



UNIVERSIDADE DA BEIRA INTERIOR

Ciências da Saúde

**Androgens and Calcium-binding Protein
Regucalcin in the Control of Prostate Cells
Metabolism and Survival: Implications in
Carcinogenesis**

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Thesis in Doctoral Degree in

Biomedicine

(3rd cycle of studies)

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Covilhã, October 2015

A todos aqueles que diariamente lutam contra o cancro e a todos os investigadores que continuam a acreditar que é possível travar esta doença.

E claro... Aos meus!

Acknowledgments

Em primeiro lugar quero agradecer à minha orientadora, Professora Sílvia Socorro, pela orientação, pelo apoio, dedicação e sensibilidade que sempre demonstrou. É um exemplo para todos nós!

Quero também agradecer ao meu co-orientador, Professor Cláudio Maia, pela orientação, pelo apoio e por estar sempre disponível para ajudar.

Agradeço ao Marco Alves, ao Professor Eduardo Cavaco e ao Professor Pedro Oliveira pela contribuição na realização dos trabalhos.

Aos meus colegas de grupo, Sara Correia, Ricardo Marques, Inês Gomes, Henrique Cardoso, Marília Figueira, Daniel Rodrigues e Nokas por toda a ajuda mesmo fora de horas, amizade e pelos bons momentos. Em especial à Sara Correia, que foi sem dúvida uma das principais responsáveis pelo gosto que tenho pela investigação, e por ser uma grande amiga!

Aos meus colegas e amigos, Margarida Gonçalves, Luís Pedro Rato, Carlos Gaspar, Micaela Almeida e Ana Mamede, que estiveram sempre disponíveis e presentes em todos os momentos que precisei. Marga e Vera obrigado por estarem sempre comigo e por fazerem parte da animação que é a minha vida!

A todas as pessoas do Centro de investigação em Ciências da Saúde, em especial à Sofia Duarte, Margarida Carrilho e Catarina Ferreira pela disponibilidade que sempre demonstraram.

Ao Pedro, que é sem dúvida o meu ponto de apoio, que me ouviu e mimou em todos os momentos e que festejou com a mesma intensidade que eu os meus sucessos! Obrigado por me fazeres feliz!

A toda a minha família, que são tantos e todos eles representam tanto para mim! Obrigado por todo o apoio e por todos os momentos fantásticos que vivemos juntos! Um agradecimento especial à minha avó, que diariamente me deixa um sorriso de bom dia!

Seria impossível descrever o quanto estou grata aos meus pais, José Alberto Vaz e Lurdes Vicente, e ao meu mano, Eduardo Vaz, por serem as pessoas que mais me incentivaram, por estarem sempre ao meu lado e por serem um exemplo para mim. São sem dúvida as pessoas mais importantes da minha vida!

Por último gostaria de agradecer à FCT pelo financiamento (SFRH/BD/70316/2010).

List of Publications

Publications included in this thesis:

- Vaz, C.V.,** Correia, S., Cardoso, H.J., Figueira M. I., Marques, R., Maia, C.J., Socorro, S. The emerging role of regucalcin as a tumor suppressor: facts and views (submitted for publication);
- Vaz, C.V.,** Marques, R.; Cardoso, H. J., Maia, C.J., Socorro, S. Suppressed glycolytic metabolism in the prostate of transgenic rats overexpressing calcium-binding protein regucalcin underpins reduced cell proliferation. (Accepted for publication after minor revisions);
- Vaz, C.V.,** Marques, R., Maia, C.J., Socorro, S. Aging-Associated Changes in Oxidative stress, Cell proliferation and Apoptosis are Prevented in the Prostate of Transgenic Rats Overexpressing Regucalcin. Translational Research. 2015; DOI: 10.1016/j.trsl.2015.08.009. IF: 5.030;
- Vaz, C.V.,** Rodrigues, D.B., Socorro, S., Maia, C.J. Effect of extracellular calcium on regucalcin expression and cell viability in neoplastic and non-neoplastic human prostate cells. Biochimica et biophysica acta. 2015; DOI: 10.1016/j.bbamcr.2015.07.006. IF: 5.019;
- Vaz, C.V.,** Marques, R., Alves, M.G., Oliveira, P.F., Cavaco, J.E., Maia, C.J., Socorro, S. Androgens enhance the glycolytic metabolism and lactate export in prostate cancer cells by modulating the expression of GLUT1, GLUT3, PFK, LDH and MCT4 genes. Journal of cancer research and clinical oncology. 2015; DOI: 10.1007/s00432-015-1992-4. IF: 3.081;
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- Marques, R., Peres, C. G., **Vaz, C.V.**, Gomes, I.M., Figueira, M.I., Cairrao, E., Verde, I., Maia, C.J., Socorro, S. 5alpha-Dihydrotestosterone regulates the expression of L-type calcium channels and calcium-binding protein regucalcin in human breast cancer cells with suppression of cell growth. *Medical oncology*. 2015; 32(9): p. 676. IF: 2.634;
- Cardoso, H.J., **Vaz, C. V.**, Correia, S., Figueira, M. I., Marques, R., Maia, C. J., Socorro, S. Paradoxical and contradictory effects of imatinib in two cell line models of hormone-refractory prostate cancer. 2015; *Prostate*. 75(9):923-35. IF: 3.565;
- Marques, R., **Vaz, C. V.**, Maia, C. J., Gomes, I. M., Gama, A., Alves, G., Santos, C. R., Schmitt, F., Socorro, S. Histopathological and in vivo evidence of regucalcin as a protective molecule in mammary gland carcinogenesis. *Experimental cell research*. 2015; Jan 15; 330(2):325-35. IF: 3.246;
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Resumo

O cancro da próstata é um dos cancros mais frequentes na população masculina e continua a ser uma considerável fonte de morbilidade e mortalidade para os homens no mundo inteiro. A progressão do cancro da próstata, que numa fase inicial está confinado à próstata, para um fenótipo mais agressivo, é caracterizada pela perda de resposta aos androgénios, ou seja, as células tumorais adquirem a capacidade de proliferar independente dos níveis de androgénios circulantes. O desenvolvimento e progressão do cancro da próstata resulta de diversas alterações genéticas e metabólicas, que conferem às células tumorais resistência e capacidade de proliferação exacerbadas. Os estudos de Warburg foram primordiais na demonstração de que as células tumorais usam preferencialmente a via glicolítica como fonte energética em detrimento da utilização da fosforilação oxidativa, o que resulta na produção de elevadas quantidades de lactato. Esta reprogramação metabólica é comum à maioria dos tumores e é atualmente considerada um “hallmark” do cancro. No entanto, as alterações que ocorrem ao nível do metabolismo glicolítico no cancro da próstata, assim como o papel dos androgénios na regulação do fluxo glicolítico, associada à progressão da doença para um estado mais agressivo, têm sido pouco estudados.

A Regucalcina (RGN) foi primeiramente descrita como uma proteína de ligação ao cálcio (Ca^{2+}), e desempenha um papel importante na homeostase intracelular deste ião. Também tem sido sugerida a sua função como gene supressor de tumor, pois esta proteína está envolvida na regulação da proliferação celular, apoptose e de diversas vias metabólicas, e parece ter propriedades antioxidantes. Para além disso, vários estudos noutros tecidos que não a próstata, demonstraram que a expressão da RGN é afetada por diversos fatores que estão associados à progressão do cancro da próstata, nomeadamente pelos androgénios, cálcio e pela idade. Foi também demonstrado que a expressão da RGN está diminuída em casos de cancro da próstata quando comparada com tecidos não tumorais, o que sugere que a perda da sua expressão poderá favorecer o desenvolvimento tumoral. Assim, tendo em consideração os vários processos biológicos que estão sob o controlo da RGN, torna-se previsível que esta proteína exerça um papel crucial na manutenção da homeostase celular. No entanto, existem poucos estudos sobre a regulação da expressão da RGN na próstata e ainda não foram detetados quaisquer avanços sobre a sua função na fisiologia prostática, nomeadamente na proliferação celular, apoptose, homeostase do Ca^{2+} e metabolismo neste tecido.

Assim, a presente tese teve como primeiro objetivo estabelecer o perfil glicolítico de duas linhas celulares tumorais da próstata representativas de uma fase mais precoce e de uma fase mais agressiva do cancro da próstata, ou seja responsivas (LNCaP) ou não responsivas (PC3) aos androgénios. Verificou-se que as linhas celulares LNCaP e PC3 apresentam um perfil glicolítico distinto. Relativamente às LNCaP, as PC3 demonstram um aumento na produção e no transporte de lactato para o meio extracelular em resultado de um

aumento da expressão de reguladores específicos desta via metabólica. Os efeitos dos androgénios na regulação do fluxo glicolítico nas células tumorais da próstata foram também estudados. O tratamento de células LNCaP com 5 α -dihidrotestosterona (DHT, 10 nM) aumentou significativamente o consumo de glucose e a sua metabolização a lactato, o que foi conseguido através do aumento da expressão dos transportadores e enzimas reguladoras da via glicolítica, num mecanismo que parece depender do recetor de androgénios (AR). Estes resultados indicam o controlo do metabolismo da glucose e do lactato como possível abordagem terapêutica para o cancro da próstata.

O efeito dos androgénios, do Ca²⁺ e da idade na expressão da RGN em células de próstata *in vitro* e *in vivo* foi igualmente avaliado. Verificou-se que tanto os androgénios como o envelhecimento regulam negativamente a expressão da RGN na próstata de rato. Concentrações fisiológicas e não fisiológicas de Ca²⁺ extracelular, e diferentes tempos de estimulação, provocaram efeitos distintos na expressão da RGN nas linhas celulares PNT1A e LNCaP, tendo sido observados efeitos mais pronunciados na sobreexpressão da RGN nas PNT1A. O conhecimento de que a RGN é uma proteína regulada pelos androgénios e pelo Ca²⁺, e de que a sua expressão se encontra reduzida com o envelhecimento, reforça a importância desta proteína como um alvo fundamental na regulação da fisiologia prostática.

De facto, o trabalho realizado nesta tese provou que a RGN exerce funções cruciais para a manutenção da fisiologia prostática, atuando a diferentes níveis fisiológicos. Através de ensaios *in vitro* e *in vivo*, verificou-se que RGN exerce um efeito negativo sobre a expressão do recetor do Ca²⁺, “calcium-sensing receptor” (CaSR), o qual é essencial na promoção dos efeitos do Ca²⁺ na proliferação. Para além disso, a sobre-expressão da RGN inibiu a proliferação celular, a apoptose e o metabolismo glicolítico na próstata de rato, através da modulação da expressão e da atividade de várias proteínas envolvidas na regulação das vias de sinalização referidas. Demonstrou-se também que a RGN reduz a peroxidação lipídica na próstata de rato, o que suporta a função da RGN como uma proteína citoprotetora.

Por último, verificou-se que níveis aumentados de RGN promovem o aumento das defesas antioxidantes, e previnem a resistência à apoptose e a proliferação descontrolada das células da próstata, características usualmente identificadas na próstata de animais envelhecidos. De uma forma geral, os resultados obtidos sugerem que a manipulação dos níveis da RGN pode ser uma boa estratégia para prevenir o desenvolvimento de doenças associadas com a idade na próstata de rato.

Em suma, o trabalho desenvolvido nesta tese, apresenta os androgénios como os “grandes amigos” das células tumorais da próstata, uma vez que estes promovem o aumento do fluxo glicolítico, o que por sua vez é uma característica associada à progressão dos tumores para fases mais agressivas da doença. Por outro lado, a perda da expressão da RGN associada ao envelhecimento leva à desregulação dos mecanismos fundamentais para a manutenção da fisiologia da próstata, o que pode contribuir para o aparecimento de alterações patofisiológicas da próstata, e para a transformação neoplásica das células. Por fim, os resultados apresentados também sugerem futuras abordagens terapêuticas para o

cancro da próstata, que passam por: i) desenvolver terapias baseadas no uso combinado de inibidores do AR e dos reguladores da via glicolítica; ii) desenvolver estratégias de manipulação da expressão da RGN de modo a explorar o seu potencial como opção terapêutica.

Palavras-chave

androgénios; apoptose; cálcio; cancro da próstata; envelhecimento; LNCaP; metabolismo glicolítico, PC3, proliferação; regucalcina; stress oxidativo.

Resumo Alargado

O cancro da próstata é um dos cancros mais frequentes na população masculina e continua a ser uma considerável fonte de morbidade e mortalidade para os homens no mundo inteiro. Vários fatores de risco para o desenvolvimento desta doença são já conhecidos, de entre os quais se destacam a história familiar, a idade e as hormonas esteroides sexuais, principalmente os androgénios. A progressão do cancro da próstata, que numa fase inicial está confinado à próstata, para um fenótipo mais agressivo, é caracterizada pela perda de resposta aos androgénios, ou seja, as células tumorais adquirem a capacidade de proliferar independente dos níveis de androgénios circulantes. Normalmente, esta representa uma etapa letal da doença, pois está associada à falha nas terapias clássicas, nomeadamente as terapias de ablação dos androgénios, restringindo deste modo as opções terapêuticas, o que em última análise leva à formação de metástases ósseas. O desenvolvimento e progressão do cancro da próstata resulta de diversas alterações genéticas e metabólicas, que conferem às células tumorais uma resistência e capacidade de proliferação desmensuráveis. Há quase cem anos atrás, em 1920, Warburg demonstrou que as células tumorais usam preferencialmente a via glicolítica como fonte energética mesmo em condições normais de oxigénio, ou seja, aumentam o consumo de glucose para produzir lactato, em detrimento da utilização da fosforilação oxidativa, que é a via mais utilizada pelas células não tumorais. Esta reprogramação metabólica é comum à maioria dos tumores e é atualmente considerada um “hallmark” do cancro. Vários estudos têm demonstrado que o cancro da próstata tem características metabólicas específicas comparativamente com as células não tumorais. No entanto, as alterações que ocorrem ao nível do metabolismo glicolítico, assim como o papel dos androgénios na regulação do fluxo glicolítico, na progressão do cancro da próstata para um estado mais agressivo, têm sido pouco estudados.

A Regucalcina (RGN) foi primeiramente descrita como uma proteína de ligação ao cálcio (Ca^{2+}), e desempenha um papel importante na homeostase intracelular deste ião. Também tem sido sugerida a sua função como gene supressor de tumor, pois esta proteína está envolvida na regulação da proliferação celular, apoptose, stress oxidativo e de diversas vias metabólicas, ou seja, em mecanismos que se encontram desregulados em casos de cancro. Para além disso, vários estudos que não na próstata, demonstraram que a expressão da RGN é afetada por diversos fatores que estão associados com a progressão do cancro da próstata, nomeadamente pelos androgénios, Ca^{2+} e pela idade. Recentemente, o nosso grupo de investigação demonstrou que a expressão da RGN está diminuída em casos de cancro da próstata quando comparado com tecidos não tumorais. Estes resultados sugerem que a RGN, também na próstata poderá ter um papel importante na manutenção da homeostase celular e que a perda da expressão desta proteína poderá favorecer o desenvolvimento tumoral. No entanto, existem poucos estudos sobre a regulação da expressão da RGN na próstata e ainda não foram detetados quaisquer avanços sobre a sua função na fisiologia da próstata,

nomeadamente na proliferação celular, apoptose, homeostase do Ca^{2+} e metabolismo neste tecido.

Assim, a presente tese teve como primeiro objetivo estabelecer o perfil glicolítico de duas linhas celulares tumorais da próstata representativas de uma fase mais precoce e de uma fase mais agressiva do cancro da próstata, ou seja responsivas (LNCaP) ou não responsivas (PC3) aos androgénios. Verificou-se que as linhas celulares LNCaP e PC3 apresentam um perfil glicolítico distinto. Relativamente às LNCaP, as PC3 demonstram um aumento na produção e no transporte de lactato para o meio extracelular em resultado de um aumento da atividade da enzima lactato desidrogenase (LDH) e da expressão do transportador de lactato, o transportador monocarboxilato (MCT) 4. Além disso, o ratio lactato/alanina também se encontra aumentado na linha celular PC3, o que sugere um aumento dos níveis de stress oxidativo nesta linha. Estes resultados indicam que a progressão do cancro da próstata para a fase independente dos androgénios está associada à aquisição de novas estratégias metabólicas por parte das células tumorais, como verificámos pelo aumento do fluxo glicolítico nas PC3. Após estes resultados tornou-se imperativo estudar os efeitos dos androgénios na regulação do metabolismo glicolítico nas células tumorais da próstata, especialmente na manipulação do lactato. O tratamento de LNCaP com 5- α dihidrotestosterona (DHT) aumenta significativamente o consumo de glucose e a sua metabolização a lactato, através do aumento da expressão dos transportadores de glucose (GLUT) 1 e 3, da fosfofrutoquinase (PFK) e MCT4, num mecanismo que parece depender do recetor de androgénios (AR). A reprogramação metabólica promovida em resposta ao tratamento com androgénios parece favorecer a sobrevivência e a proliferação celular. De uma forma geral, estes resultados indicam o controlo do metabolismo da glucose e do lactato como possível abordagem terapêutica para o cancro da próstata.

O segundo objetivo passou por determinar o efeito dos androgénios na expressão da RGN *in vivo*, e explorar o efeito da sobre-expressão da RGN na proliferação celular, apoptose, no metabolismo glicolítico e stress oxidativo na próstata de ratos adultos. Verificou-se que os androgénios regulam negativamente a expressão da RGN na próstata de rato. Através de um modelo animal transgénico que sobre-expressa a RGN (Tg-RGN), constatou-se que a próstata dos animais Tg-RGN apresenta um menor índice de proliferação relativamente aos ratos wild-type (Wt), em conjunto com uma maior expressão do inibidor do ciclo-celular, o p21 e uma redução na expressão do oncogene H-ras. Para além disso, a sobre-expressão da RGN inibe a apoptose na próstata, através da redução da expressão da caspase-8, da atividade da caspase-3, e do aumento no ratio das proteínas Bcl-2/Bax. A RGN parece exercer uma dupla função pois reduz tanto a proliferação como a apoptose, o que estabelece a sua função como reguladora do crescimento celular na próstata. Os animais Tg-RGN apresentam reduzidos níveis de peroxidação lipídica na próstata relativamente aos Wt, o que sugere o papel protetor da RGN contra o stress oxidativo. Quanto a alterações ao nível metabólico, a próstata dos ratos Tg-RGN apresenta menores quantidades de glucose e lactato, bem como diminuída expressão do GLUT3, PFK, LDH e MCT4, o que indica o papel da RGN na redução do

fluxo glicolítico na próstata. Estes dados indicam que de facto a RGN é uma proteína fundamental para a manutenção da fisiologia prostática.

