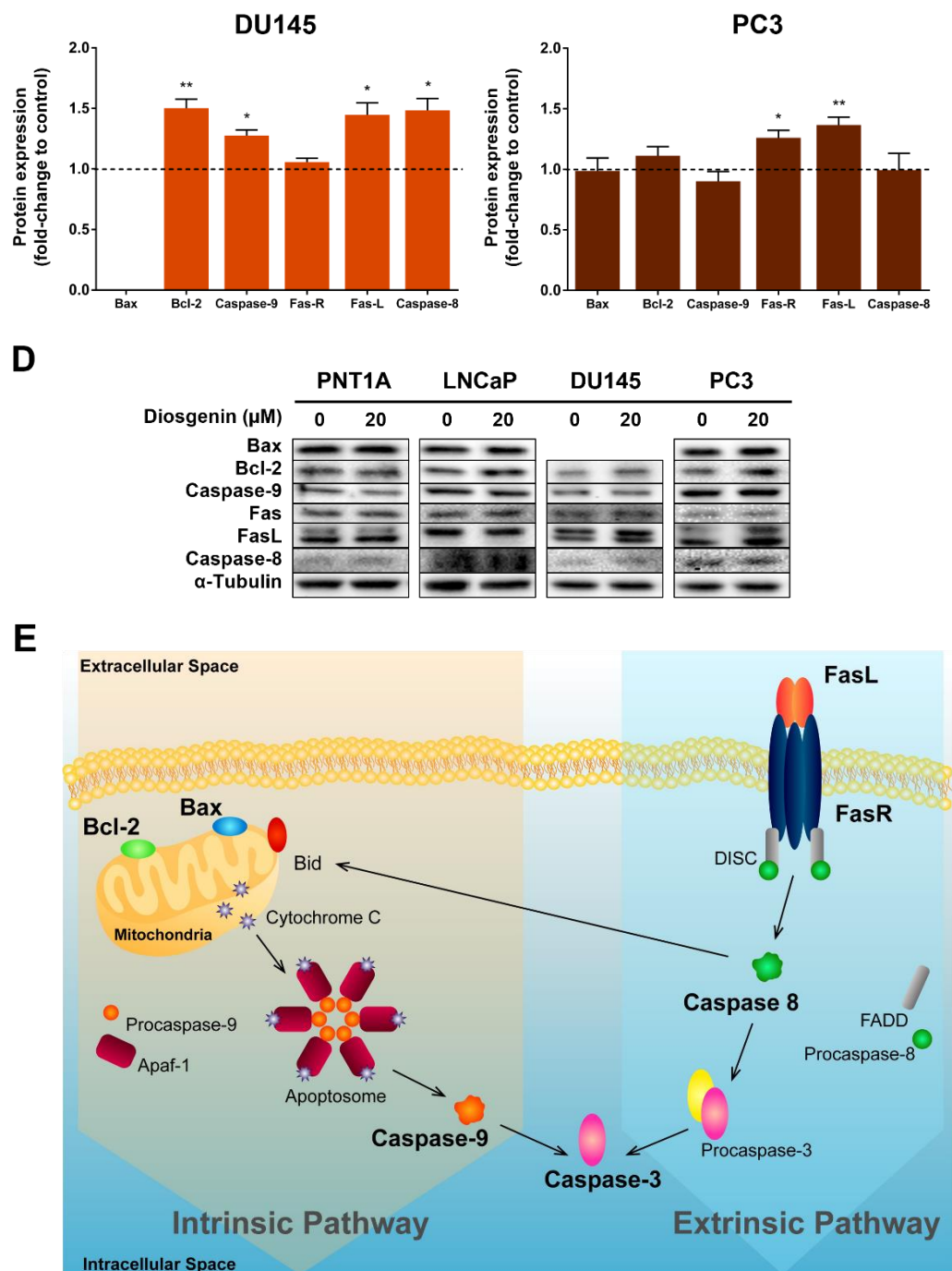


Figure 8.5. Effect of diosgenin alone or in combination with G1 in non-neoplastic (PNT1A) and neoplastic (LNCaP, DU145 and PC3) human prostate cells apoptosis. Cells were treated with 20 μ M diosgenin and 1 μ M G1 for 24 h. (A) Caspase-3 like activity determined spectrophotometrically. (B) Bax and Bcl-2 protein ratio. (C) Protein expression of regulators of the intrinsic and extrinsic pathways of apoptosis determined by WB analysis after normalization with α -tubulin. Results are expressed as fold-change relative to the control group (0 μ M Diosgenin, dashed line). Error bars indicate mean $\pm$ S.E.M. * P < 0.05 ** P < 0.01 *** P < 0.001. (D) Representative immunoblots. (E) Schematic representation of apoptotic pathways. Two central pathways are involved in apoptosis activation: the intrinsic pathway and extrinsic pathway. When intrinsic pathway is activated, Bax acts leading to cytochrome c released from mitochondria, which binds to apoptotic protease activating factor-1 (Apaf-1). Then, it binds to procaspase-9 and cleaves it originating its active form caspase-9, which in turn cleaves and activates procaspase-3 into

the effector caspase-3. The extrinsic pathway of apoptosis is activated by the interaction between Fas-R and Fas-L, resulting in the formation of the death-inducing signaling complex (DISC), which contains the Fas-associated death domain protein (FADD) and procaspase-8. Upon recruitment by FADD, procaspase-8 is activated by self-cleavage, and then activates other downstream caspases, namely, caspase-3, the endpoint of both intrinsic and extrinsic pathways. Furthermore, caspase-8 can cleave (Bcl-2 interacting protein (BID), that translocates to mitochondria, triggering cytochrome C release, and following the intrinsic pathway.

To investigate whether diosgenin can act via GPER, PC3 cells were transfected with siRNA targeting GPER and scramble siRNAs (control) and caspase-3-like activity was evaluated (Fig. 8.6). GPER siRNA transfection significantly reduced GPER protein expression by approximately 30% (Fig. 8.6A). *GPER* knockdown suppressed the effect of diosgenin increasing caspase-3-like activity in PC3 cells (Fig. 8.6B).

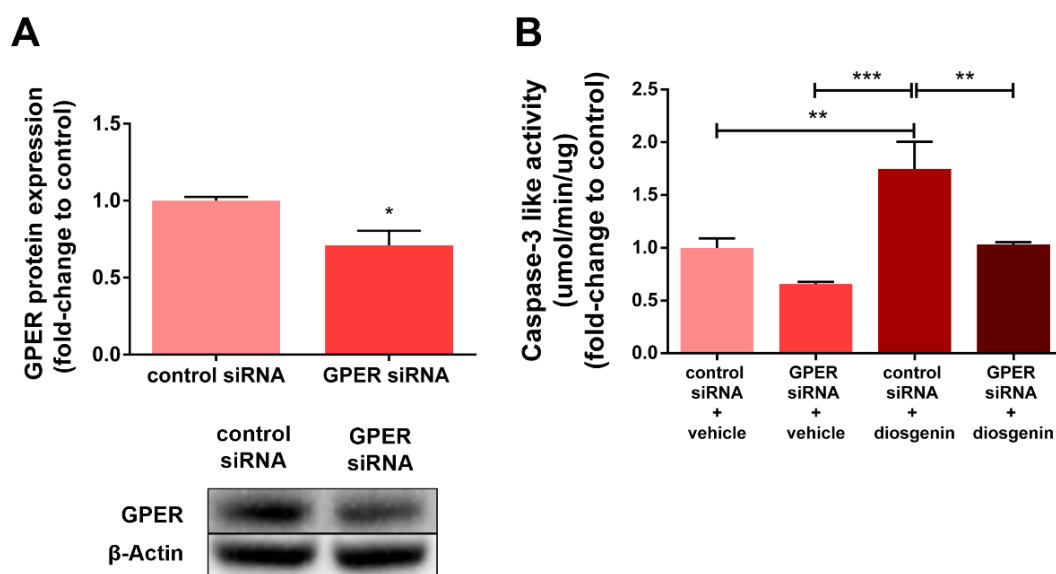


Figure 8.6. *GPER* knockdown abrogates the diosgenin effects inducing apoptosis of human neoplastic PC3 cells. Cells were transfected with 40 nM of a nontargeting siRNA (control siRNA) or a siRNA targeting *GPER* (*GPER* siRNA), using Lipofectamine RNAiMAX. (A) *GPER* protein expression determined by WB analysis after normalization with β -actin. Representative immunoblots are shown in bottom panel. (B) Caspase-3 like activity determined spectrophotometrically. Results are expressed as fold-change to the control siRNA + vehicle group. Error bars indicate mean $\pm$ S.E.M. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

8.3.5. Diosgenin modulated the glycolytic metabolism of prostate cells

Metabolic reprogramming by a switch from oxidative phosphorylation to glycolysis, even in aerobic conditions, is a well-known characteristic of cancer cells [64, 65], which in the case of PCa is mainly associated to the advanced stages of disease [66-68]. These metabolic alterations were established as a cancer hallmark in the last decade, as they highly sustain cancer cell growth and invasiveness [66, 69]. Therefore, we aimed to investigate the effect of diosgenin influencing the glycolytic profile of prostate cells. Stimulation with diosgenin significantly increased glucose consumption in PNT1A and CRPC cells (3.45 ± 0.61 , 1.74 ± 0.03 and 1.49 ± 0.04 fold-change relative to control in PNT1A, DU145 and PC3 cells, respectively, Fig. 8.7A), with no significant effect in LNCaP cells (Fig. 8.7A). Also, G1 augmented glucose consumption in all cell lines (3.34 ± 0.37 ; 1.93 ± 0.12 ; 1.76 ± 0.07 and 1.31 ± 0.06 fold-change relative to control in PNT1A, LNCaP, DU145 and PC3 cells, respectively, Fig. 8.7A). Moreover, the combination of diosgenin and G1 showed to improve the diosgenin and/or G1 - induced augment of glucose consumption in CRPC cells (2.62 ± 0.02 and 1.69 ± 0.03 fold-change relative to control in DU145 and PC3 cells, respectively, Fig. 8.7A). In PNT1A, the combined diosgenin and G1 treatment had no effect on glucose consumption comparatively to the compounds alone (Fig. 8.7A).