Seguidamente, analisou-se a expressão da RGN em linhas tumorais (LNCaP e PC3) e não tumorais (PNT1A) da próstata, bem como a relação entre o Ca^{2+} e a expressão da RGN. A expressão da RGN está significativamente reduzida nas linhas tumorais relativamente às não tumorais. Concentrações fisiológicas e não fisiológicas de Ca^{2+} extracelular (Ca^{2+}_o), e diferentes tempos de estímulo, provocam efeitos distintos na expressão da RGN nas linhas celulares PNT1A e LNCaP. Mais ainda, o estímulo com elevadas concentrações de Ca^{2+}_o despoleta diferentes efeitos no que respeita à proliferação, entre as linhas celulares PNT1A e LNCaP, um fenómeno que parece estar relacionado com a expressão diferencial da RGN nestas linhas celulares. Os efeitos do Ca^{2+}_o na regulação da proliferação celular, diferenciação e apoptose estão associados com a atividade do seu recetor, “calcium-sensing receptor” (CaSR). Neste trabalho demonstrou-se que a RGN exerce um efeito negativo sobre a expressão do CaSR, o que foi constatado após a elaboração de ensaios *in vitro*, inibindo o gene da RGN, e *in vivo* usando os animais Tg-RGN e os respetivos Wt. Os resultados apresentados neste tópico apontam para a RGN como uma proteína regulada pelo Ca^{2+} , e reguladora das vias de sinalização dependentes do Ca^{2+} .

Esta tese tem como último objetivo, caracterizar o padrão de expressão da RGN na próstata de rato ao longo da idade, e determinar o impacto da sobre expressão desta proteína na próstata de ratos mais velhos (9 M) no que respeita à proliferação celular, apoptose e stress oxidativo. Verificou-se que a expressão da RGN decresce com a idade na próstata de rato. E, curiosamente, a sobre-expressão da RGN na próstata aumenta o potencial antioxidante e a atividade da proteína antioxidante, glutathione-S-transferase, em resposta ao aumento dos níveis de peroxidação lipídica, característico do envelhecimento. Os ratos Tg-RGN (9M) apresentam elevados níveis de p53 e reduzida expressão do sistema SCF/c-Kit, bem como aumento da expressão de genes indutores da apoptose, nomeadamente Bax, FasL, FasR e caspase-8 e a atividade da caspase-3. Estes resultados sugerem que a RGN pode ter uma função importante na prevenção do desenvolvimento de doenças na próstata associadas com o envelhecimento, como é o caso do cancro da próstata, promovendo o aumento das defesas antioxidantes e prevenindo a resistência à apoptose e a proliferação desmedida das células, o que é usualmente característico em células da próstata de animais envelhecidos e em tumores.

Em suma, o trabalho desenvolvido nesta tese, apresenta os androgénios como “grandes amigos” das células tumorais da próstata, uma vez que estes promovem o aumento do fluxo glicolítico, o que por sua vez é uma característica associada à progressão dos tumores para a fase mais agressiva da doença. Por outro lado, a perda da expressão da RGN com a idade leva à desregulação de mecanismos fundamentais para a manutenção da fisiologia da próstata, contribuindo para alterações patofisiológicas da próstata associadas com o envelhecimento, e para a transformação neoplásica das células. Ainda mais, este trabalho lança novos desafios para o desenvolvimento de futuras terapias para o cancro da

próstata e para prevenir a evolução da doença, baseadas no uso combinado de inibidores do AR e dos reguladores da via glicolítica, e em estratégias de manipulação da expressão da RGN de modo a utilizá-la como proteína terapêutica.

Abstract

Prostate Cancer (PCa) is one of the most common cancers in men and continues to be a source of considerable morbidity and mortality worldwide. The progression of PCa from an early stage, confined to prostate, to a more aggressive phenotype is characterized by the loss of androgen-responsiveness, which means that tumor cells gain the ability to grow independently of the circulating androgens. Furthermore, the neoplastic transformation of prostate cells and tumor progression to more aggressive stages require several genetic and metabolic alterations. Warburg firstly demonstrated that tumor cells predominantly use glycolysis for obtaining energy in detriment of oxidative phosphorylation, with the production of high amounts of lactate. This metabolic adaptation is common to the majority of cancers and is now established as a hallmark of cancer. However, the alterations in the glycolytic metabolism that occur in the progression to the androgen-independent stage of PCa as well as the role of androgens in regulating the glycolytic flux are poorly studied.

Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein that plays a determinant role in the maintenance of intracellular Ca^{2+} homeostasis. RGN also has been suggested as a tumor suppressor protein, since it is involved in the regulation of proliferation, apoptosis, and diverse metabolic pathways, and its antioxidant properties also have been reported. In addition, androgens, Ca^{2+} and age, all well-known factors implicated in PCa development, regulate RGN expression in different tissues but their action in prostate remains to be elucidated. It was also demonstrated that RGN is under expressed in human PCa cases comparatively to normal tissues, which suggest that the loss of RGN may be an event favoring tumor development. Moreover, considering the biological processes under the influence of RGN it is also predictable that it may exert a crucial role in the maintenance of cell tissue homeostasis. Notwithstanding, little is known about the factors that regulate RGN expression in the prostate, and no studies exist regarding the role of RGN in the regulation of cell proliferation, apoptosis, Ca^{2+} homeostasis, and metabolism in this tissue.

The present thesis firstly established the glycolytic profile of androgen-responsive (LNCaP) and non-responsive (PC3) PCa cells. We demonstrated that LNCaP and PC3 cells display a distinct glycolytic profile, with PC3 presenting higher production and export of lactate as a result of increased expression of target regulators of this metabolic pathway. The role of androgens in the regulation of the glycolytic flux in PCa cells was also studied. Treatment of LNCaP cells with 5 α -dihydrotestosterone (DHT, 10nM) significantly increased glucose uptake, glycolysis rate and the export of lactate by upregulating the expression of important regulators of glycolysis, in a mechanism that seems to involve the androgen receptor (AR). These results highlighted the importance of controlling glucose and lactate metabolism as possible therapeutic approaches in PCa.

The effect of androgens, Ca^{2+} and age on the expression of RGN in prostate cells *in vitro* and *in vivo* was investigated. The expression of RGN in rat prostate was downregulated

by androgens and aging. Administration of extracellular Ca^{2+} to human prostate cells differentially regulated the expression of RGN in LNCaP and PNT1A cells in a time- and dose-dependent manner, with effects more pronounced in the up-regulation of RGN expression in PNT1A cells. The fact that androgens and Ca^{2+} regulate RGN levels in prostate cells, and the evidence of RGN down-regulation with aging, strongly points this protein as an important target in the regulation of prostatic physiology.

Indeed, the work of this thesis also proved the role of RGN in controlling the crucial mechanisms involved in prostate cells homeostasis. RGN exerted a negative effect on the expression of Ca^{2+} sensing receptor, which was essential in the promotion of Ca^{2+} -dependent proliferation, as demonstrated by *in vitro* and *in vivo* assays. In addition, overexpression of RGN suppressed cell proliferation, apoptosis and the glycolytic metabolism in rat prostate, as a result of the regulation of the expression and activity of several proteins involved in these pathways. RGN also decreased lipid peroxidation in rat prostate, which supports their cytoprotective function.

Finally, we verified that augmented levels of RGN improved the antioxidant defenses, prevented the age-induced resistance to apoptosis, and the excessive proliferation usually found in the prostate of aged animals. The obtained results suggest that the manipulation of RGN levels may prevent the development of aged-associated diseases in rat prostate.

In conclusion, the main findings of this dissertation pointed androgens as the “good friends” of PCa cells, since they increase the glycolytic flux in PCa cells, which is stimulated with the progression of disease. In contrast, loss of RGN expression with aging led to deregulation of different mechanisms crucial for the maintenance of prostate physiology, and thus it may contribute to the emergence of aged-associated pathophysiologic alterations, and to the neoplastic transformation. The results presented here also suggest future approaches for the management of PCa, which could passing by: i) the development of new therapeutic strategies based on the use of combined therapies, targeting AR and glycolytic metabolic regulators; and ii) the manipulation of RGN expression as a therapeutic option.

Keywords

age; androgens; apoptosis; calcium; glycolytic metabolism; LNCaP; oxidative stress; proliferation; PC3; prostate cancer; regucalcin;

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List of Abbreviations

¹⁸F-FDG	2-deoxy-2-fluoro-D-glucose (FDG) labeled with fluorine-18
¹H-NMR	Proton Nuclear Magnetic Resonance
2DG	2-deoxyglucose
ABTS®	2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]
ADX	adrenalectomy
AMPK	5-AMP-activated protein kinase
AR	androgen receptor
AT	annealing temperature
ATP	adenosine triphosphate
Bcl-2	B cell lymphoma 2
Bp	base pairs
Ca²⁺	calcium
Ca²⁺_o	extracellular calcium
CaM	calmodulin
CCl₄	carbon tetrachloride
chr	chromosome
CT	calcitonin
CTR	calcitonin receptor
CypA	cyclophylin A
CZ	central zone
DHT	5α-dihydrotestosterone
DPPH	1,1-diphenyl-2-picryl-hydrazyl
E₂	17β-estradiol
EGFR	epidermal growth factor receptor
ER	estrogen receptor
ERMA	energy restriction-mimetic agents
F-1,6-BP	fructose-1-6-bis-phosphate
F-2,6-P₂	fructose-2,6-bisphosphate
F-6-P	fructose-6-phosphate
FA	fatty acid
FasL	Fas ligand
FASN	fatty acid synthase
FasR	Fas receptor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLUT	glucose transporter
GP	glycogen phosphorylase

GP	G protein-coupled estrogen receptor
GS	glycogen synthase
GST	glutathione-S-transferase
GSTP1	glutathione S-transferase-pi gene
HCC	hepatocellular carcinoma
HGPIN	high-grade prostatic intraepithelial neoplasia
HIF	hypoxia-inducible transcription factor
HK	hexokinase
HMIT	proton-coupled myoinisitol transporter
HRPC	hormone-refractory PCa
IHC	Immunohistochemistry
InsR	insulin receptor
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
M	month
MAPK	mitogen-activated protein kinase
MCT	monocarboxylate transporter
MCU	mitochondrial Ca ²⁺ uniporter
MDA	malondialdehyde
MR	mineralocorticoid receptor
mTOR	mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)- 2-(4-sulfophenyl)-2M) tetrazolium
NAD⁺	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
ORCHX	orchiectomized
PCa	prostate cancer
PCA3	prostate cancer antigen 3
PET	positron emission tomography
PFK	phosphofructokinase
PFKB2	cardiac isoform of PFK
PI3K	phosphatidylinositol 3-kinase
PIA	proliferative inflammatory atrophy
PIN	prostatic intraepithelial neoplasia
PMCA	plasma membrane ATPase
pNA	p-nitroaniline
PSA	prostate specific antigen
PTEN	phosphatase and tensin homolog
PTH	parathyroid hormone

PTHr	parathyroid hormone receptor
PZ	peripheral zone
qPCR	quantitative real-time PCR Polymerase chain reaction
RGN	regucalcin
RGN-KO	RGN-knockout mice
ROS	reactive oxygen species
SCF	stem cell factor
SCO2	synthesis of cytochrome c oxidase protein
SERCA	sarco/endoplasmic reticulum Ca ²⁺ ATPase
SLC2A	solute carriers 2A
SMP30	senescence marker protein 30
SnoN	ski-novel protein
SOD	Superoxide dismutase
T	testosterone
T3	3,3', 5-triiodo-L-thyronine
T4	thyroxine
TAC	total antioxidant capacity
TBA	thiobarbituric acid
TBARS	Thiobarbituric Acid Reactive Substances
t-BPH	tert-Butyl hydroperoxide
TCA	tricarboxylic acid
TGF- β	transforming growth factor - β
Tg-RGN	transgenic rats overexpressing RGN
TIGAR	TP53-induced glycolysis and apoptosis regulator
TMPRSS2	type II transmembrane serine protease
TNF-α	tumor necrosis factor- α
TR	thyroid hormones receptor
TZ	transition zone
VEGF	vascular endothelial growth factor
WB	Western blot
Wt	wild-type
β2M	β 2-microglobulin

Chapter 1

General Introduction

Brief overview of prostate anatomy-physiology

Prostate cancer: etiology and progression

General Introduction

Brief overview of prostate anatomy-physiology

The human prostate is a glandular structure with a size of a walnut and anatomically located immediately below the urinary bladder, where it surrounds the initial portion of the urethra [1, 2]. In the posterior and lateral sides, prostate is enclosed by a fibrous capsule, whereas the anterior fibromuscular stroma binds the anterior and apical surfaces of the gland [2]. The structural division of prostate gland has been largely discussed and three main zones are considered, namely, the peripheral zone, the central zone, and a smaller transition zone (Figure 1.1) [3, 4]. The peripheral zone comprises the largest region of the prostate with approximately 70% of glandular tissue, and the central and the transition zones account for the remaining 30% [5, 6]. The transition zone usually suffers a hypertrophy development with aging, becoming the most prominent prostate zone [7]. However the prostate gland also seems to comprise a fourth non-glandular region, the anterior-fibromuscular zone [8]. Alternatively, the prostate can also be divided in anterior, dorsal, lateral and ventral lobes [8].

The glandular structures are irregularly shaped due to the folds formed by the epithelium, which is composed by two histologically distinct layers (Figure 1.1). The secretory luminal layer consists of tall columnar cells responsible for the production of prostatic secretions, the keratinocyte growth factor receptor and prostate specific antigen (PSA), and express androgen receptor (AR), cytokeratin 8 and 18. This layer of cells is underpinned by a continuous layer of cuboidal epithelial cells, the basal cells, which in turn is lined by a basement membrane allowing the division between basal cells and stroma (Figure 1.1). Basal cells have the ability to produce keratin, p63, cytokeratin 5 and 14 and selectively express the insulin-like growth factor receptor, epidermal growth factor receptors (EGFR), and estrogen receptor (ER) enabling them to maintain cell growth [3, 6, 9]. It have been hypothesized that the basal layer contains a stem cell like reservoir with the capacity to differentiate into the columnar secretory cells and that the basal cells may be involved in the control of the molecular trafficking between luminal cells and the extracellular space [10, 11]. A third type of cells located among the epithelial cells were also found in prostate, the neuroendocrine cells, and are believed to be involved in the regulation of secretory activity and cell growth of prostate [6, 9, 12]. Within the stromal cell population, fibroblastic and smooth muscle cells are thought to produce growth factors that support the development and function of the epithelial cells [9].

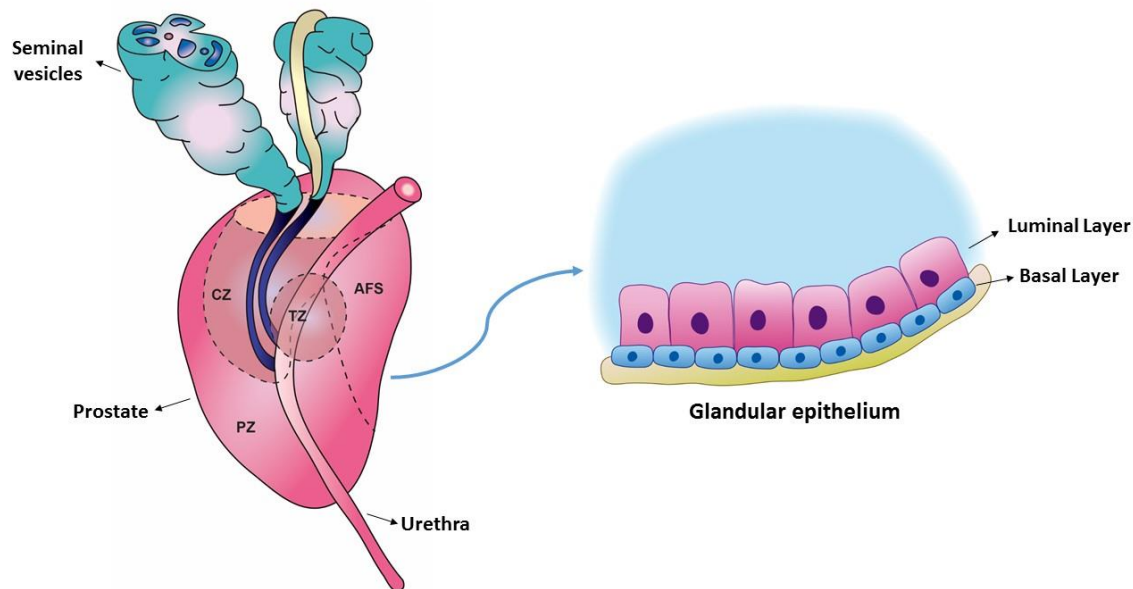


Figure 1.1. Schematic representation of prostate anatomy. Prostate gland surrounds the bladder neck and the initial portion of the urethra. The anterior and apical surfaces are bounded by anterior fibromuscular stroma (AFS). In addition, three main zones can be observed, the transition zone (TZ) that surrounds proximal prostatic urethra, the central zone (CZ) that surrounds the ejaculatory ducts, and peripheral zone (PZ), constituting approximately 70 % of the prostate gland. At a closer look, two distinct histological epithelial layers can be distinguish in prostate glandular tissue: the luminal layer, which is constituted by tall columnar cells responsible for the production of prostatic secretions, the keratinocyte growth factor receptor and prostate specific antigen; and the basal layer, in which basal cells express several growth receptors and estrogen receptor, being involved in the maintenance of cell growth. Also in the basal layer, a stem cell like reservoir seems to exist, and this cells have the ability to differentiate into the columnar secretory cells. In addition, a third type of cells, neuroendocrine cells (not shown), located among the epithelial cells seems to be involved in the regulation of secretory activity and cell growth of prostate.

Human prostate morphogenesis and growth occur during fetal life and puberty and the size of prostate is maintained at maturity until the fourth decade of life, when its growth is reinitiated [6]. The initial outgrowth and differentiation of epithelium is mediated by mesenchymal cells. Both in embryogenesis and in differentiated prostate the interaction between stromal and epithelial cells is crucial, and androgens play an essential role in the growth and development of the embryonic epithelium into differentiated prostate [9, 13].

During the period of prostate growth an increased rate of cell proliferation over death occurs. In the adult, the size of prostate gland is maintained through a homeostatic balance between the process of cell renewal and cell death. This balance is regulated by hormones secreted by the endocrine system, mainly androgens, of which testosterone is the major circulating form. In peripheral tissues in general, and particularly in prostate, testosterone is preferentially metabolized to 5 α -dihydrotestosterone (DHT) by the activity of 5 α -reductase isoenzyme, mainly the type 2 [14, 15]. Androgens mostly act through binding to its receptor, the AR, which is a major transcription factor of the nuclear receptor superfamily that

regulates the transcription of target genes involved in the control of prostate cell proliferation and differentiation [16, 17].

Also, sex steroids estrogens seem to be involved in the maintenance of cellular homeostasis in the prostate gland [18]. The prostatic concentration of DHT seems to decrease with aging while the concentrations of testosterone and 17 β -estradiol (E₂) are age-independent. This causes an age-dependent change in the ratio of serum sex steroid concentration in favor of estrogens, which is believed to play a role in the emergence of benign prostatic hyperplasia (BPH) and development of prostate cancer (PCa) [19-21].

The main function of the prostate gland is to secrete several factors, including PSA, prostatic acid phosphatase, prostaglandins and citric acid, which strongly collaborate in fertilization, and sperm motility, survival and delivery to the female reproductive tract. The prostatic secretions also contribute for neutralizing the acidic environment of duct deferent and female vagina [22]. The maintenance of the secretory functions of the prostate are also dependent of androgens and their receptor [23].

Prostate cancer: etiology and progression

Prostate cancer is a multifactorial disease arising from mutations in cancer susceptibility genes, action of environmental agents that influence the acquisition of somatic genetic changes, and several other systemic or local factors including behavior/diet, hormones and growth factors resulting in cell growth or survival advantages [17, 24]. Epigenetic changes contribute also to the development and progression of this disease [25]. In addition to genetic changes, the intercellular communication of tumor cells with the stroma is essential to local cancer growth and metastization [26].

Prostate cancer can be classified in hereditary and sporadic PCa, and 85% of all PCa belong to the sporadic class [17]. In sporadic PCa, usually a heterogeneous pattern of oncogenes activation occur, and some candidate genes and activated pathways with a role in PCa initiation and progression to a more aggressive stage were identified. These molecular players will be discussed later in this chapter.

Histologically PCa progresses from precursor lesions, termed prostatic intraepithelial neoplasia (PIN) arising primarily in the peripheral zone of prostate. In contrast, the non-malignant overgrowth of prostate epithelial cells - BPH which is common among aging man, occurs almost exclusively within the transition zone [27]. PCa is initially confined to the prostate and at initial diagnosis the majority of tumors show an androgen-dependent cell proliferation [28]. However, this disease tend to progress and metastasize, becoming independent of circulating androgens to growth, which usually corresponds to a lethal stage of the disease [28].

Epidemiology

Prostate Cancer is one of the most common cancers in men worldwide in terms of new cases diagnosed and cancer-related deaths. In the last years, an increasing number of new PCa

diagnoses and also of cancer survivors have been reported in developed countries contrarily to what occurs in developing countries, which is due to the aging and growth of the population, and the improvement and advances in the detection and treatment methods [29, 30]. In fact, the highest rates of PCa incidence have been reported in North America, Oceania, and western and northern Europe, whereas the death rates seems to increase in less developed regions, such as parts of South America, the Caribbean, and sub-Saharan Africa [31]. In 2030, it is expected that PCa incidence growth to 1.7 million new cases and 499.000 new deaths worldwide [31].

In the United States, it is estimated that 3 million American man lives with PCa, in 2014 newly 233 000 cases would be diagnosed with 29.480 estimated deaths, and this type of cancer will remain on the top of cancer diagnosis in 2030 [30, 32, 33]. The medium age of the men diagnosed with PCa is 66, and the majority of the survivors (62%) are over 70 years.

In the European Union, 742.500 deaths were predicted in 2014, of which 70.100 occurred by PCa. In a positive way, the predicted rate for PCa decreased relatively to 2009, falling by 10% [34].

In Portugal, PCa represented the second most incident type of cancer in 2008 (12.3%), and in 2012 accounted for 1.814 deaths (1.7%) [35, 36].

Risk factors

In the last years, several risk factors for the development of PCa have been described (Figure 1.2) though the specific causes on the etiology of this disease have remained poorly understood. The risk factors considered to promote and contribute for PCa can be divided in two classes: endogenous (age, family, genetic factors, ethnicity, hormones and oxidative stress) and exogenous (dietary factors, physical inactivity, obesity, environmental factors, occupation, smoking) [37]. Nevertheless, familiar and genetic factors, and also aging, have been indicated as the major risk factors for the emergence of PCa [17]. The risk of developing familial PCa is directly associated with the number of family members diagnosed and the degree of relatedness, and is inversely correlated with the age at which the relatives were affected [38-40].

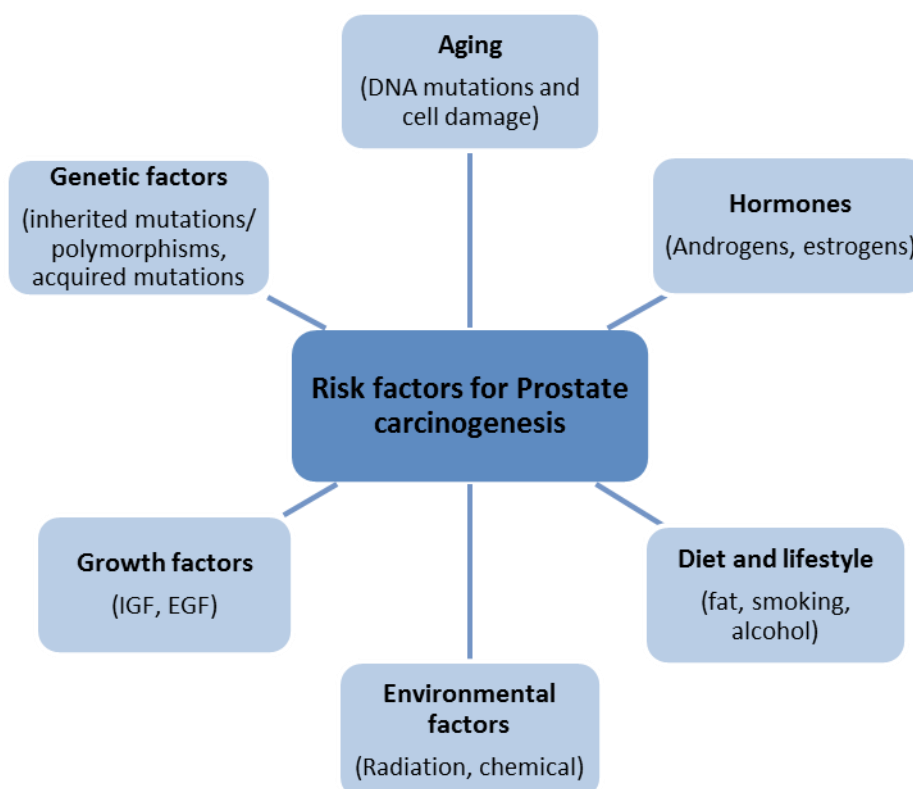


Figure 1.2. The diverse exogenous and endogenous factors contributing for prostate carcinogenesis. Legend: IGF, Insulin-like growth factor; EGF, epidermal growth factor.

Genome-wide analysis and genetic epidemiologic studies allowed to identify a set of susceptibility genes involved in the development of PCa, most of them with a dominant mode of inheritance [41]. RNASEL and macrophage scavenger receptor are the most promising susceptibility genes discovered, exerting important functions in apoptosis, inflammation and susceptibility to infection, and alterations in these genes seem to be related to the progression and poor prognosis of PCa [17, 42-45]. Nevertheless, although found in low frequency, mutations in ELAC2, OGG1, CHEK2, BRCA2, PON1 and GDF15 genes, some of them with important known functions in DNA repair, cell cycle regulation, and cellular defense against oxidative stress and inflammation, have also being considered susceptibility genes for PCa [17, 46-48]. Besides the mutations in the individual genes referred, also type II transmembrane serine protease (TMPRSS2), an androgen-regulated gene involved in epithelial cell growth, frequently suffers chromosomal rearrangement that leads to a fusion with ETS family members. TMPRSS2-ERG and TMPRSS2-ETV are both fusion oncogenes that appear to be related to the development of PCa [49, 50]. In addition, the existence of polymorphisms in 8q24, 17q12 and 17q24.3, 2p15, 10q11, 11q13 and Xp11 chromosomes (chr) seems to be moderately associated with PCa [51-53]. In the last decades, the investigation of epigenetic changes demonstrated the existence CpG islands hypermethylation in multiple genes sites associated with progression of PCa [25, 54].

Prostate cancer has a high incidence in aged men, which is not surprising since aging is associated with a physiologic decline. At senescent stages, the cellular defenses against

oxidative stress and inflammatory processes, and the mechanisms of DNA repair are continuously impaired. Consequently increased DNA mutations and cell damage accumulate in the organism, which enormously increases the susceptibility for the development of aged-associated diseases, as is the case of PCa [17, 55, 56].

Another risk factor that has been considered for PCa is the ethnicity. Different incidence rates of PCa have been found between diverse ethnic groups and countries, and the highest rates were found among the African American men in the USA contrastingly to the observed in Asian countries [57, 58]. The causes of this discrepancy of PCa risk associated with the ethnicity of the population have been attributed to genetic variants, external factors as diet and environment and, although controversial, to altered hormonal levels, including androgens [59-61]. In fact, deregulation of androgen metabolism, diet and lifestyle and also enhanced infection and inflammation have been considered important factors of risk in the development of PCa [17].

The importance of androgens in the physiology of prostate, regulating its development, maturation and maintenance, is perfectly recognized. The deregulation of androgen metabolism seems to be involved in the onset of PCa [17]. A great number of studies have been performed in an attempt to understand the relationship between the levels of androgens (circulating and intraprostatic) and PCa, and although a positive association between high serum levels of androgens and risk of PCa was suggested, this is a matter that needs further clarification [14, 17]. Accordingly, conflicting reports exist concerning to the existence of mutations and polymorphisms in genes associated with the metabolism of androgens and the PCa development [62, 63]. Nevertheless, it is widely accepted that the androgen and AR signaling pathways are crucial for the development and progression of PCa. Due to its importance this issue will be explored in a specific topic of this chapter below.

Several epidemiologic studies suggest the existence of infectious components on the pathogenesis of some PCa, which can be associated with the exposition of the prostate to infection agents via sexual and urine activity [64, 65]. A persistence of inflammation caused by infection or tissue injury may result in an unbalanced ratio between inflammatory response and tissue repair, in which an increased production of reactive species and delivery of cytokines and growth factors contribute for cellular and DNA damage in proliferative cells and, in turn, for the development of PCa [66-69]. Indeed, innumerable studies have linked the chronic prostatic inflammation with the incidence of BPH and PCa [69, 70].

Finally, dietary factors and lifestyle also seem to be related to the etiology and incidence of PCa. Ingestion of foods enriched in antioxidants, vitamin C, D and E, soy, lycopene and selenium and poor intake of fats may have a good impact protecting against the development of tumor, and the opposite is also true [17, 71-75]. Although controversial, smoking and excessive alcohol consumption are both lifestyles that may contribute for tumor appearance [17].

Stages of tumor development

Although the precise mechanisms involved in the initiation of PCa are not entirely known, there is evidence indicating that PCa may arise from pre-cancerous lesions originated by the inflammatory processes that occur during tissue injury [76-78]. Located at the basement membrane of prostatic gland, a niche of 1-2% of specific stem cells seem to be involved in the processes of prostate regeneration after injury. A pool of the adult prostatic stem cells may eventually be restored and progress into transit-amplifying/intermediate cells to reconstitute the normal epithelium, by differentiating into luminal secretory epithelial cells or neuroendocrine cells [9, 78-81]. These prostatic stem cells have been viewed as a minority of epithelial cells that could generate the PCa progenitor cells. Moreover, the persistent activation of androgens, estrogens and other growth factors signaling pathways in these cells may lead to the development of an heterogeneous population of PCa progenitor cells with an unlimited proliferation capacity, aberrant differentiation and decreased cell death, which in turn may result in the generation of PIN lesions or ultimately in PCa (Figure 1.3) [78-80]. In fact, PCa progresses from the PIN precursor lesions and the high-grade PIN (HGPIN) is considered the most likely precursor of PCa (Figure 1.3) [27, 82]. HGPIN has been characterized by abnormal proliferation within the prostatic acini and duct, in which basal and luminal cells show dysplastic features including nuclear hyperchromasia, nuclear crowding and prominent nucleoli [83, 84]. The volume of HGPIN in prostates with cancer increases with progression of pathologic stage, Gleason grade, and invasion [82, 85].

It has been proposed that the majority of the alterations that lead to development of PCa occur in the transition to HGPIN, since similar chromosomal modifications were observed both in HGPIN and PCa, with gain of chr 8q, 7 and Xq, and loss of chr 8p, 10q and 13q [86-88]. In addition, the same genetic changes and the activation of several oncogenic cascades are involved in the transition to HGPIN and PCa and to more aggressive stages.

An alternative precursor of PCa is the proliferative inflammatory atrophy (PIA). PIA is defined as the discrete foci of proliferative glandular epithelium occurring in association with inflammation, and the presence of the stromal and epithelial cell layer with a stromal atrophy as well as a mononuclear and/or polymorphonuclear inflammatory cell layer can be found [89, 90]. PIA is frequently viewed only as a primordial precursor, in which genetic alterations may lead to the development of HGPIN lesions [66, 88]. However, it also presents some molecular alterations characteristic of neoplastic transformation, namely high expression of glutathione S-transferase alpha 1, cyclooxygenase-2 and B cell lymphoma 2 (Bcl-2), reduced levels of NK3 homebox 1 (NKX3.1), glutathione S-transferase-pi gene (GSTP1) hypermethylation, early chromosomal abnormalities and shortening of telomere length [91-94].

Prostate cancer mostly arises in the peripheral zone of the prostate and initially is represented as a small foci of intraductal dysplasia, but with time it differentiates and evolves to an invasive stage [95, 96]. PCa is characterized by an effacement of the basal cell layer with consequent derangement of basal lamina, decreased epithelial polarity and an

inefficient glandular acini connection [97]. PCa can be confined to the prostate, spread into adjacent tissue or lead to a metastatic disease, first to the pelvic lymph nodes and eventually to distant lymph nodes, brain, liver, lungs and bones (Figure 1.3) [26, 98, 99]. A supportive tumor microenvironment is essential in controlling local tumor growth, invasion and metastasis [26]. A high incidence of bone metastasis is observed in the advanced stages of this disease, and may be related to the tumor microenvironment and the characteristics of the bone matrix that may facilitate the establishment of metastasis [100, 101].

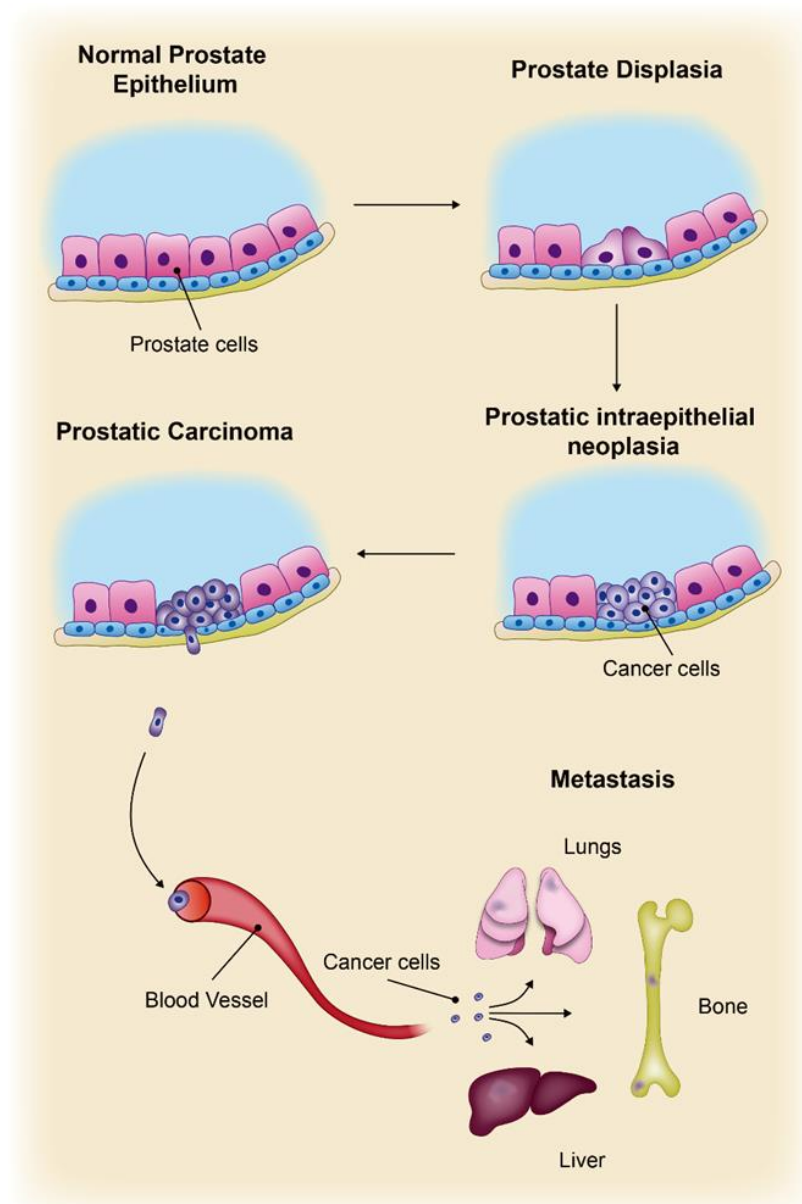


Figure 1.3. Schematic representation of the proposed model of the cellular events associated with development and progression of prostate cancer. The pathophysiology of prostate comprises multiple steps and starts with the appearance of pre-malignant lesions and the transformation of prostate epithelial cells, which in turn may generate cancer progenitor cells and prostate intraepithelial neoplasia phenotype can emerge. The constant activation of distinct growth signaling pathways in prostate cancer cells, including the activation of androgen receptor, leads to the development of local and invasive carcinoma and ultimately to the metastazitation to different organs, including liver, lungs and bones.