The increased consumption of glucose in diosgenin- or G1-treated CRPC cells was accompanied by the enhanced lactate production. Diosgenin increased lactate production by 1.97 ± 0.09 and 1.50 ± 0.03 fold-change relative to control in DU145 and PC3 cells, respectively (Fig. 8.7B). For G1 variation was as 1.97 ± 0.02 and 1.51 ± 0.09 fold-change relative to control in DU145 and PC3 cells, respectively (Fig. 8.7B). Furthermore, the combined diosgenin and G1 treatment strongly increased lactate production in all the cells lines by approximately 2-3 times compared to the control group (Fig. 8.7B). Nevertheless, the activity of LDH, the enzyme responsible for the conversion of pyruvate to lactate, only was changed in diosgenin plus G1-treated LNCaP cells (approximately 2.5 fold-changed compared to the control group (Fig. 8.7C). LDH expression was reduced by diosgenin in PNT1A cells (0.71 ± 0.03 fold-change relative to control, Fig. 8.7D and E). On the other hand, diosgenin increased LDH protein levels in PC3 cells (1.45 ± 0.14 fold-change relative to control); no changes were perceived on the expression of LDH in LNCaP or DU145 cells (Fig. 8.7D and E).

GLUT1 expression was reduced by diosgenin in PNT1A and PC3 cells (0.80 ± 0.03 , 0.89 ± 0.02 and 0.61 ± 0.15 fold-change relative to control, respectively, Fig. 8.7D and E), but diosgenin-treated LNCaP and DU145 cells display augmented GLUT1

expression (1.54 ± 0.13 and 1.44 ± 0.05 fold-change relative to control, respectively, Fig. 8.7D and E).

GLUT2 expression was increased by diosgenin treatment in the non-neoplastic PNT1A cells (2.00 ± 0.38 fold-change relative to control, Fig. 8.7D and E) and LNCaP cells (1.75 ± 0.28 fold-change relative to control), whereas no changes were observed in DU145 or PC3 cells (Fig. 8.7D and E).

Regarding GLUT3, diosgenin reduced its expression in PNT1A cells (0.80 ± 0.03 0.89 ± 0.02 and 0.88 ± 0.02 fold-change relative to control, respectively Fig. 7D and E) and augmented it in LNCaP and PC3 cells (2.06 ± 0.26 and 1.17 ± 0.04 fold-change relative to control, respectively, Fig. 8.7D and E). No effect was observed in DU145 cells (Fig. 8.7D and E).

PFK1, is an enzyme that catalyzes a limiting step of glycolysis, converting fructose 6-phosphate to fructose 1,6-bisphosphate [70]. Stimulation with diosgenin reduced PFK1 expression in PNT1A and PC3 cells (0.68 ± 0.02 and 0.81 ± 0.04 fold-change relative to control, respectively, 8.7D and E). On the other hand, diosgenin increased PFK1 expression in LNCaP cells (1.60 ± 0.09 fold-change relative to control, 8.7D and E). No changes were observed in PFK1 expression in diosgenin-treated DU145 cells (Fig. 8.7D and E).

The expression of MCT4, the monocarboxylate transporter responsible for lactate export, was reduced by diosgenin in PNT1A (0.64 ± 0.03 fold-change relative to control) and PC3 cells (0.63 ± 0.10 fold-change relative to control) (Fig. 8.7D and E). Contrarily, DU145 displayed increased expression of MCT4 after treatment with diosgenin (1.52 ± 0.05 fold-change relative to control); no changes were observed in LNCaP cells (Fig. 8.7D and E). Diosgenin was also found to augment GS2 in all cell lines (PNT1A: 1.56 ± 0.02 ; LNCaP: 2.03 ± 0.33 ; DU145: 1.91 ± 0.25 and PC3: 1.56 ± 0.14 fold-change relative to control, Fig. 8.7D and E).