At the initial stages of PCa, a reactive microenvironment is created upon PCa and stromal cell layer interaction, in which androgens play a crucial role promoting cell proliferation and survival [102]. Androgen action is mediated through AR, which appears to have structural and functional alterations during progression of PCa [103]. In primary PCa, only a few AR gene mutations have been found, and at initial stages androgen ablation therapies lead to tumor regression in the majority of patients [103, 104]. However, PCa cells may acquire additional genetic alterations in comparison with the early stages of the disease; among them are mutations in the AR gene, which are frequently found in metastatic PCa [103, 105-107]. At this advanced stage, PCa cells can survive and proliferate without the signals that in normal conditions are delivered by the circulating androgens [104]. It corresponds to the so-called recurrent androgen-independent PCa phenotype or hormone-refractory PCa (HRPC), and at this stage the classical androgen ablation therapies tend to fail [13, 103, 104]. Therefore, the loss of androgen dependence restricts the therapeutic options and is usually associated with lethal forms of disease [107, 108]. However, the androgen insensitivity and the ability of tumor cells to grow even without circulating androgens depends on the activation of AR intracellular signaling pathways by mechanisms independent of hormone-binding to receptor [104]. Others refer that the androgen independence characteristic of the advanced stages of PCa may result from the clonal survival and expansion of transformed prostate epithelial stem cells and also by the activity of AR-independent mechanisms [109]. The diverse pathways involved in the development and progression of PCa from androgen dependent to androgen independent stages will be present further ahead.

Molecular pathways of prostate carcinogenesis: androgen dependence /independence

There are several pathways involved in androgen-independent progression of PCa, and many antiapoptotic gene products, growth factors and their receptors are differently expressed in the different stages of disease progression, contributing to tumor growth and cell survival, migration and invasiveness. Several differentially expressed genes have been identified as having a preponderant role in the initiation of PCa, namely the tumor suppressor proteins, phosphatase and tensin homolog (PTEN), p27, NKx3 and Rb, the transcription factor Myc, GSTP1, which exert an important role in preventing oxidative stress damage, and also hepsin, a cell-surface serine protease [42, 110]. The activation of the signaling cascades mediated by AR, ER, EGFR, human epidermal growth factor receptor 2, sonic hedgehog and Wnt/ β -catenin and cytokines (MIC-1 and IL-6) may confer the aggressive phenotypes that are observed in HGPIN and adenocarcinomas (Figure 1.4), and the differential expression of cell survival factors, such as Bcl-2, p53, Bax, syndecan-1 and clusterin are among the most frequent genetic alterations associated with aggressiveness of PCa [42, 76, 111, 112]. Moreover, the down-regulation of elements in several apoptotic signaling cascades, such as ceramides and caspases, was also observed in metastatic PCa cells [76].

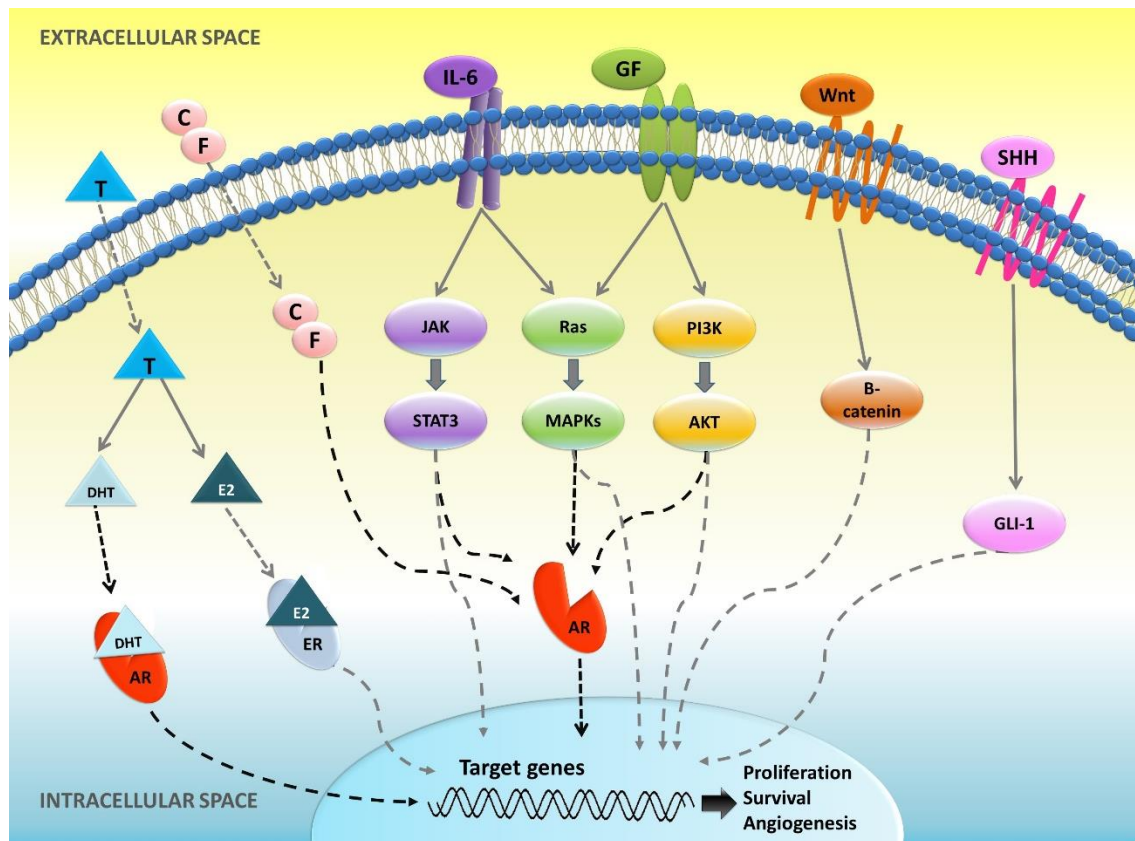


Figure 1.4. Schematic representation of the proliferative and anti-apoptotic putative signaling pathways activated in prostate cancer by the androgen receptor (AR), estrogen receptor (ER), growth factors family members (GF), IL-6, sonic hedgehog (SHH) and Wnt. Testosterone (T) can be converted to 5α-dihydrotestosterone (DHT) and 17β-estradiol (E₂) through the enzymatic activity of 5α-reductase and aromatase, respectively. The diverse pathways and factors such as corticosteroids (C), flutamide (F) or other anti-androgens, which also may be involved in the activation of AR are shown. Legend: JAK, Janus kinase; STAT, Signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3'-kinase.

Indeed, PCa growth occurs by the unbalanced ratio between cell proliferation and apoptosis. Androgens are viewed as the main regulators of this ratio, and in patients who androgen ablation had failed, a high percentage of tumors present overexpression of AR, which is rarely observed at the early stages of PCa. The androgen/AR pathway may be hyperactivated through different mechanisms, which have been extensively reviewed over years [108, 113]: (a) enhanced AR sensitivity to low androgens levels by increasing the expression of AR or elevating of the local production of androgens by increasing the activity of 5α-reductase; (b) alteration of the normal androgen response by way of mutations in AR gene that lead to decreased specificity for ligand, with AR becoming responsive to other steroid hormones, growth factors or antiandrogens; or by changes in the expression of co-regulators of the AR; and (c) several growth factors, including the epidermal growth factor, keratinocyte growth factor and the insulin-like growth factor-1 can activate AR and AR target genes in the absence of androgens [76]; HER-2/neu (and eventually other receptor tyrosine kinase), which is usually overexpressed in PCa, could be an important mechanism for the progression to HRPc, because it can activate the AR by indirect activation of mitogen-

activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways, or inactivation of the tumor suppressor gene PTEN [114]. Akt is also involved in the inactivation of pro-apoptotic Bad and procaspase-9 proteins [115].

Non-genomic activity of androgens/AR typically involves the rapid induction of conventional second messenger signal transduction cascades, including the increase of intracellular calcium (Ca^{2+}) levels, which in turn activates PKA, PKC, and MAPK. This stimulation of second messenger signaling pathways may ultimately modulate the transcriptional activity of AR or other transcription factors, contributing for PCa progression [116]. Therefore, the regulation of cell proliferation and apoptosis is also dependent of Ca^{2+} signaling and the re-arrangement of Ca^{2+} pumps, $\text{Na}^+/\text{Ca}^{2+}$ exchangers and Ca^{2+} channels is intimately related to the enhanced proliferation and reduced susceptibility to die, and so with the development and progression of PCa to an androgen-independent stage [117].

Other alternative or complementary pathways to the activation of AR signaling in the androgen-independent stages, include Bcl-2 upregulation and/or combined loss of p53/Rb or PTEN/NKx3.1 tumor suppressor genes [118-120].

Also, the stimulation of the AR transcription rate may promote cell proliferation at low androgens levels. On the other hand, the diminution or loss of AR activity promote growth and selection of cell clones that proliferate in an AR independent manner [114]. A large part of the current knowledge on the physiology of androgen-dependent and androgen-independent PCa stages comes from *in vitro* studies using different PCa cell line models. The majority of studies, classically and routinely have been using PC3 and DU145 cell lines as androgen nonresponsive models, and the LNCaP cell line as androgen-responsive, mimicking, respectively, advanced and early stages of metastatic PCa. PC3 and DU145 cells are derived from bone and brain metastasis, respectively, and are associated with a relatively high proliferation rate [121, 122]. The LNCaP cell line was originated from a lymph node metastasis, express a functionally active mutated form of the AR [123] and are androgen-responsive in terms of cell proliferation [124]. The several genetic alterations involved in the development of PCa directly affect the cellular response to hypoxia, the ability of tumor cells to recruit new blood vessels, as wells as metabolic alterations.

Metabolic adaptation of prostate cancer cells

The development and progression of tumors result from a complexity of altered mechanisms that allow cells to acquire a malignant phenotype with an unlimited cell proliferation. In addition to the mechanisms described above, it is perfectly established that tumors cells adjust their energy metabolism to fuel the uncontrolled cell proliferation characteristic of cancer [125]. Therefore, this reprogramming of energy metabolism is considered a hallmark of cancer [126]. Warburg's studies first established that tumor cells have an abnormal increase of glucose consumption to lactate production, instead of following the mitochondrial pathway to obtain energy, even in the presence of oxygen, contrastingly with what occur with normal cells [127]. This process is usually called "aerobic glycolysis", and has been

considered a crucial metabolic switch in PCa, which will be detailed in Chapter 2. In fact, tumor cells have high rates of glucose consumption and utilization relatively to normal cells, a feature that has been useful in cancer diagnosis with the utilization of the glucose analog 2-deoxy-2-fluoro-D-glucose (FDG) labeled with fluorine-18 (^{18}F -FDG) with positron emission tomography as imaging method [128-130]. Some reports exist indicating higher FDG consumption in PCa cases and in PCa cell lines, while other studies demonstrated that PCa have low FDG activity [131-133]. Therefore, other metabolites have been suggested for use in PCa detection through the PET system, including, ^{18}F -choline, ^{11}C -choline, ^{11}C -acetate and ^{18}F -fluorodihydrotestosterone [134-138].

In the case of PCa, the importance of other metabolic pathways besides glycolysis has been described. As a matter of fact, normal prostate cells produce, accumulate and secrete high levels of citrate [139], while in PCa low levels of citrate were detected [140, 141]. It was proposed that PCa cells use the oxidation of citrate as an energy source, instead of maintaining the secretory activity of citrate, resulting in an energy-generating system being essential for PCa development [139, 141]. Citrate can also serve as precursor for lipogenesis, an essential process in tumor cells for the membrane biogenesis [141].

Interestingly, increasing evidence has been pointing fatty-acid metabolism as a dominant source of energy in cancer cells, and the metabolic products of fatty acid metabolism are rapidly consumed by dividing cells, which allow growth and survival of neoplastic cells [142]. In addition, glutamine seems to be used as carbon source to maintain the Tricarboxylic acid (TCA) cycle and a major role of glutaminolysis in the development and progression of PCa also has been suggested [142, 143].

In sum, PCa cells have the ability to reprogram their metabolism to assure the energy requirements for proliferation and growth. However, the molecular mechanisms that drive and control the metabolic reprogramming in PCa still are largely unknown. Further research is needed to accomplish the existing gaps and find the best strategies to target the metabolic pathways that are involved in the progression of this disease.

Diagnostic and therapeutic options

The main diagnostic methods used for detection of PCa include digital rectal examination, the serum concentration of PSA and transrectal ultrasound guided biopsies followed by histologic examination [144-146]. In the cases in which clinical doubtfulness of PCa persists after negative results from biopsies, the magnetic resonance imaging technique is usually used to analyze a possible previously located PCa followed by a biopsy of the suspicious area [146]. In the last decades, we have witnessed a growing interest in discovering biomarkers that can be used for PCa detection, monitoring and also for the identification of recurrent disease. Although PSA continues to be considered a valuable biomarker and indicator for clinical practice in PCa diagnosis, it seems to have some limitations, which includes in several cases poor specificity [147-149]. Therefore, other biomarkers have been searched in tissue samples or body fluids (e.g. blood and urine) and PCa antigen 3 (PCA3), TMPRSS2-ERG gene fusions,

circulating tumor cells, exosomes, bone turnover markers and also metabolic related molecules, among other, are the most promising biomarkers for PCa detection [150-156]. The establishment of biomarkers with high sensibility and sensitivity and easily measured is a crucial point to guide therapeutic decision [146].

In patients with localized PCa, the standard method for treatment is the complete removal of the prostate gland and some surrounding tissue, called as radical prostatectomy, or alternatively the radiation therapy, external beam radiation or internal radiation, designated as brachytherapy [146, 157, 158]. Both treatment approaches often are used in combination with hormone therapy or chemotherapy [158]. Androgen-deprivation therapy, with “chemical castration” suppressing the production of testosterone or the use of anti-androgens inhibiting the AR actions, is actually the principal treatment for patients with locally advanced PCa, metastatic PCa and recurrent biochemical disease after failure of treatment [113, 158, 159]. However as previously referred, HRPc can emerge, and another option to treat these cases is the usage of some chemotherapeutic and immunotherapeutic agents, in which docetaxel and cabazitaxel and sipuleucel-T are the therapeutic agents approved by FDA for PCa treatment, and seems to be effective in improving the life-quality of patients with pain, although they do not have great results in survival outcomes [113, 160-162]. In this aggressive stages of tumors, it is believed, as discussed above, that AR continues constantly active, and new strategies have been emerging in an effort to inhibit AR signaling pathways. The abiraterone acetate is an inhibitor of androgen biosynthesis that seems to increase survival time [163, 164]. Nevertheless, and although these therapies are recognized as effective in the treatment of PCa, the optimal sequencing to use these treatments is not entirely defined and the truth is that many of these cases eventually progress or relapse. In the last years, other therapeutic approaches have been studied, and has gone through the analysis of the molecular pathways involved in the progression to androgen-independent tumor cells [113, 158]. The molecular targeting of the oncogenic and apoptotic cascades involved in the progression of PCa and the metabolic alterations founded in more aggressive stages of PCa represent new promising therapeutic approaches, which will be discussed in Chapter 2.

The deep knowledge about the molecular pathways involved in the progression of PCa hopefully may lead to the identification of selective biomarkers and development of new and effective therapies, in combination or alone, against the metastatic and androgen-independent prostate tumors.

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Chapter 2

Glucose uptake and androgen responsiveness of prostate cancer cells

This chapter was published in:

Vaz, C.V., Alves, M.G., Marques, R., Oliveira, P.F., Maia, C.J., Socorro, S. *Glucose Uptake and Androgen Responsiveness of Prostate Cancer Cells. In: Glucose Uptake: Regulation, Signaling Pathways and Health Implications*, Nova Science Publishers, Inc, New York, USA. 2013; ISBN: 978-1-62618-670-5

Glucose uptake and androgen responsiveness of prostate cancer cells

Abstract

Prostate cancer (PCa) onset and development require a sequence of genetic changes and additional metabolic adaptations to ensure survival of the modified cells and to finally achieve a malignant phenotype. This concept may be associated with the “Warburg phenomenon”, which consists of an increased glycolytic capacity and reduced mitochondrial oxidative phosphorylation, maintained even in the presence of high oxygen concentrations. It has been proposed that this alteration may be a metabolic mechanism of tumor cells to survive and growth under hypoxic conditions through the modulation of key enzymes and transporters involved in the glycolytic metabolic pathways. The progression of PCa is characterized by a transition from an androgen-dependent to an androgen-independent phenotype, which corresponds to early and advanced stages of PCa respectively, and an increase of glucose uptake and consumption is required for rapid proliferation of androgen-dependent cells. Identifying the metabolic alterations that occur in the transition of androgen-dependent to androgen-independent stage is a fundamental step for the development of new diagnosis and therapeutic approaches of PCa.

Introduction

Despite intensive scientific research in the field, and remarkable advances in diagnosis and treatment, prostate cancer (PCa) continues to be a source of considerable morbidity and mortality for men worldwide. For example, the American Cancer Society estimates that over 215000 new diagnoses and 32000 deaths occur per year in the United States of America [1]. Environmental issues, namely diet and other life style factors, host immune and inflammatory responses, and endogenous hormones along with genetic factors, all concur to the pathogenesis of prostate and to PCa [2, 3]. Anyway, the disease results from the progression of acquired phenotypes that are continuously generated by aberrant changes in gene expression and function [4]. A large number of genes, including oncogenes, tumor suppressor genes and hormonal receptors, among others, are altered in prostate tumor tissues, and have been implicated in the etiology of PCa [5, 6].

Androgens are important regulators of cell proliferation exerting their actions mainly through the activation of the classical intracellular androgen receptor (AR) [7-9]. The size of adult prostate gland is maintained by a homeostatic balance between the processes of cell renewal and death. This balance is essentially regulated by androgens, which are known to play a key role in the control of prostate cell growth and evolution of PCa [10]. Histologically PCa progresses from precursor lesions, termed prostatic intraepithelial neoplasia (PIN) arising primarily in the peripheral zone of prostate. In contrast, the non-malignant overgrowth of prostate epithelial cells - BPH - which is common among aging man, occurs almost exclusively

within the transition zone [11]. PCa is initially confined to the prostate and at initial diagnosis the majority of tumors show androgen-dependent proliferation [12]. Therefore, the AR is commonly used as therapeutic target in PCa treatment and androgen deprivation approaches result in tumor regression [13]. However, as disease progresses, PCa is characterized by a transition from the androgen-dependent to the androgen-independent phenotype [14], and these tumor cells are able to survive and progress even in the absence of circulating androgens. Unfortunately, as the new androgen-independent status emerges, androgen ablation therapies fail and tumor recurs to a lethally resistant phenotype [15, 16]. Understanding the pathways that lead to androgen-independence and characterizing the physiology of androgen-independent PCa cells is a crucial step towards developing new and effective therapies for this lethal form of PCa, the so-called hormone refractory prostate cancer.

The uncontrolled cell proliferation typical of cancerous tissues is associated with increased energy requirements. An increased glucose consumption is a basic feature of prostate malignant cells, and their glycolytic pathways must provide the energy needed for abnormal cell proliferation and growth [17, 18]. Increased rates of glycolysis may also confer adaptive advantages if the resulting excess of pyruvate is available for lipid synthesis, or provides essential anabolic substrates, such as ribose, for nucleic acid synthesis [19]. In addition, PCa has been considered to have a unique metabolism and the enhanced glycolytic metabolic profile attributed to the advanced stages of PCa results from adaptation of cells to hypoxia, being strongly linked with oncogene activation and silencing of tumor suppressor genes [20, 21]. Also, a relationship between androgens and the metabolic changes associated with progression of PCa has been established [22, 23]. Moreover, it has been proposed that progression to an androgen-independent stage may be associated to distinct metabolic phenotypes [24]. Detailing the metabolic reprogramming and the characteristic metabolic profile of PCa cells, and also unraveling the molecular hallmarks associated with transition to androgen-independent stages will undoubtedly contribute for development of new metabolism-based approaches in diagnosis and treatment of PCa.

Metabolic reprogramming and the “Warburg effect” in prostate cancer cells

The energetic substrate adenosine triphosphate (ATP), required for maintenance of cell functions, is produced through aerobic glycolysis by the conversion of glucose via pyruvate into acetyl-CoA, which in non neoplastic cells is completely oxidized in the mitochondria to carbon dioxide and water, through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation [18]. As an adaptive condition cancer cells exhibit alternative metabolic strategies to ensure energy production. The first and the well-known energetic metabolism alteration in tumor cells, was proposed by Warburg in 1920 [17] and it has been known as the “Warburg effect”. It assumes that glycolysis is predominantly used for ATP production in detriment of oxidative phosphorylation, producing high levels of lactate, an alteration

maintained even in the presence of high oxygen levels (“aerobic glycolysis”) [17, 25]. Glycolysis is the conversion of glucose to pyruvate which can be subsequently transformed in lactate. Glucose enters into the cell via glucose transporters (GLUTs), and then is metabolized to fructose-1,6-bisphosphate, which in turn originates pyruvate (Figure 2.1). Hexokinase (HK) and phosphofructokinase (PFK) are both important glycolytic enzymes involved in this process. The activity of lactate dehydrogenase (LDH) converts pyruvate into lactate, which is extruded to the extracellular space through monocarboxylate transporters (MCTs) [25]. Tumor cells may have to survive in conditions of fluctuating oxygen tension and the increase in glycolytic flux (Figure 2.1) to generate ATP, may be a metabolic strategy to ensure their survival and growth in environments with low oxygen concentrations [26].

Tumor cells, as highly proliferating cells consume elevated amounts of glucose and glutamine. Glucose is mostly converted to pyruvate through glycolysis, which is frequently excreted as lactate, an important step for regeneration of nicotinamide adenine dinucleotide (NAD⁺) for glycolysis. Lactate production contributes to the acidification of the microenvironment, which favors tumor invasion and suppresses anticancer immune effectors [27, 28]. The lactate produced by tumor cells, eventually enters in stromal cells following oxidative phosphorylation pathway or regenerating pyruvate that in turn, may be used by cancer cells [25, 29]. This process generates complementary metabolic pathways allowing cancer cells survival and growth. On the other hand, glucose can also be used as a source of carbon to produce ribose, used in DNA and RNA synthesis, through the pentose phosphate pathway. In addition, this process generates nicotinamide adenine dinucleotide phosphate (NADPH) that ensures antioxidant defenses of the cell and contributes to fatty acid (FA) synthesis [20]. Pyruvate can also be converted to acetylCoA (Figure 2.1) that tends to be introduced in a “truncated” TCA cycle in the form of citrate, which is exported back to the cytosol, and acetyl-CoA becomes also available for the synthesis of FA, cholesterol and isoprenoids [19]. De-novo FA and sterol synthesis are both characteristic of early and advanced PCa. A number of studies have demonstrated a diminished citrate content in PCa [19], consistent with the regulation by androgens of a number of key enzymes involved in FA and cholesterol synthesis, which exhibit increased activity in PCa cells [30]. PCa express high levels of fatty acid synthase (FASN), a key metabolic enzyme catalyzing the terminal steps in the synthesis of long chain saturated FAs [31]. The PCa cell lines, PC3 and DU145, showed significantly increased mRNA levels of sterol regulatory element-binding proteins SREBP-1a and SREBP-2 in comparison with LNCaP, but diminished mRNA levels of SREBP-1c, the isoform linked to lipogenesis. Although no significant differences in FASN mRNA levels were verified, acetyl-CoA carboxylase, an enzyme that also mediates lipid synthesis, was significantly more expressed in both PC3 and DU145 cells relatively to LNCaP [24]. The increase in lipid synthesis is advantageous for tumor cells in terms of growth advantage, self-survival and drug resistance.

Interestingly, in cancer cells glycolytic intermediaries are provided for anabolic reactions, including glucose 6-phosphate for the synthesis of glycogen, ribose 5-phosphate

essential for sustention of protein and DNA synthesis, and pyruvate to produce non-essential amino acids such as alanine [20]. In cancers with a predisposition to use aerobic glycolysis to produce energy, the emergence of this new metabolic phenotype may arise from the selection of cells that have adapted to the tumor microenvironment and/or from anomalous signaling induced by genetic changes. It is not totally clear how this is an advantage for tumor growth in a different microenvironment, but increased levels of both lactate and alanine were detected in PCa compared with benign prostate tissues [32], which indicates enhanced glycolytic activity or the Warburg effect (Figure 2.1). Although, the underlying molecular mechanisms need to be detailed, it is evident that the resulting alterations confer adaptive, proliferative, and survival advantages to the tumor cells.

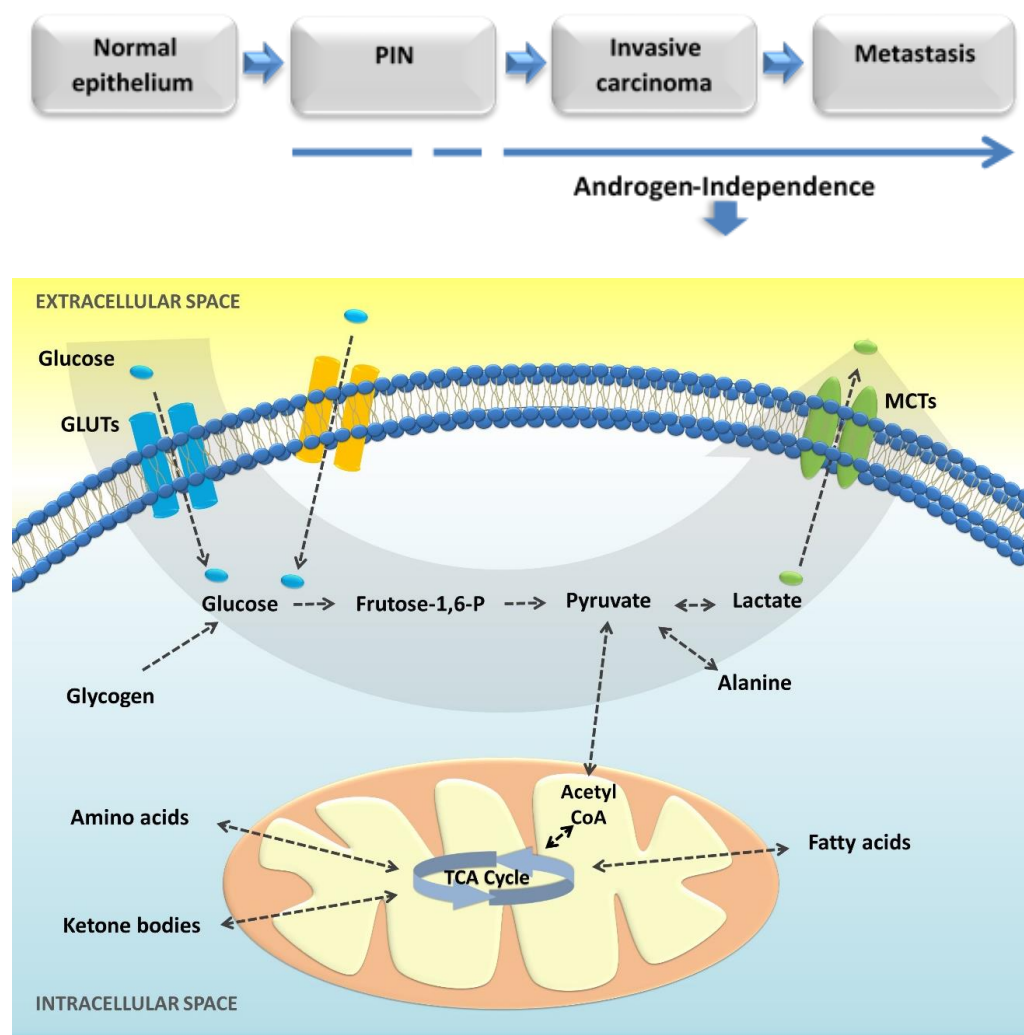


Figure 2.1. Schematic diagram of progression stages of prostate cancer (PCa) and the overall cellular metabolism. The stages of progression are indicated, together with the alterations in the metabolic pathways associated with the androgen-independent stages of PCa. Tumor cells display enhanced glycolytic capacity due to increased uptake of glucose from the extracellular space. After enter the cell via glucose transporters (GLUTs), glucose is metabolized to fructose-1,6-P, which in turn originates pyruvate. Pyruvate can be converted to lactate or alternatively originate alanine. The produced lactate is transported to the extracellular milieu by monocarboxylate transporters (MCTs). Gray-shaded arrow highlights the glycolytic metabolic pathway. Legend: TCA, tricarboxylic acid.

Mechanisms of metabolic reprogramming

The mechanisms that underlie tumor metabolic reprogramming have been investigated and one of the main events involved in these metabolic changes resides in the activation of hypoxia-inducible transcription factor (HIF), an important transcription factor that is activated by hypoxic stress, due to different oxygen concentrations in the tumor environment [33]. Also, the activation of oncogenes or the inactivation of tumor suppressor genes, inflammatory response, metabolic factors, and oxidative stress contribute to HIF expression [34-36]. HIF controls the transcription of several genes involved in cell survival, increases metabolic resistance to hypoxia and apoptosis, favors tumor invasion, stimulates angiogenesis by upregulating vascular endothelial growth factor (VEGF) and regulates glucose metabolism [37]. HIF-1 enhancement promotes the conversion of glucose to pyruvate by upregulating GLUT1, HK1 and HK2 (catalyzers of the initial step of glycolysis), PFK-1 and PFK-2 (involved in an irreversible reaction of glycolysis), and LDHA and MCT4 (involved in lactate production and transport) [26, 35]. In addition, HIF-1 decreases the conversion of pyruvate to acetyl-CoA by regulating pyruvate dehydrogenase kinase 1, which inhibits pyruvate dehydrogenase [38, 39]. All these mechanisms lead to the stimulation of the glycolytic pathway.

The majority of cancers, including PCa, present a distinct glycolytic metabolic profile under regulation of HIF [40]. In PCa, HIF-1 expression is upregulated at early phases of the neoplastic development [41] but it is also highly correlated with the risk of metastases [37]. In addition, DU145 and PC3 PCa cells, which represent more aggressive states of this disease, show higher levels of HIF-1, as well as HIF target genes such as VEGF isoforms, than LNCaP cells [24, 42]. In addition to HIF-1 upregulation, also oncogenes and tumor suppressor genes have been recognized to be important in the metabolic reprogramming at different levels. The tumor suppressor gene p53, regulates metabolism at multiple steps, including the enhancement of glutamate synthesis, through upregulation of glutaminase 2 [43], and also the inhibition of FA synthesis and glycolysis. p53 regulates positively the synthesis of SCO2 (synthesis of cytochrome c oxidase protein), which interferes with the mitochondrial electron transport chain [44] and activates TIGAR (TP53-induced glycolysis and apoptosis regulator) inhibiting glycolysis by decreasing the levels of fructose-2,6-bisphosphate (F-2,6-P₂) [45].

The oncogenes Ras and myc that are often mutated and hyperactivated in a set of cancers, including PCa, increase glycolysis and lactate production by enhancing the expression of various metabolism related genes [46-48]. Myc is also the main oncogene implicated in glutamine addiction of cancer cells, through the upregulation of glutamate synthesis [49]. The mammalian target of rapamycin (mTOR) pathway, is hyperactivated in PCa regulating glycolysis, through HIF-1 dependent and independent pathways, and lipogenesis through the transcription factors SREBP and Myc. In addition, PI3K/Akt and MAPK pathways are also involved in glycolysis and lipid biosynthesis, and are deregulated and constitutively activated in human PCa [31]. 5-AMP-activated protein kinase (AMPK), other control point in different metabolic pathways is also deregulated in PCa. AMPK-mediated phosphorylation events participate in the regulation of glucose and lipid metabolism, through inhibiting

metabolic functions that requires ATP consumption, which includes FA, sterols, glycogen and protein synthesis, and increases glucose uptake and mitochondrial activity, controlling lipogenic and PI3K/mTOR pathways [31].

Glycolytic metabolism in PCa: What is known?

Tumor cells need a constant supply of glucose as an important source of energy, as well as a precursor of glycoproteins, triglycerides and glycogen. PCa has been recognized to have unique metabolic features associated with neoplastic development and the rapid proliferation of androgen-nonresponsive PCa cells, which include an increase in lactate and alanine levels and an increase of glucose consumption [32, 50-52]. There are strong evidences that glycolysis is enhanced in tumor cells due to modulation of the expression of key enzymes and transporters involved in this metabolic pathway (Figure 2.1) [53].

In this topic we focus on the description of the glycolytic phenotype of PCa cells and their metabolic strategies to ensure tumor growth and survival. The low rate of ATP production coupled with high-energy requirements leads to an increased need of glycolysis, a process that starts with glucose uptake. This increased glucose consumption is required for the rapid proliferation of both androgen-dependent and independent PCa cells [54], and has been proved to be useful and frequently used for cancer detection by positron emission tomography (PET), through measurement of the uptake of a glucose analogue, 2-deoxy-2-fluoro-D-glucose labeled with fluorine-18 (^{18}F -FDG) [55]. Studies have demonstrated an higher FDG consumption in PCa cases with a higher Gleason score [56]. Furthermore, it was reported a decreased uptake of FDG in the prostate tissues of PCa patients undergoing androgen ablation therapy [22], which suggests that glucose metabolism differs between early and advanced stages of PCa. Also *in vitro* studies showed a higher FDG uptake in androgen-responsive PC346C cells derived from a non-progressive prostate primary tumor [23, 57]. Moreover, under androgen deprivation, a diminished FDG uptake was observed in androgen-dependent LNCaP cells [23], which was lower than that observed in androgen-independent PC3 cells [58]. Others have also described that glucose uptake is increased in PCa allowing rapid cell proliferation, and that androgen-independent PC3 and DU145 cells present a high rate of glucose consumption [54].