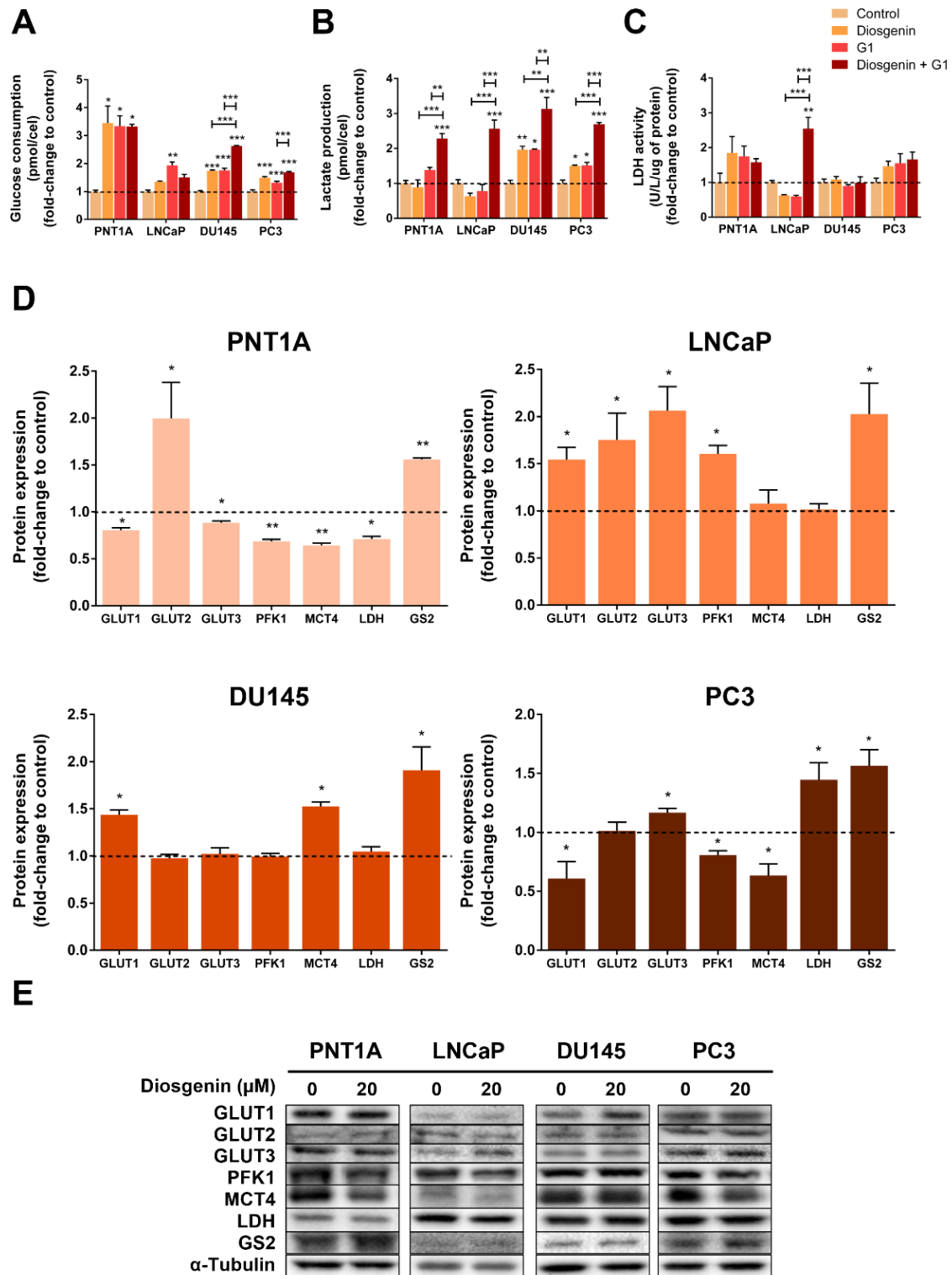


Figure 8.7. Diosgenin alone or in combination with G1 in modulating the glycolytic profile of non-neoplastic (PNT1A) and neoplastic (LNCaP, DU145 and PC3) human prostate cells. Cells were treated with 20 μM diosgenin and 1 μM G1 for 24 h. (A) Glucose consumption, (B) Lactate production and (C) LDH activity, determined spectrophotometrically. (D) Protein expression of glycolytic metabolism regulator proteins determined by WB analysis after normalization with α-tubulin. Results are expressed as fold-change to the control untreated group (0 μM diosgenin and 0 μM G1, dashed line). Error bars indicate mean ± S.E.M. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$; (E) Representative immunoblots.

8.4. Discussion

The present work investigated the effects of diosgenin and the contribution of this natural compound in counteracting PCa cell features. We started by characterizing the concentration-response curve to diosgenin in non-neoplastic (PNT1A) and neoplastic (LNCaP, DU145 and PC3) human prostate cells. Diosgenin reduced prostate cell viability in a concentration-dependent manner, with IC₅₀ values ranging from 14.02 to 66.10 μ M. These findings resemble preceding studies though the diosgenin IC₅₀ values herein reported were slightly lower [28-30, 71, 72].

Interestingly, CRPC DU145 and PC3 cells, representing more aggressive stages of PCa, were the most sensitive to diosgenin, which also reduced LNCaP viability but in less extension and did not affect the viability of non-neoplastic PNT1A cells. These results underlie the potential therapeutic interest of diosgenin in targeting the more aggressive stages of PCa, namely, when the tumour becomes resistant to androgens, which is further supported by the ability of other steroidal saponins to reduce CRPC cells growth [73]. Moreover, lower diosgenin concentrations (5 μ M) showed to reduce the viability of DU145 cells but not of LNCaP cells [71], confirming the sensibility of CRPC cells.

Reports indicate that some phytoestrogens could reduce PCa growth through interaction with GPER [50, 74]. Therefore, we analyzed diosgenin's effects in combination with the GPER agonist G1. Although not altering PNT1A cells' viability, diosgenin reduced the viability of PCa cells, an effect that was much higher in the presence of G1. Moreover, diosgenin only increased PNT1A viability when combined with G1. Curiously, diosgenin augmented the expression of GPER in both non-neoplastic and neoplastic cells (not significantly in PC3), which may explain the enhanced results of G1 plus diosgenin suppressing cell viability compared with the isolated compounds. Other phytoestrogens, such as genisten, were found to regulate classical ERs and GPER, up- or down-regulating its expression depending on the tissue [75-79]. However, to the best of our knowledge, this is the first report of diosgenin controlling GPER expression.