The transport of glucose across the plasma membrane may be the first rate-limiting step for glucose metabolism. Glucose is a hydrophilic compound and crosses the plasma membrane by mediation of GLUTs, which belong to the gene family of solute carriers 2A (SLC2A) [59]. This family consists of 14 glucose transporters, in which GLUT14 appears to be a duplication of GLUT3, and GLUT13 is a proton-coupled myoinisitol transporter (HMIT). GLUTs can be grouped in three different classes based on their sequence similarities. The class I includes the well characterized GLUT1-4, identical 48-63 % to each other in human. GLUT5, 7, 9 and 11 belong to Class II, are 36-40% identical and all demonstrate transport activity for fructose. Class III is composed for the remaining GLUT6, 8, 10, 12 and the HMIT [59]. GLUTs exhibit variable hexose affinity and are metabolic- and hormone-regulated, displaying tissue-

specific expression. They regulate the bidirectional transport of glucose (or fructose) to the intracellular and extracellular space, according with the gradient concentration [60, 61]. Given that glucose and (eventually) fructose are required substrates to increase the energy production necessary for tumor proliferation, several authors have speculated that GLUT family might play a fundamental role in cancer progression. In fact, an aberrant expression of GLUT family members has been reported in cancer cases [60]. Although GLUT1, 3, 5, 7 and 10 have been detected in nonneoplastic prostate [62-64], a total loss of GLUT5 expression was observed in PCa [62]. In contrast, GLUT1 is usually upregulated in different cancer tissues, and GLUT1 mRNA expression is correlated with FDG uptake in a rat model of PCa [65]. In PCa, expression of GLUT1 gene was significantly higher in comparison with BPH tissue and correlated with differentiation grade and Gleason score [66, 67]. In addition, over-expression of GLUT1 was observed in some specimens of highly proliferative intraductal PCa [68]. Effert et al investigated the expression of GLUT1 in LNCaP, PC3 and DU145 PCa cell lines and GLUT1 was detected in all cells, but the expression in the poorly-differentiated cell lines, DU145 and PC3, was higher relatively to the androgen-dependent LNCaP cell line [66]. Another report studied GLUTs expression in human PCa cell lines, LNCaP and its derivatives (C4, C4-2 and C4-2B), which are associated with increased tumor progression and androgen independence. All tested cell lines present GLUT1 and GLUT12 mRNA and protein expression, however no relationship was established between GLUT1 and GLUT12 expression and tumor progression or androgen independence grade [69]. The presence of GLUT12 in malignant prostate tissues, and in BPH cells surrounding cancerous ducts was demonstrated, but not in BPH cells from normal prostate tissues [69]. It was shown that androgens induce the expression of GLUT1 in LNCaP but not in PC3 cells through activation of the HIF-1 [70, 71]. Recently, the presence of GLUT4 in PCa cells was demonstrated, and its expression was higher in androgen-independent PCa cells [72]. These findings suggest a role for GLUT1 GLUT4 and GLUT12 for PCa progression.

Once inside the cells glucose is phosphorylated by the enzyme HK. Changes in the expression pattern of HK isoform occur in several tumor cells in comparison to nonneoplastic cells, which seems to be part of the mechanisms involved in the highly glycolytic flux presented by tumor cells [47]. In mammalian cells four different isoforms of HK (HK-1-4, or glucokinase) have been identified, which differ by their kinetic properties and tissue-specific expression [73, 74]. HK-2 is frequently overexpressed in proliferating tumor cells and seems to be important in the initiation and maintenance of high rates of glucose catabolism [47]. Another key control step in the glycolytic flux is the conversion of fructose 6- phosphate into fructose 1,6-bisphosphate by PFK-1, and is often considered a rate-limiting step in glycolysis. The activity of this enzyme is controlled by F-2,6-P₂, which biosynthesis and degradation are modulated by the bifunctional PFK-2 [47, 75]. PC3 cells displayed higher mRNA levels for HK-2 [24]. In DU145 cells an increase in expression of the glycolytic enzymes, HK, PFK-1 and PFK-2 was observed [47]. Importantly, it was reported that androgens upregulate the expression of HK-2 and PFK-2 in LNCaP cells, which mediated the increased uptake of glucose [76]. HK and PFK2 seems to be AR regulated genes [77]. In addition, an increased expression of aldolase

and pyruvate kinase (PK), both important enzymes in glycolysis, was observed in PCa cells [24]. A significant increase in lactate and alanine concentration has been described as one of the metabolic changes associated with PCa development [32, 51, 52, 78]. It was described that PC3 and DU145 cells produced higher amounts of lactate than LNCaP cells [24], and higher lactate production/concentration ratios have been associated with more aggressive cancer stages and metastasis [52, 78, 79]. Accordingly, LDHA has been used as a marker of neoplastic transformation [47]. The lactate produced from glucose results from pyruvate conversion, a process achieved by the activity of LDH enzyme, a tetramer of A and B subunits [80]. *In vitro* studies showed an increase in LDH mRNA levels in androgen-independent PC3 cells comparatively to androgen-dependent LNCaP cells [24]. MCTs represent a large gene family (SLC16) composed by 14 members [81]. MCT1 to MCT4 proteins are proton symporters and mediate the transmembrane transport of lactate, pyruvate and ketone bodies [81]. So, MCTs are responsible for lactate export, associated with hyperglycolytic phenotype found in cancer cells, and for pH regulation, important to acid-resistant phenotype. MCTs have been associated with PCa progression and it has also been reported a correlation between MCT4 expression and a poor prognosis in PCa [82, 83]. MCT1 and MCT4 are expressed in around 90% of PCa cases, whereas no expression was found in nonneoplastic prostate tissues, PIN lesions or in non-tumor regions adjacent to primary PCa tissues [82]. The expression of MCT1 and MCT4 was associated with high pretreatment PSA levels, high Gleason score, high pathological stage, and nodal involvement [82].

In another study, MCT1 expression was confirmed in normal samples and significantly lower in tumor samples, and it was associated with higher PSA levels, absence of perineural invasion, and presence of biochemical recurrence [83]. On the other hand, MCT2 and MCT4 were significantly more expressed in tumor cells than in nonneoplastic tissues [83]. MCT4 expression was associated with higher PSA levels, advanced tumor stage, higher Gleason score, presence of perineural invasion, and presence of biochemical recurrence [83]. Besides the lactate, also alanine results from pyruvate conversion. PCa has been shown to produce higher levels of alanine compared with nonneoplastic prostate tissue [32, 50, 51]. A study performed by proton and ^{13}C HR-MAS spectroscopy using pyruvate as a metabolic substrate demonstrated a high fractional enrichment of alanine in PCa cells [84]. The lactate/alanine ratio is associated with the NADH/NAD⁺ equilibrium in the cell because the conversion of pyruvate to lactate and alanine is coupled with NAD⁺ and NADH reactions. Thus, lactate/alanine ratio reflects the cell redox state [85]. The study of this ratio is particularly attractive since it has been suggested that antioxidant therapy prevents the progression from androgen-dependent to androgen-independent PCa [86].

Glucose uptake and possible target therapies/hallmarks in prostate cancer

The development of novel chemopreventive and chemotherapeutic strategies preventing prostate carcinogenesis and progression of PCa into more aggressive stages is of great clinical interest either reducing costs or improving health outcomes for patients. PCa cancer,

similarly to tumors of other tissues, is highly dependent on glucose metabolism presenting a distinct glycolytic profile associated with hallmarks of disease progression (Figure 2.2), which strongly supports the demand for new anticancer treatments targeting glycolysis [23, 24, 54, 56]. Indeed, intense research efforts have targeted the metabolic pathways altered in PCa development and progression. Also, therapeutic strategies aiming at reach nonmetabolic pathways of PCa, including the inhibition of cell-autonomous growth signals (suppressing the PI3K/Akt/mTOR pathway), restoration of apoptosis (one example is the reestablishment of p53 function) and inhibition of angiogenesis (by inhibition of HIF), may also suppress cancer-specific metabolic programs and normalize the metabolic state (Figure 2.2) [25].

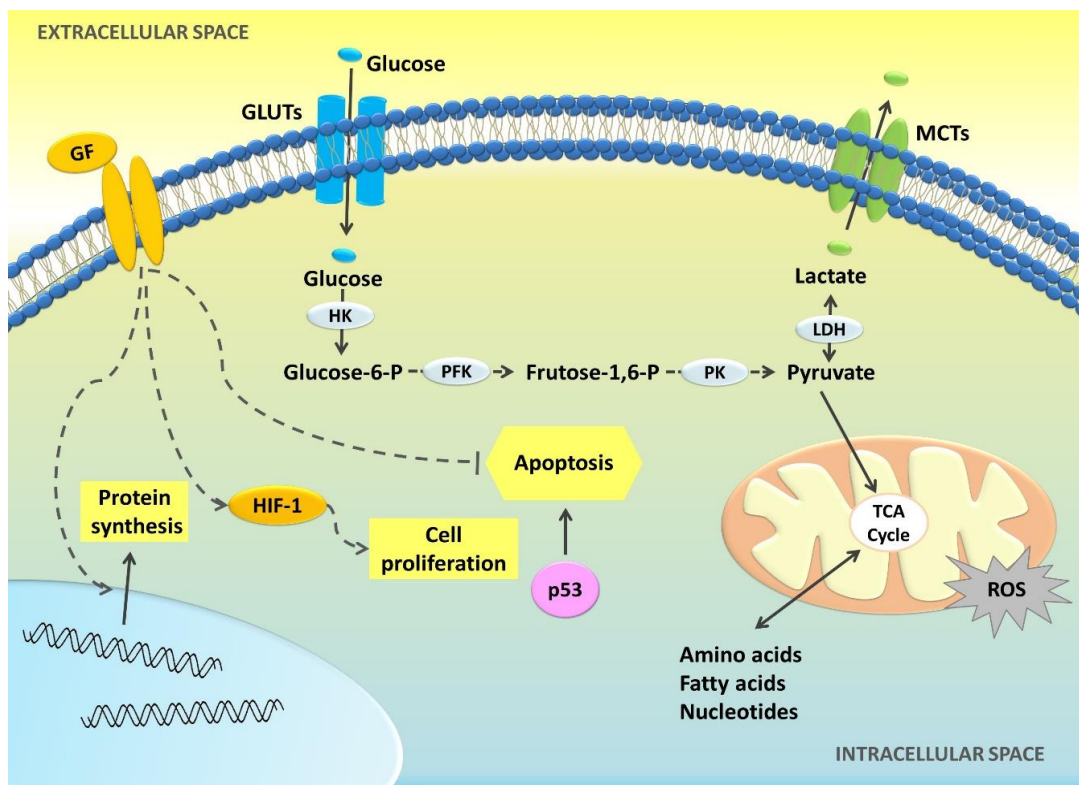


Figure 2.2. Schematic illustration of the main cellular metabolic pathways in prostate cancer (PCa) with emphasis in the hallmarks of disease development and progression, which could be important therapeutic targets. Prostate tumorigenesis is a multistep process that reflects the progressive transformation of normal cells into malignant derivatives. PCa cells present a distinct glycolytic profile, associated with the stage of progression, which strongly supports the increased cellular energetic demands. The prevalence of high glycolysis in PCa cells results primarily from overexpression of the high affinity glucose transporters (GLUTs), glycolytic enzymes and lactate transporters (MCTs). Cell-autonomous growth signals, increase of angiogenesis (via HIF-1 α) and suppression of apoptosis (with loss of p53 function) are also important features of PCa. Elevated oxidative stress in the prostatic microenvironment occupies also a central role in PCa progression by leading to inflammation and induction of lipid, protein and DNA damage that causes genomic instability. Dotted-arrows represent direct or indirect activation and blunt dotted-arrow indicates an inhibitory effect. Legend: GLUTs, glucose transporters; HK, hexokinase; LDH, lactate dehydrogenase; MCTs, monocarboxylate transporters; PFK, phosphofructokinase; PK, pyruvate kinase; ROS, reactive oxygen species; HIF-1 α , Hypoxia-Inducible Factor; TCA, tricarboxylic acid.

In addition, the inhibition of the processes and enzymes involved in cancer metabolic reprogramming may have a fundamental role in limiting cancer cell-specific bioenergetic requirement and anabolic reactions, and hence reversing the neoplastic phenotype [25, 31]. The prevalence of a high glycolytic state in cancer cells results primarily from overexpression of the high affinity glucose transporters GLUTs and the phosphorylation of glucose by the HK-2 enzyme. It has been demonstrated by clinical application of PET imaging measuring the incorporation of ^{18}F -FDG in cancer tissues, in which 2-deoxyglucose (2DG) competes with glucose for transmembrane transport and is then phosphorylated by HK-2, but due to the lack in 2' hydroxyl group cannot be metabolized, which leads to accumulation in the cells [87]. In addition, treatment with 2DG leads to intracellular ATP depletion and induces autophagy in PCa cells [88]. 2DG has been tested as an anticancer drug, and a recently terminated phase I clinical study in PCa patients defined a dose of 45 mg/kg to be used in Phase II trials [89].

Targeting glucose metabolism with agents such as 2DG may have a potential to competitively reduce glucose uptake and change the tumor glycolytic profile [88, 90]. In addition, studies based on glucose deprivation or using glucose uptake inhibitors (product-based energy restriction-mimetic agents-ERMA) have been reported in a therapeutic/preventive perspective and a link between ERMA antitumor effects [54, 91-93].

Another strategy to inhibit glucose metabolism may be through the inhibition of expression or functionality of GLUT family members (Figure 2.2). Specially GLUT1 has a high affinity for glucose and therefore, once expressed, play an essential role ensuring efficient glucose uptake when glucose becomes a limiting resource [94]. As previously described, GLUT1 and more recently GLUT12, were demonstrated to be overexpressed in PCa, with GLUT1 being differentially expressed in tumors with different grades of disease evolution [66, 69]. Thus, the use of GLUT inhibitors may constitute a good anticancer strategy in PCa in different stages of the disease. The silybin/silibinin is a GLUT inhibitor recently identified, which inhibits tumor formation and is actually being tested on ongoing clinical Phase I trials for PCa treatment [95]. Nevertheless, the development of specific inhibitors for GLUTs isoforms, namely for GLUT1, can be a useful strategy for PCa treatment, targeting stages of disease specifically overexpressing these transporters. Lactate production and extrusion to the extracellular space contributes to the acidification of the microenvironment and is associated with the glycolytic metabolic profile presented by PCa cells (Figures 2.1 and 2.2) [24]. This perturbation selects cells that present an acid-resistant phenotype and it has been considered an essential condition for cancer cell survival [96]. The inhibition of these adaptive mechanisms could be potentially used as a target to kill or to diminish the proliferation rate of cancer cells, increase the immune response against tumor cells and increase sensitivity to chemotherapeutic agents [97]. Other interesting target playing an essential role producing lactate and restoring the NAD for maintenance of the high-rate glycolytic profile is LDHA (Figure 2.2).which can be a potential therapeutic option in PCa [98]. These results are particularly interesting knowing that in other human cancer cells, LDHA

silencing by RNA interference impairs tumor initiation, maintenance and progression [99, 100].

The transport of lactate across the membrane is mediated through MCTs. MCTs play an essential role in pH regulation acting as transporters of lactate in the metabolic symbiosis created between glycolytic tumor cells and oxidative tumor cells, which produce and consume lactate respectively [101]. Importantly, MCT expression has been correlated with the progression of PCa [82]. MCT1, MCT2 and MCT4 expression was demonstrated to be increased in tumor cells, and specifically, MCT4 levels were correlated with a poor prognosis in PCa [82, 83]. In addition, MCT4 silencing results in decreased cancer cell migration through a mechanism involving interaction of MCT4 with $\beta 1$ -integrin [102]. This suggests that MCT1 and MCT2 should be explored as marker of PCa tumor and MCT4 as a marker of a more aggressive stage of PCa [83]. The contribution of lactate to the malignant phenotype, together with the up-regulation of MCTs in PCa cells makes MCT inhibition a tempting strategy for PCa treatment. Moreover, inhibitors of enzymes that occupy a central role in cancer-specific metabolism, such as HK, PFK and PK, all important enzymes in the glycolytic process (Figure 2.2) and overexpressed in PCa, might be targets for cancer therapy [24, 47]. Other interesting concept is the function of redox metabolism and reactive oxygen species (ROS) in tumor progression. Production and removal of ROS is intimately related with glucose and glutamine metabolism (Figure 2.2). In cancer, ROS may act as signaling molecules that promote proliferation or as mediators of cell death [103]. Some of the effects of metabolic reprogramming may due to regulation of ROS, and the use of antioxidant therapies have been suggested to prevent the progression from androgen-dependent to androgen-independent PCa [86].

Other attractive and possible anticancer target is HIF-1, which plays a critical role in upregulating glycolysis as an adaptation of tumor cells to hypoxia and is also upregulated in different stages of PCa [24, 37]. In addition to glucose metabolism, unique genetic and biochemical features in amino acid metabolism, lipid metabolism and nucleotide metabolism of cancer have been reported as potential targets for PCa cancer imaging and therapies [31, 104, 105]. However, the heterogeneity of tumor cells and environment conditions leads to the existence of a range of metabolic pathways used by invasive tumor cells, implying the use of multiple agents that inhibit energy production through aerobic and anaerobic pathways. The most therapeutic strategies in research associate the use of glycolytic inhibitors with chemo-radiotherapeutic agents, increasing the cytotoxicity of such agents [106, 107].

Conclusion

Cancer is a metabolic disease, and not surprisingly an altered glucose metabolism has been described in primary and metastatic PCa. Moreover, an enhanced glycolytic profile is characteristic of PCa cells, resulting from the necessity of these cells to produce enough energy to survive when resources are limited. PCa cells also use metabolic intermediates to the biosynthetic pathways, including lipid and nucleic acid synthesis, which support cell

proliferation. Currently, irrefutable and promising clinical and experimental evidences highlight the potential of therapeutic strategies inhibiting the targets that participate in the metabolic reprogramming, in order to suppress growth of established invasive PCa. Targeting the metabolic processes alone or in combination with other anti-cancer drugs may represent an improvement in the treatment of advanced PCa.

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Chapter 3

The emerging role of regucalcin as a tumor suppressor: facts and views

This chapter was submitted for publication:

Vaz, C.V., Correia, S., Cardoso, H.J., Figueira M. I., Marques, R., Maia, C.J., Socorro, S. The emerging role of regucalcin as a tumor suppressor: facts and views.

The emerging role of regucalcin as a tumor suppressor: facts and views

Abstract

Regucalcin (RGN) was first described as a calcium (Ca^{2+})-binding protein that plays a relevant role in the maintenance of intracellular Ca^{2+} concentrations. However, due to its downregulated expression with aging, RGN is also known as senescence marker protein-30. RGN protein is an X-chromosome gene product, whose transcription is regulated by various transcription factors, as well as, by a myriad of hormonal and non-hormonal factors. Besides the well-known role in Ca^{2+} homeostasis, RGN also has been linked to the control of intracellular signaling pathways, and several basic biological processes, such as cell proliferation and apoptosis. The role of RGN suppressing cell proliferation is associated with the regulation of expression of oncogenes and tumor suppressor genes. Also, RGN has been viewed as a protein involved in the control of cell metabolism and oxidative stress, having antioxidant activity, by reducing the production of reactive oxygen species and increasing the antioxidant defenses. It results clear that all the existent knowledge implicates RGN in the control of the main biological processes actually recognized as hallmarks of cancer. Moreover, it has been shown that tumor onset and progression are underpinned by the loss of RGN expression, whereas RGN overexpression had a protective role against the development of chemically-induced tumors. This review describes the mechanisms that control the tissue expression of RGN and discusses the experimental evidence that indicate RGN as a new tumor suppressor protein.

Keywords: apoptosis; cancer; cell proliferation; metabolism; oxidative stress; regucalcin; SMP30; tumor suppressor.

Introduction

Regucalcin (RGN) was firstly identified in 1978 by M. Yamaguchi as a Ca^{2+} -binding protein that do not contain the typical EF-hand Ca^{2+} -binding motif [1]. The RGN protein is encoded by an X-chromosome gene composed of seven exons, which is assigned to the segments p11.3-q11.2 and q11.1-12 of the human and rat chromosomes, respectively [2-4]. Besides the full-length product of RGN gene, a 33 kDa and 299 amino acid residues protein [3, 5], mechanisms of alternative splicing originate RGN protein variants with deletion of exon 4 or exons 4 and 5 [6, 7], for which the functional role needs to be determined.

The overall structure of RGN protein encompasses 24 β -stands which form 6 β -sheets, with the ability to bind Ca^{2+} ions and other [8, 9]. However, the major documented role for RGN is indeed Ca^{2+} -binding and the regulation of Ca^{2+} homeostasis, through modulating the activity of Ca^{2+} -pumps at plasma membrane, endoplasmic reticulum and mitochondria [10].

Over the years, the RGN functions have surpassed the regulation of intracellular Ca^{2+} concentrations, and a broad physiological role has been indicated for this protein. Its involvement in the regulation of several proteins involved in intracellular signaling pathways, namely kinases, phosphatases, phosphodiesterase, synthases, and proteases was demonstrated [10-12]. Also, it was shown that RGN is a cytoplasmatic protein with the ability to translocate to the nucleus [6, 13], where it seems to influence DNA fragmentation, and DNA and RNA synthesis, associated with the control of cell death and proliferation [12]. The relevant role of RGN in cell tissue homeostasis was also proved by studies that demonstrated a dual function of RGN in controlling both proliferation and apoptosis by modulating the expression and activity of important regulators of these processes [12, 14, 15]. Also, it has been described that RGN is involved in the control of metabolism and oxidative stress, acting as an antioxidant protein [12, 16]. Interestingly, accumulating evidence has been shown that loss of RGN expression underpins the onset and progression of several types of human cancers [6, 12, 17-21]. On the other hand, RGN overexpression *in vivo* was effective restricting the development of carcinogen-induced tumors, which strongly supports the protective role of RGN against tumor development and progression [15]. Despite the importance of these findings, they are not entirely surprising if we consider the multifunctional nature of RGN protein. As mentioned above, the biological actions of RGN have been implicated in the control of cell proliferation and cell death, metabolism, and oxidative stress, all well-known process recognized as hallmarks of cancer. The present review systematically describes the myriad of factors that contribute to the maintenance of RGN tissue expression levels, and discusses the recent experimental advances that sustain the role of RGN as a new tumor suppressor protein.

Regulation of RGN tissue levels: hormonal and non-hormonal factors

The most distinctive feature of RGN tissue expression pattern is its downregulation with aging, which has been described in several tissues of aged rat, namely, liver, kidney and testis [22, 23]. For this reason, RGN protein is also known as senescence marker protein 30 (SMP30) [22-24].

The molecular mechanisms that drive the diminished expression of RGN with advanced age are poorly understood though a role for the age characteristic increase in oxidative stress has been proposed. Lipopolysaccharide (LPS), tert-Butyl hydroperoxide (t-BPH), 1,1-diphenyl1-2-picryl-hydrazyl, and carbon tetrachloride, all known agents that elicit oxidative stress, were able to decrease RGN expression in rat liver and heart, and cell line models (Figure 3.1) [25-27]. Moreover, the effect of t-BPH was blocked by the antioxidant, N-acetylcysteine [25]. Contrarily, the caloric restriction for 6 to 24 months through its well established anti-oxidant and anti-aging actions prevented the marked decrease of RGN expression with aging in rat liver and kidney, as well as the LPS effects inducing the production of reactive oxygen species (ROS) [28].

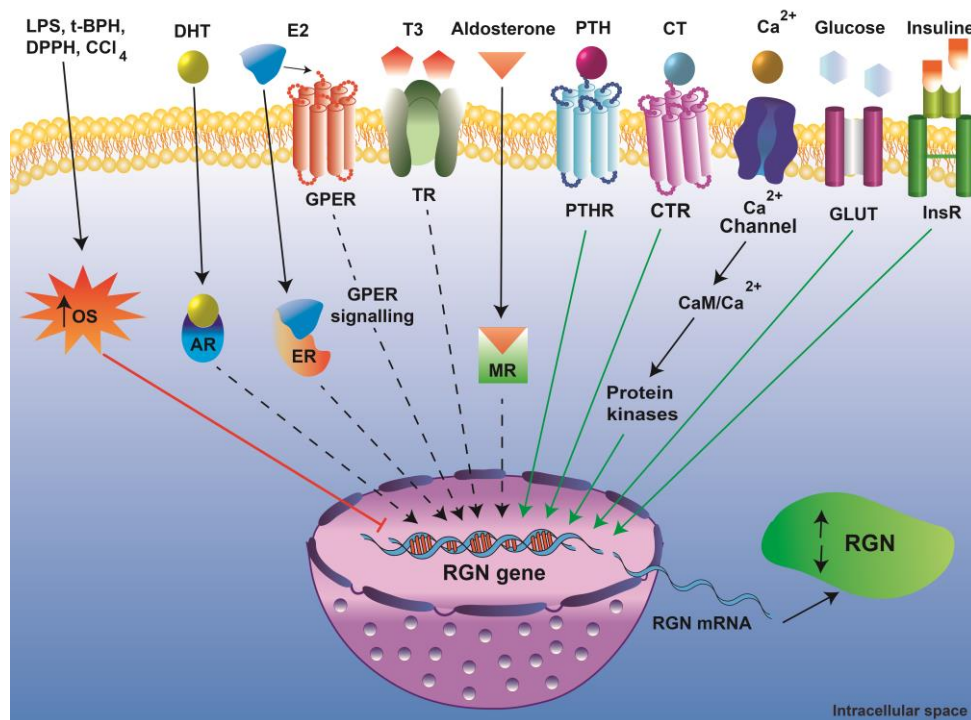


Figure 3.1. Schematic illustration of the diverse factors that regulate regucalcin (RGN) expression in tissues and cell lines. Legend: Head-arrows lines and bar-ended lines indicate activation and inhibition, respectively. Dashed arrows indicate that up- or down-regulation depends on the dose, time of stimulation and specific cell type. LPS, lipopolysaccharide; t-BPH, tert-Butyl hydroperoxide; DPPH, 1,1-diphenyl-2-picryl-hydrazyl; CCl₄, carbon tetrachloride; OS, oxidative stress; DHT, 5 α -dihydrotestosterone; AR, androgen receptor; E₂, 17 β -estradiol; ER, estrogen receptor; GPER, G protein-coupled estrogen receptor; T₃, Triiodothyronine; TR, thyroid hormones receptor; MR, mineralocorticoid receptor; PTH, parathyroid hormone; PTHR parathyroid hormone receptor; CT, calcitonin; CTR, calcitonin receptor; Ca²⁺, calcium; CaM, calmodulin; GLUT, glucose transporter; InsR, insulin receptor.

Considering the decline in gonadal hormones with the aging process, it is not surprisingly that RGN has been identified as a sex steroid hormones target gene. Estrogens and androgens have been both described as regulators of RGN expression in several tissues and cell lines (Figure 3.1). Ovariectomy enhanced RGN expression in rat mammary gland, an effect abrogated by the administration of 17 β -estradiol (E₂) [29]. However, the effect of castration on RGN expression was not observed in the liver of male and female rats [30, 31]. Even so, other studies demonstrated that estrogens increase the expression levels of RGN in rat and mice liver, as well as, in mouse osteoblastic cells [32-34], while decreasing the expression of RGN in rat kidney and in bulbourethral gland, prostate and testis of veal calves and beef cattle [35, 36]. Interestingly, in MCF-7 cells, the effect of E₂ on RGN expression was time-dependent, since at early-time points (6 and 12 h) E₂ treatment increased the mRNA levels of RGN whereas at longer exposure times RGN expression was diminished [6]. These effects seem to involve a membrane-bound estrogen receptor since the administration of E₂-BSA conjugated maintained the estrogenic effects in the regulation of RGN expression in MCF-7 cells (Figure 3.1) [6].

Besides estrogens, also androgens were demonstrated to regulate the expression of RGN in a tissue-specific manner. DHT administration increased RGN levels in the testis of veal calves and beef cattle [36], and in rat seminiferous tubules cultured *ex vivo* [23]. However, the suppressive effect of DHT on the modulation of RGN expression was reported in MCF-7 human breast cancer epithelial cells, and also in LNCaP human prostate cancer cells, and was dependent on the androgen receptor (AR) signaling (Figure 3.1)[6, 37].

The involvement of adrenal hormones in the regulation of RGN in the rat kidney was also demonstrated (Figure 3.1). Both adrenalectomy (ADX) and saline ingestion, a hypertensive factor, promoted a reduction in RGN expression, and the suppressive effect of ADX on RGN was not restored by saline ingestion and dexamethasone administration [38-40]. Nevertheless, dexamethasone induced a stimulatory effect on RGN mRNA expression in kidney tissue, an effect not observed with hydrocortisone treatment [35]. The mineralocorticoid aldosterone suppressed RGN expression in rat kidney cortex, whereas an opposite effect was verified in rat renal proximal tubular NRK52E cells [35], which indicates the intricacy and the multifactorial nature of the regulation of RGN expression *in vivo*.

Vitamin D is another important steroid hormone that exerted a suppressive effect on RGN expression in mouse osteoblastic MC3T3-E1 cells but did not changed RGN levels in NRK52E cells [33, 40].

Also, parathyroid and thyroid hormones, which play important functions in multiple physiological mechanisms, including in the maintenance of Ca^{2+} homeostasis, seem to modulate RGN expression in kidney and liver tissues and in several types of cell lines. Both Calcitonin and parathyroid hormone (PTH) stimulated RGN expression in the liver of thyroparathyroidectomised rats and in MC3T3-E1 and NRK52E cells, respectively (Figure 3.1) [33, 40, 41]. The effect of PTH seems to be mediated by intracellular signaling factors, such as cAMP, protein kinase C and/or MAP kinase [40]. In addition, PTH stimulated the nuclear translocation of RGN gene promoter region-related protein-p117 transcription factor in NRK52E cells leading to the stimulation of RGN gene transcription [40]. Relatively to the thyroid hormones, 3,3', 5-triiodo-L-thyronine (T3) exerted a dual effect over RGN expression in the liver of female rats. RGN expression was stimulated at early hours after administration of T3, decreasing severely with the time of exposure [42]. Accordingly, T3 diminished RGN levels in breast cancer cells with long periods of incubation (Figure 3.1) [43]. No effect on RGN expression was perceived in response to thyroxine (T4) treatment in rat liver [32].

Other factors that contribute to the regulation of RGN tissue levels include glucose and the metabolic hormone, insulin [44]. Although insulin did not changed RGN levels in hepatoma cells *in vitro*, it was described that both glucose and insulin up-regulated the expression of RGN in rat liver, suggesting a role for RGN in the control of liver metabolism (Figure 3.1) [45].

It is noteworthy the existence of controversial effects observed in the studies of hormonal stimulation, which clearly showed the complexity of pathways regulating the RGN

expression levels. Moreover, they highlighted the importance of clarifying the network of factors that control RGN levels in each specific tissue.

Calcium is an essential ion in the control of important cell signaling pathways, and doubtless it is the most important non-hormonal factor regulating the expression of RGN (Figure 3.1). In rat and mice liver, as well as, in rat kidney cortex, the intraperitoneal and oral administration of CaCl_2 markedly increased RGN mRNA expression [45-49]. Also, the levels of Ca^{2+} and consequently the expression of RGN were increased in the fetal liver after a single administration of Ca^{2+} to the maternal rats [50]. This effect of Ca^{2+} in increasing RGN expression was also demonstrated *in vitro*, in H4-II-E hepatoma cells treated with Bay K 8644, a Ca^{2+} channel agonist [44]. It has been proposed that this regulation of RGN *in vivo* and *in vitro* may be partly mediated by the Ca^{2+} /Calmodulin (CaM) signaling pathway, and in H4-II-E cells may also involve the activation of protein kinases and tyrosine kinase [44, 49, 51, 52].

All hormonal and non-hormonal factors listed above as regulators of RGN expression, have a crucial role in maintaining cell tissue homeostasis, which supports the involvement of RGN in the regulation of the basic biological processes that contribute to the equilibrium of tissue growth.

Experimental *in vitro* and *in vivo* evidences linking RGN with the hallmarks of cancer

Several hallmarks of cancer have been identified taking into account the capabilities that tumor cells acquire during the development and progression of tumor [53]. The continuous increase of oxidative stress, the aberrant capacity to proliferate and escape to apoptosis, the reprogramming of energy metabolism and the suppression of immune system are all mechanisms that promote tumor cell survival and invasion. Curiously, RGN is a multifunctional protein that has been identified as an exciting target in cancer studies due to its emerging roles as a regulator of oxidative stress, metabolism, cell proliferation and apoptosis [12, 15, 16]. Therefore, the following topics of this review will discuss the relationship of RGN with the hallmarks of cancer.

Oxidative stress

In vitro and *in vivo* experiments have linked RGN protein with the protection against oxidative stress (Figure 3.2). Overexpression of RGN seems to exert a protective effect against cell damage promoted by t-BPH or nitric oxide (NO). RGN enhanced the viability of P19 embryonic carcinoma cells in response to t-BPH induced cytotoxicity [54]. In several rat tissues (brain, liver, kidney and heart) and cell lines (H4-II-E, NRK52E and MC3T3-E1) RGN exerted a suppressive effect over the activity of NO synthase, the enzyme responsible for NO production [55-59]. In some cases, this decrease was observed also in the presence of TFP and EGTA, which significantly inhibit NO synthase activity, and was completely abolished in the presence of anti-RGN monoclonal antibody (Figure 3.2) [11, 55-58, 60-63]. In addition, RGN has the ability to decrease lipid peroxidation and the formation of ROS, increasing the activity of

important antioxidant defense enzymes, such as glutathione S-transferase (GST) and superoxide dismutase (SOD) in HepG2 cells and in rat heart and liver (Figure 3.2) [11, 63, 64].

Among its multiple biological functions, RGN was also identified as a gluconolactonase, an enzyme involved in the biosynthetic pathway of L-ascorbic acid (Vitamin C) in the liver (Figure 3.2) [65]. Vitamin C is a well-known antioxidant and is synthesized in rat but not in humans, which are obligated to obtain it from the diet. The RGN-knockout mice (RGN-KO) that is severely depleted of vitamin C was shown to be an elegant model to study the role of RGN in modulating cell oxidative status. It was demonstrated that the complete lack of vitamin C in RGN-KO animals lead to the development of scurvy symptoms [65] and pulmonary emphysema [66]. Moreover, several factors associated with increased oxidative stress, such as the NADPH oxidase activity and ROS, as well as, dityrosine and carbonyls levels were enhanced in the brain of RGN-KO mice, concomitantly with the reduction of glutathione peroxidase activity [67, 68]. Also, protein carbonyls and total glutathione were significantly increased in the lung of RGN-KO after exposure to cigarette smoke, suggesting again the protective role of RGN against oxidative stress [69].

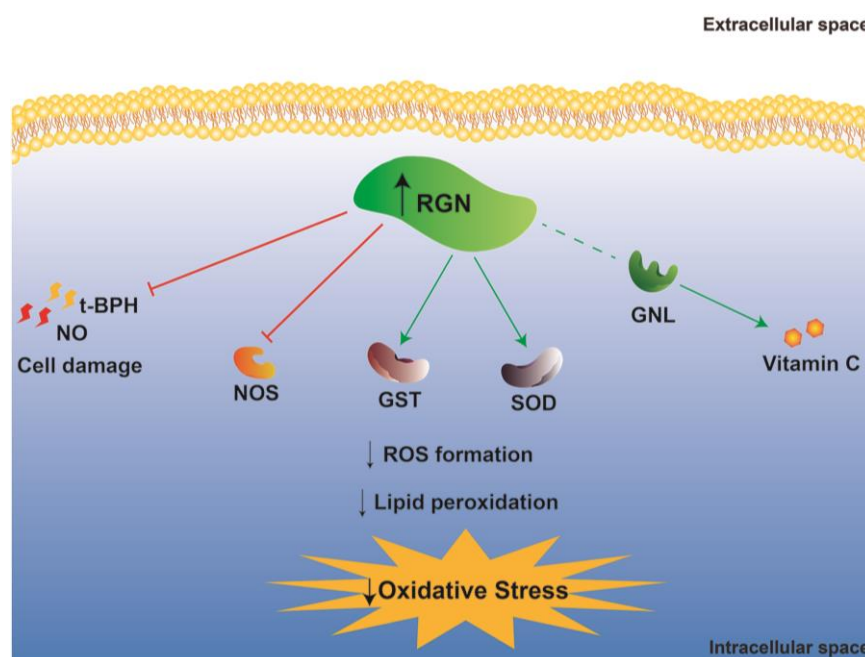


Figure 3.2. Schematic representation showing the different actions of regucalcin (RGN) as cytoprotective protein. RGN diminishes oxidative stress levels by decreasing lipid peroxidation and the production of reactive oxygen species (ROS), inhibiting nitric oxide synthase (NOS) and stimulating the activity of antioxidant enzymes, such as, glutathione-S-transferase (GST) and superoxide dismutase (SOD). RGN protects against cell damage promoted by t-BPH (tert-Butyl hydroperoxide) or NO. Also, RGN was identified as gluconolactonase (GNL), an enzyme involved in the biosynthetic pathway of L-ascorbic acid (Vitamin C), a well-known antioxidant. Legend: Head-arrows lines and bar-ended lines indicate activation and inhibition, respectively.