The reported changes on cell viability caused by diosgenin treatment were underpinned by the altered expression of cell cycle and cell survival regulators. The oncoprotein c-Myc is a strong promoter of cell cycle progression, leading to cell proliferation [80]. It is also known that the stability of c-Myc is regulated by phosphorylation at the NH₂-terminal. ERK-mediated phosphorylation at Ser 62 stabilizes c-Myc, whereas glycogen synthase kinase-3-mediated phosphorylation at Thr 58 accelerates ubiquitin-mediated degradation of the oncoprotein [81-84]. We have

found that diosgenin decreased the expression of phosphorylated c-Myc in PNT1A cells but increased it in LNCaP and DU145, which suggests that different mechanisms are being activated.

c-Myc is also known to suppress the expression of other cell cycle regulators, namely, p21, a cell cycle inhibitor that inhibits the activity of most cyclin-dependent kinases, and tumour suppressor p53 [85-89]. The expression levels of p53 were augmented in PNT1A and LNCaP cells, and all PCa cell lines showed increased expression of p21 in response to diosgenin treatment. These results are in agreement with the reduced viability found in PCa cells lines upon exposure to this phytoestrogen. Moreover, they sustain the hypothesis that the increased phosphorylated-c-Myc is being signaled for degradation, and not repressing p21 expression [90-92]. Further investigation is needed to corroborate this hypothesis. Nevertheless, in other cancer cell types, it was shown that diosgenin could revert the TNF effects, increasing back p21 levels [93].

In PCa also were reported the effects of diosgenin inducing apoptosis [29, 72, 94], but the mechanism of action was not solved. Accordingly, we found that diosgenin treatment augmented the activity of caspase-3, an endpoint of apoptotic signaling [95] in PNT1A, LNCaP and PC3 cells. This effect was enhanced in the presence of G1, which suggests an interplay between diosgenin and GPER actions. As discussed above, this response could be related to the increase of GPER levels by diosgenin, which may explain the enhanced effects when both compounds are combined. Furthermore, diosgenin-induced apoptosis of PC3 cells, not with a significant increase of GPER expression, was abrogated by the *GPER* knockdown, which proves that diosgenin actions driven apoptosis require GPER. Although other studies have identified the involvement of GPER in phytosteroids effects triggering apoptosis in cancer cells [96, 97], this is the first report linking diosgenin with GPER activation in PCa.

Altered caspase-3 activity was followed by the altered expression of apoptosis regulators. Despite the increase of caspase-3 activity, Bax/Bcl-2 ratio was reduced by diosgenin in LNCaP and PC3 cells, which can be explained as diosgenin increased Bcl-2 expression in LNCaP cells, and slightly increased it, but without statistical significance, in PC3-treated cells. The pro-apoptotic/antiapoptotic Bax/Bcl-2 ratio was not determined in DU145 cells, which do not express the Bax protein [98-100], and remained unchanged in PNT1A-treated cells despite the reduced Bax expression.

PNT1A cells also displayed reduced caspase-9 expression, whereas showing increased expression of Fas-R and caspase-8, mediators of the extrinsic pathway of apoptosis [101]. Fas-R, Fas-L and caspase-8 protein expression also were increased in

LNCaP and PC3-stimulated cells, which supports that diosgenin induces apoptosis by activating the extrinsic pathway.

In the case of DU145 cells, it cannot be excluded that both the intrinsic and extrinsic apoptotic pathways are activated in response to diosgenin treatment, as these cells displayed increased expression of caspase-9, but also Fas-L and caspase-8. Another possibility is that the augment observed in caspase-9 may be explained by the cross activation of the intrinsic pathway through caspase-8 [102], which is reasonable to consider as diosgenin showed to inhibit FLIP and activate caspase-8 in the Fas-related apoptotic pathway in human thyrocytes [103].

A well-known characteristic of cancer cells is its metabolic reprogramming, using glycolysis instead of oxidative phosphorylation, even in aerobic conditions [64, 65]. Concerning PCa, this switch is mainly associated to the advanced stages of disease with the metabolism of primary tumors being less glycolytic and dependent on oxidative phosphorylation [66-68, 104, 105]. Since these metabolic alterations sustain cancer cell growth and progression, being established as a cancer hallmark [66, 69], we investigated the effect of diosgenin in the glycolytic profile of prostate cells. The steroid sapogenin enhanced glucose consumption in PNT1A, DU145 and PC3 cell lines. Also, in LNCaP diosgenin slightly increased glucose consumption, although without significance. These results are in line with the augmented expression of GLUTs transporters. In fact, the distinct GLUTs isoforms were differentially regulated by diosgenin depending on the cell line, suggesting that each cell line may use different transporters for glucose uptake.

Entering the glycolytic pathway, glucose is converted into pyruvate, the final product of glycolysis [70]. PFK1 enzyme catalyzes the conversion of fructose 6-phosphate into fructose 1,6-bisphosphate, in a limiting step of glycolysis, indicating the rate of glycolytic flux [70]. Curiously, this enzyme was only augmented in LNCaP cells and was reduced by diosgenin treatment in PNT1A and PC3 cells, with no changes observed in DU145 cells. These results suggest that in LNCaP cells, glucose enter the glycolytic pathway, but there are no conversion of resultant pyruvate to lactate. The pyruvate produced in glycolysis can be used in other pathways, namely, entering the mitochondria to be oxidized to acetyl CoA and combined with oxaloacetate to start the tricarboxylic acid (TCA) cycle and following for oxidative phosphorylation [106]. Our results in PNT1A and PC3 suggest that glucose was not being driven for glycolysis. Instead of enter the glycolytic cascade, glucose can be converted into glycogen through the enzyme GS [107], which was increased in all diosgenin-treated cell lines, suggesting that glucose is being stored. Nevertheless, the increased glucose consumption was accompanied by the augmented lactate production in CRPC cells, which was supported

by the augmented expression levels of LDH, the enzyme responsible for the conversion of pyruvate into lactate [108] in PC3 cells; and the increased expression of MCT4, the transporter responsible for lactate export to the extracellular space [108, 109] in DU145. In CRPC, the increased lactate production was not followed by changes in the expression of PFK and LDH in case of DU145. Moreover, in PC3 cells, PFK1 and MCT4 showed to be down-regulated by diosgenin. Thus, these results suggest also that lactate could be produced from other sources, and not from glucose [110, 111] in CRPC cells. Possible alternative sources of pyruvate include malate transformation by cytosolic malic enzyme, or aminoacids like alanine, serine, threonine, glycine, cysteine and tryptophan [112]. Previous research found the ability of diosgenin to reduce the overall glycolytic flux and oxygen consumption rate, also inhibiting the mitochondrial oxidative phosphorylation capacity and suppressing the non-mitochondrial respiration of colorectal cancer cells [113]. Contrarily to our results, this study also found that diosgenin inhibited the glucose uptake and lactate production, with reduction of GLUT3, GLUT4 and pyruvate carboxylase genes, and without alterations in GLUT1, GLUT2 and phosphoglycerate kinase genes [113].

Interestingly, the combined treatment of diosgenin plus G1 enhanced the effects in glycolytic metabolism of the compounds alone. Taken together, these results propose that diosgenin modulates metabolism of glucose, but not driving it through glycolysis. Instead, a storage of glucose in response of diosgenin can be hypothesized, but further investigation is needed.

In conclusion, our results showed that diosgenin diminished cell viability, increased apoptosis and modulated the glycolytic metabolism of PCa cells, supporting the anti-tumorigenic role of this compound in PCa. Overall, the effects of diosgenin were enhanced when combined with G1, which is supported by the ability of diosgenin to augment GPER expression. Moreover, we showed for the first time that GPER is required for diosgenin activation of apoptosis in PCa cells.

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Chapter 9

Concluding remarks and future perspectives

9. Concluding remarks

PCa continues to deserve attention because of its high prevalence and increasing incidence worldwide. Despite continuous research, and clinical and technological advances, the existent options for PCa management are not always effective. Understanding the particularities of prostate tumors and the mechanisms that sustain the escape to therapy remains a relevant scientific challenge. The diversity of the cellular, structural, and molecular changes involved in PCa make it a multifaceted disease. PCa development and progression are laid down by a set of genetic events, such as somatic mutations, chromosomal alterations, alternative splicing and also epigenetic changes. These gene alterations are the drivers for increasing oncogenes expression and/or reducing tumor suppressor genes, which lead to dysregulation of a panoply of cellular processes, including abnormal proliferation, suppression of cell death, EMT transition, and acquisition of migration and invasion capabilities. The bioenergetic metabolism is also reprogramed, accompanying the onset and progression of PCa, as a strategy to maintain the energy supply required for maintaining proliferative activity and tumor growth.

Furthermore, PCa is a hormone-dependent cancer, and thus highly dependent on hormone actions, which control all the processes mentioned above, adding an increasing complexity to the approaches for tackling the disease. At the initial stages, PCa is highly regulated by androgens, which contribute to cell proliferation and survival. However, in advanced phases of disease, PCa cells develop resistance to androgens and become skilled at surviving and proliferating even if the circulating androgens levels are low. This represents the establishment of the so-called CRPC, that is characterized by the failure of ADT. Therefore, it still is necessary to find out therapies that slow down the progression of disease to the CRPC, as well as it is mandatory to define effective treatment approaches depending on the PCa stage.

In the scenario of PCa, as hormone-dependent cancer, also estrogens have something to say. Huggins and Hodges were the first to provide the foundation for the use of hormones in therapy, demonstrating that PCa is a hormone-sensitive cancer, stimulated by androgenic activity and inhibited by suppression of androgens levels or estrogens administration. In fact, estrogens were used in PCa treatment for decades, being an effective therapy for delaying the progression of metastatic PCa and were discontinued only because of the adverse side effects.

Despite the clinical utility of estrogens as therapeutic options and the efforts to measure circulating levels of these hormones in men, most studies failed to establish a relationship between PCa and serum E₂ levels. As discussed in Chapter 2, there is a scientific base for this lack of liaison, which is probably due to the high variability of E₂ concentrations reported as a consequence of methodological issues, age group, ethnicity or physiological and pathological conditions, such as inflammation, obesity, specificities on aromatase activity or other factors that were not sufficiently covered by the different studies.

Nevertheless, strong evidence of the beneficial effects of estrogens against PCa comes from the fact that Eastern countries, with a plant-based diet and higher consumption of phytoestrogens, present a lower incidence of disease. Considering this, the interest in the therapeutic potential of these compounds has gained interest in the last years and represents a pharmacological challenge in developing new approaches that maintain the efficacy of estrogens-like compounds controlling PCa development with mitigation of side effects. In Chapter 2, we reviewed exhaustively the reported effects of several phytoestrogens in PCa. Control of PCa cell proliferation and tumor growth, reduction of migration and invasiveness capabilities, suppression of EMT, angiogenesis and metastization, and also the regulation of the bioenergetic metabolism of PCa cells were among the effects on the dependency of phytoestrogens activity. Considering its tumor-suppressor properties, phytoestrogens, such as chrysin, daidzein, genistein, quercetin, coumestrol, resveratrol and equol, have been pointed out as possible alternative therapies for PCa, inclusively with tests in humans performed. Some of the most promising estrogen-like agents have been shown to reduce PSA levels and lower PCa risk and incidence when used in diet, which highlights its potential for use in PCa therapy, alone or in combination with conventional treatments such as chemotherapy, radiation therapy and immunotherapy, enhancing their efficacy and/or mitigating the systemic adverse effects. Another promising phytosteroid is diosgenin, a sapogenin with estrogen-like properties that seems to have a broad action as an anti-cancer molecule in several types of cancer. However, its action in PCa is less explored. Its similar structure to cholesterol and other steroids makes it an attractive compound for pharmaceutical industry. In fact, it is already used for the synthesis of sex hormones and corticosteroids for application as anti-inflammatory, androgenic and contraceptive drugs, increasing the interest in this compound for clinical use. As discussed below, we investigated the diosgenin effects in PCa confirming its anti-cancer activity in a novel perspective.

Despite the evidence pointing out estrogens as protector agents against PCa, on the other side of the coin, the opposite effects of these hormones cannot be ignored.

Many reports showed that the effects of estrogens augment cell proliferation, resistance to apoptosis, migration and invasion, and promote other events involved in tumor growth and progression, contrastingly with others reporting that estrogens counteract PCa development and metastization by reducing cell growth, promoting cell cycle arrest, increasing apoptosis, and suppressing migration, invasiveness and angiogenesis. This duality of actions has intrigued the scientific community and was mainly associated with the diversity of receptors activated by estrogens. The pro-carcinogenic actions of estrogens were extensively linked to the activation of ER α , whereas anti-carcinogenic effects were ER β -dependent. Nevertheless, the intricacy of estrogens' actions is far from being totally enlightened. The discovery of GPER, the membrane-bound ER, increased the complexity of estrogenic responses. Apart from classical nuclear ERs, many studies evaluating the GPER' role in cancer also described a duality of effects balancing between pro- and anti-tumorigenic, as reviewed in Chapter 3.

In Chapter 7 of this thesis, we explored the role of GPER in PCa. The effect of GPER activation over a broad range of cancer hallmarks in human prostate cells was investigated. Our results confirmed that GPER expression is higher in PCa cases compared to BPH, and that GPER immunoreactivity was inversely correlated with PSA levels. These findings raise curiosity about the potential role of GPER as a predictive biomarker of biochemical recurrence after radical prostatectomy, together with PSA. In accordance with the expression pattern in human samples, GPER protein levels were also higher in neoplastic LNCaP cells than in the non-neoplastic PNT1A. Nevertheless, CRPC cells displayed reduced expression of GPER compared to the androgen-responsive cell line, with GPER protein levels being modulated by androgens. The evaluation of GPER expression in human samples of metastatic PCa tumors would be interesting in upcoming works to decipher this hypothesis of using GPER as a possible biomarker for tumor progression. We also studied the subcellular localization of GPER in prostate cells, finding it at the cell membrane, as expected, but also in the endoplasmic reticulum and, residually, in the nucleus. Moreover, we verified that GPER activation, by its specific agonist G1, altered the receptor location at the cell membrane and endoplasmic reticulum of prostate cells, which is a relevant finding, especially considering GPER as a pharmacological target.

Concerning the hallmarks of cancer herein evaluated in PCa, G1 treatment showed a multiplicity of benefic effects driven by GPER, also supporting the protective role of estrogens in this type of cancer. GPER activation by G1 showed to reduce cell viability and proliferation, arrest cell cycle and increase apoptosis (Fig. 9.1). It was also able to reduce migration and invasion, concomitantly with EMT attenuation (Fig. 9.1),

depending on the cell line. Moreover, GPER activation by G1 increased energy metabolism and oxidative stress in the PCa cells (Fig. 9.1).

Compiling GPER effects over a broad range of cancer hallmarks, including metabolic reprogramming, was very important since we must know what happens in PCa cells' behavior as a totality and not in an isolated characteristic. The integrated information we found herein highlight that even considering all the benefits of GPER as an anti-tumoral agent in PCa, the metabolic alterations driven by its activation should be addressed when considering GPER as a target for treatment in PCa. Moreover, further investigation would clarify the role of GPER as a metabolic regulator in PCa and the underpinning mechanisms depending on the cell type, stage and aggressiveness of disease.

Considering the diversity of ERs' effects and their ability to trigger a panoply of signaling pathways, efforts must be made to disclose the mechanisms of action and the downstream molecular targets of estrogens/ERs actions. With this rationale, we aimed to characterize the liaison of estrogens with the SCF/c-KIT system. The tyrosine kinase receptor c-KIT and its ligand SCF activate several signaling pathways involved in cancer development. Their expression was proven to be increased with PCa progression, indicating its significant role in the pathophysiology of PCa and its association with the severity of disease and appearance of metastasis. Because of this pattern, c-KIT was suggested as an attractive biomarker of PCa progression, contributing to improve clinical management of the disease. Furthermore, this protein system seems to be regulated by hormones, as reviewed in Chapter 4, but almost nothing is known in terms of its regulation by estrogens in PCa.

In Chapter 6, we contributed to disclose the role of E₂ in regulating the expression of SCF/c-KIT system *in vitro* and *in vivo*, and its impact for prostate cell proliferation and apoptosis. Herein, we demonstrated that E₂ down-regulated the expression of the SCF/c-KIT system both in human PCa cell lines and in rat prostate *in vivo*. Moreover, this regulation of SCF and c-KIT was accompanied by the effects of E₂ suppressing proliferation and inducing apoptosis in rat prostate (Fig. 9.1), supporting the protective role of estrogens against PCa and the relevance in c-KIT and SCF regulation by estrogens. Interestingly, the effect of E₂ has some nuances concerning the expression of c-KIT and SCF depending on the PCa cell line used, which we hypothesized to be related to the differential activation of ERs. So, in the future, it would be crucial to disclosure the effect of ERs in regulating SCF and c-KIT with PCa progression.

Considering the ability of some natural compounds to mimic estrogens actions by binding and activating the classical nuclear ERs and GPER and the increasing

interest in studying these phytoestrogens in PCa therapy, we also made our contribution to the scientific debate with this thesis Chapter 8. We investigated the effect of diosgenin on shaping prostate cells behavior. It was found that it can diminish the viability of androgen-sensitive and CRPC cells (Fig. 9.1) without affecting the viability of non-neoplastic PNT1A cells. This is quite an important finding as it is a desirable characteristic in any cancer therapy, and a drawback in most of them. Moreover, diosgenin was able to induce apoptosis and also modulated glycolytic metabolism of PCa cells (Fig. 9.1), supporting the anti-tumorigenic role of diosgenin in PCa and its interest as a therapeutic compound for PCa alone or in combination with other therapies. The effects of diosgenin were enhanced when combined with G1 (Fig. 9.1), supported by the ability of diosgenin to augment the expression of GPER, which raises curiosity about its use in therapy together with GPER agonists. Furthermore, we demonstrated that diosgenin activation of apoptosis in PCa is mediated by GPER, which has been shown to happen with other natural compounds. Further investigation would be performed regarding other diosgenin actions in the interplay with GPER, namely, by evaluating its impact on other cancer hallmarks and disclosing the molecular mechanisms underlying its anti-tumorigenic effects in PCa.

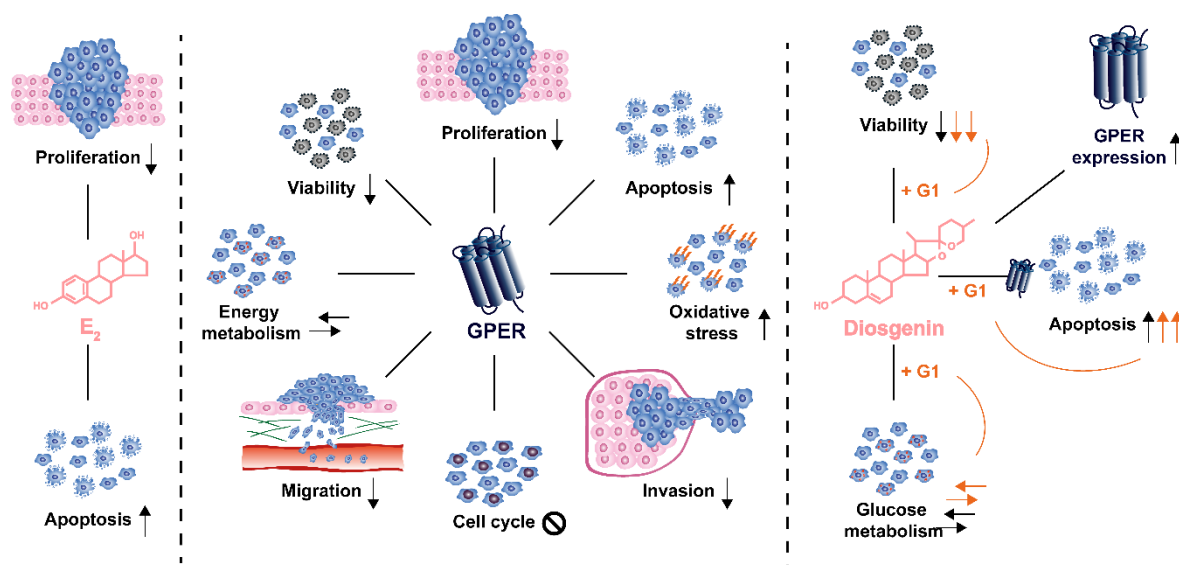


Figure 8.1. Systematic illustration of the effects of E_2 , GPER activation by G1 and diosgenin in prostate cells behavior. E_2 reduced proliferation and increased apoptosis of rat prostate cells. GPER activation by its agonist G1 reduced viability and suppressed proliferation, migration and invasion of PCa cells, also increasing apoptosis, arresting cell cycle, augmenting oxidative stress and changing the energetic metabolism depending on the cell line. Diosgenin caused a reduction in PCa cells viability with increased apoptosis and changes in glucose metabolism. Diosgenin augmented GPER levels, and the effects of diosgenin were enhanced by combination with G1. Moreover, GPER is required for diosgenin induced-apoptosis.

The present thesis revisited the role of estrogens in PCa, contributing to answering the central question: “are estrogens angels or demons in PCa?”. The work presented here would pave the way to further investigate and implement new and safe estrogen-based therapies. Altogether, the findings reported corroborate the line of evidence of estrogens as PCa protectors and support the research that has been made to find safe approaches for re-introducing estrogens in PCa therapy, rescuing it from hell to heaven.