It is perfectly established that the appearance of age-related diseases, such as neurodegenerative diseases and cancer, are intimately related with an increase in oxidative stress levels. RGN also seems to have an important function in preventing the oxidative stress

deregulation associated with the appearance of some of neurodegenerative diseases such as, Alzheimer's and Parkinson's [70, 71]. Increased levels of RGN were detected in rat brain and also in CTX TNA2 rat astrocytes cells after treatment with kainate, a neurodegenerative agent [72]. In line with this results, it was demonstrated in mice that RGN-deficiency increases the sensitivity to injury induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a common neurotoxin used for induction of Parkinson's disease in rat models, concomitantly with an increase of oxidative stress [70]. This suggest that RGN may protect against age-associated deterioration of cell function associated with increase of oxidative stress.

Altogether, the available data suggest that RGN has a cellular protective role decreasing the effects of noxious stimuli and, thus, avoiding the cell escape to malignant transformation and/or preventing age-related deterioration of cell function.

Calcium homeostasis

Several reports have discussed the relationship between RGN and Ca^{2+} homeostasis in different tissues and cell lines. RGN regulates the cytosolic Ca^{2+} concentration by modulating the activity of Ca^{2+} pumps in plasma membrane and endoplasmic reticulum, respectively the plasma membrane ATPase (PMCA) and sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA); the nuclear outer membrane SERCA; and the uptake of Ca^{2+} by the mitochondria by regulating the activity of mitochondrial ATPase and mitochondrial Ca^{2+} uniporter (MCU) channel (Figure 3.3) [12]. The effect of endogenous RGN on the activation of PMCA in rat liver was suggested, since PMCA activity induced by Ca^{2+} administration in rat liver was abolished in the presence of anti-RGN antibody [73]. Other *in vitro* and *ex vivo* studies demonstrated that RGN significant increases the activity of PMCA, SERCA and mitochondrial ATPase in HepG2 and COS-7 cell lines, rat heart and brain, and also in rat liver plasma membranes and nuclei, kidney cortex basolateral membranes and in liver and heart microsomes mixtures (Figure 3.3) [74-79]. The opposite effect was observed in brain microsomes overexpressing RGN [80]. In addition, RGN has the ability to regulate Ca^{2+} flux into the mitochondria by stimulating MCU channel, and the use of MCU inhibitors blocked the increased activity of mitochondrial ATPase induced by RGN in diverse rat tissues [81-83].

The effect of RGN enhancing the activity of these enzymes may be related to its capacity to regulate SH groups, since the activity of PMCA, SERCA, mitochondrial ATPase enzyme and MCU channel- induced by RGN were inhibited and increased in the presence of N-ethylmaleimide and DTT, respectively [77, 78, 84-86]. Also, RGN can regulate the activity of Ca^{2+} pumps and transporters by direct binding to the plasma membrane, which was demonstrated by the clear decrease of RGN-induced activity after treatment with digitonin or thapsigargin [84-86]. In addition, vanadate treatment significantly diminished the RGN-induced SERCA activity in kidney microsomes and liver nucleus, which suggest that RGN is involved in the phosphorylation of these enzymes [78, 84]. Interestingly, RGN seems to modulate CaM effects on the activity of PMCA, nuclear SERCA and mitochondrial ATPase in HepG2 cells or rat liver (Figure 3.3) [74, 84, 87].

Other studies performed in kidney cells demonstrated that RGN had a suppressive effect on the expression of other proteins involved in intracellular Ca^{2+} -signaling pathways, including the Ca^{2+} -sensing receptor and L-Type Ca^{2+} -channels, but no effects were observed on Ca^{2+} -channel activity (Figure 3.3) [88].

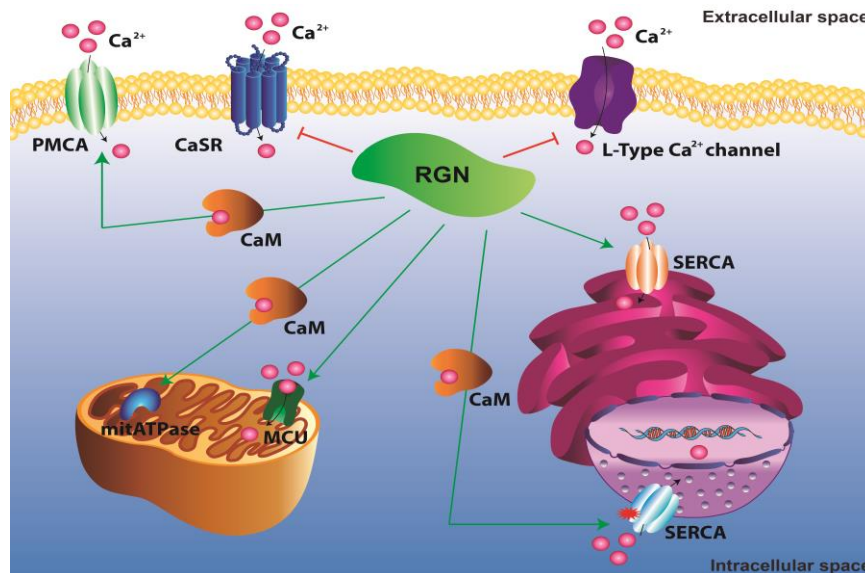


Figure 3.3. Schematic representation of the mechanisms by which regucalcin (RGN) controls calcium (Ca^{2+}) homeostasis. RGN regulates the cytosolic Ca^{2+} concentration by modulating the activity of Ca^{2+} pumps, including plasma membrane ATPase (PMCA) and sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA); the nuclear outer membrane SERCA; and the uptake of Ca^{2+} by the mitochondria by regulating the activity of mitochondrial ATPase and mitochondrial Ca^{2+} uniporter (MCU) channel. RGN modulates the calmodulin (CaM) effects on the activity of PMCA, nuclear SERCA and mitochondrial ATPase, and is involved in the phosphorylation of SERCA in the nucleus. RGN also regulates other proteins involved in intracellular Ca^{2+} -signaling pathways, including the Ca^{2+} -sensing receptor (CaSR) and L-Type Ca^{2+} -channels. Legend: Head-arrows lines and bar-ended lines indicate activation and inhibition, respectively

It is well known that Ca^{2+} plays an important role in the regulation of physiological processes like cell proliferation, differentiation, and apoptosis in many tissues, and the deregulation of Ca^{2+} homeostasis has been intimately associated with development and progression of tumors. The knowledge about the proteins involved in the control of Ca^{2+} homeostasis, and particularly the mechanisms of action of RGN in controlling the pumping activity and the intracellular Ca^{2+} levels needs clarification since targeting Ca^{2+} signaling may be an interesting strategy to control tumor growth.

Proliferation and apoptosis

Regucalcin is widely expressed in the cytoplasm but it can be translocated to the cell nucleus [6], in a mechanism mediated by the protein kinase C signaling pathway [89]. Taking in account the sub-cellular localization of this protein, not surprisingly many evidences exist associating RGN function with the control of important cell signaling pathways, DNA replication, cell proliferation and apoptosis (Figure 3.4). RGN exerts a suppressive effect on

the activity of various modulators of intracellular Ca^{2+} signaling and protein phosphorylation/dephosphorylation processes in the nucleus and cytoplasm of different cell types and tissues, including Ca^{2+} /CaM-dependent protein kinase, protein kinase C, NO synthase, cAMP phosphodiesterase, calcineurin and others phosphatases increased in proliferating cells [17] (Figure 3.4). In addition, RGN is also involved in the inhibition of protein synthesis and activation of protein degradation, exerting its effect through the regulation of the activity of aminoacyl-tRNA synthase and cysteinyl proteases [90, 91]. In the cell nucleus, RGN has the ability to bind proteins and DNA, and inhibit both DNA and RNA synthesis associated with diminished proliferative activity (Figure 3.4) [92]. Other *in vitro* and *in vivo* studies have been demonstrating the regulatory effect of RGN over cell proliferation. Overexpression of RGN decreased the proliferation index in rat H4-II-E and NRK52E cells, in human MIA PaCa-2 and Pt45P1 pancreatic cancer cells, and also in mammary gland tissue [15, 93-95]. The restricted cell proliferation promoted by RGN overexpression was underpinned by the enhanced expression of several tumor suppressor genes (namely, p21, p53 and Rb) and suppressed levels of oncogenes and cell cycle-regulators (such as cdk, chk2, c-Jun, H-ras, c-src and myc) (Figure 3.4) [94, 95]. In addition, it was proposed that RGN counteracts cell proliferation through the modulation of protein kinases and phosphatases activity and the regulation of diverse signaling pathways namely, nuclear factor- κB , ERK, phosphatidylinositol 3-kinase (PI3K), Ca^{2+} signaling and nuclear transcription activity *in vitro* [93-95].

Contrastingly, augmented levels of RGN increased cell survival in testicular cells, cardiomyocytes, and in NRK52E, HepG2, H4-II-E and mouse embryonic carcinoma P19 cells, in response to Ca^{2+} ionophore A23187, that induces intracellular Ca^{2+} overcharge, thapsigargin, actinomycin D, H_2O_2 , t-BPH, Bay K 8644, insulin, transforming growth factor (TGF)- β , tumor necrosis factor (TNF)- α , insulin-like growth factor or LPS, all known inducers of oxidative stress, DNA fragmentation and cell death [54, 55, 96-100]. The inhibitory effect of RGN on apoptosis induced by the panoply of agents mentioned above may be related to its suppressive effect on the expression and activity of various protein kinases, NO synthase, Ca^{2+} -dependent endonuclease, Bax, cytochrome C and caspase-3, and also with the stimulation of anti-apoptotic Bcl-2 (Figure 3.4) [14]. All these findings are corroborative of the involvement of RGN as a protein that protects from cell death induced by damaging factors, thus, contributing to the maintenance of healthy tissues. Nevertheless, RGN also seems to be able to modulate cell death in NRK52E and HepG2 cells, and also in the mammary gland tissue, even without any treatment with apoptosis-inducers agents. NRK52E and HepG2 cells transfected with RGN showed increased expression of Bcl-2 and AKT and augmented activity of AKT, respectively [99, 101]. Previous work of our research group using a transgenic rat model overexpressing RGN protein (Tg-RGN) demonstrated that the mammary gland of Tg-RGN animals displayed higher activity of caspase-3, and an increased Bax/Bcl-2 mRNA ratio, suggesting that RGN activates the apoptotic pathway in this tissue [15]. Further research is warranted to deeply characterize the peculiarities of apoptotic regulation by RGN in different cell and tissue contexts.

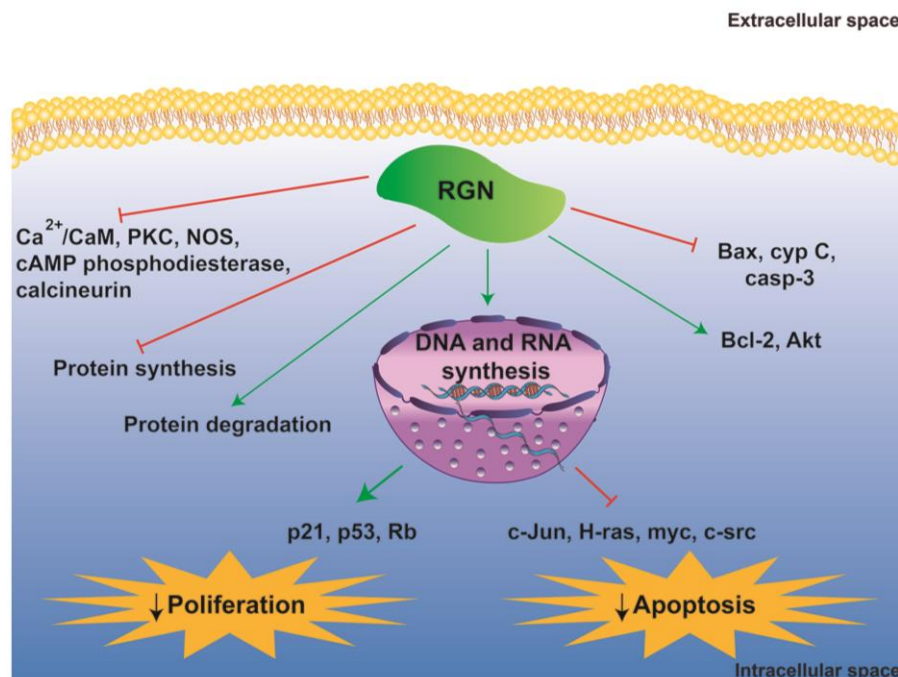


Figure 3.4. Schematic representation of the mechanisms by which regucalcin controls intracellular signaling, proliferation and apoptosis. RGN inhibits Ca^{2+} /calmodulin-dependent protein kinase ($\text{Ca}^{2+}/\text{CaM}$), protein kinase C (PKC), nitric oxide synthase (NOS), cAMP phosphodiesterase and calcineurin. RGN has the ability to inhibit protein synthesis and activate protein degradation, and in the cell nucleus, it inhibits both DNA and RNA synthesis. In addition, RGN enhances the expression of several tumor suppressor genes (namely, p21, p53 and Rb) and suppresses the levels of oncogenes and cell cycle-regulators (such as c-Jun, H-ras, c-src and myc), which leads to the inhibition of cell proliferation. In addition, RGN inhibits caspase 3 activity and the expression of Bax and cytochrome (Cyp) and increases the expression of apoptosis inhibitors Akt-1 and Bcl-2, suppressing apoptosis. Legend: Head-arrows lines and bar-ended lines indicate activation and inhibition, respectively

On the other hand, several types of experimental evidence demonstrated that suppression of RGN expression increases cell susceptibility to apoptotic death. In MCF-7 human breast cancer cells repression of RGN expression by T3 was underpinned by increased apoptosis [43]. Also, the hepatocytes from RGN-KO mice were highly sensitive to apoptosis induced by $\text{TNF-}\alpha$ plus actinomycin D, which seems to involve the activation of caspase-8 but not the NF- κB and Fas-mediated apoptosis [102].

Being a protein that regulates both cell proliferation and apoptosis, it is highly expected that RGN plays a relevant role in the maintenance of the cell tissue homeostasis avoiding the characteristic uncontrolled cell proliferation of tumor cells that results from the deregulation of cell survival/death pathways. This duality of effects over cell fate is typical for other well-known tumor suppressor proteins, such as p53. It is widely accepted the function of p53 as a cell cycle regulator, inhibiting cell proliferation, and as a protein involved in the modulation of apoptosis, inducing the expression of Bax and other pro-apoptotic factors, and activating the mitochondrial pathway of apoptosis [103, 104]. Also, the apoptosis regulator survivin exerts a dual role in controlling both apoptosis and cell cycle,

which depends on its location at specific cell compartments; in the nucleus it favors cell cycle progression whereas when present in the mitochondria survivin inhibits the apoptotic pathway [105].

Another emblematic example is the ski-novel protein (SnoN), which has been viewed as a protein capable to act as a tumor suppressor or a promoter of oncogenic transformation in tumorigenesis [106, 107]. In mice mammary gland, the overexpression of SnoN protein significantly increased the development of adenocarcinoma, and accordingly SnoN negatively regulated the effect of the TGF- β signaling promoting cell death, thus its role as a proto-oncogene was suggested [106, 107]. On the other hand, the function of SnoN as tumor suppressor gene also has been discussed, since it seems to stabilize p53 conformation, inducing premature senescence [107].

Metabolism

Several lines of evidence have been demonstrating that alterations in RGN expression levels are related with development of metabolic disorders, and the deregulation of diverse metabolic pathways, from glucose uptake to its metabolization, has been described in animal models as well as in cell cultures with under- or overexpression of RGN. Overexpression of RGN enhanced the mRNA expression of glucose transporter (GLUT)2 in H4-II-E cells, which suggests the role of RGN enhancing glucose consumption [108]. After enter the cell, glucose may be stored as glycogen or metabolized following the sequential enzymatic reactions of glycolysis. Young and 50-weeks old Tg-RGN animals displayed a significant decrease in glycogen content in the liver [109], which is supported by the previous findings describing that RGN suppresses the activity of glycogen phosphorylase a, an enzyme that catalyzes a rate-limiting step in glycogenolysis [110]. On the other hand, it was reported that RGN increased the consumption of glucose and lactate production in bone marrow cell cultures [111]. However, RGN also seems to inhibit glycolysis, gluconeogenesis and oxidative phosphorylation, reverting the effect promoted by Ca^{2+} and/or CaM in increasing the activity of diverse -enzymes related with these metabolic pathways, such as pyruvate kinase [112], fructose 1,6-diphosphatase [113], glucose-6-phosphatase [114] and mitochondrial succinate dehydrogenase in rat and rabbit liver (Figure 3.5).

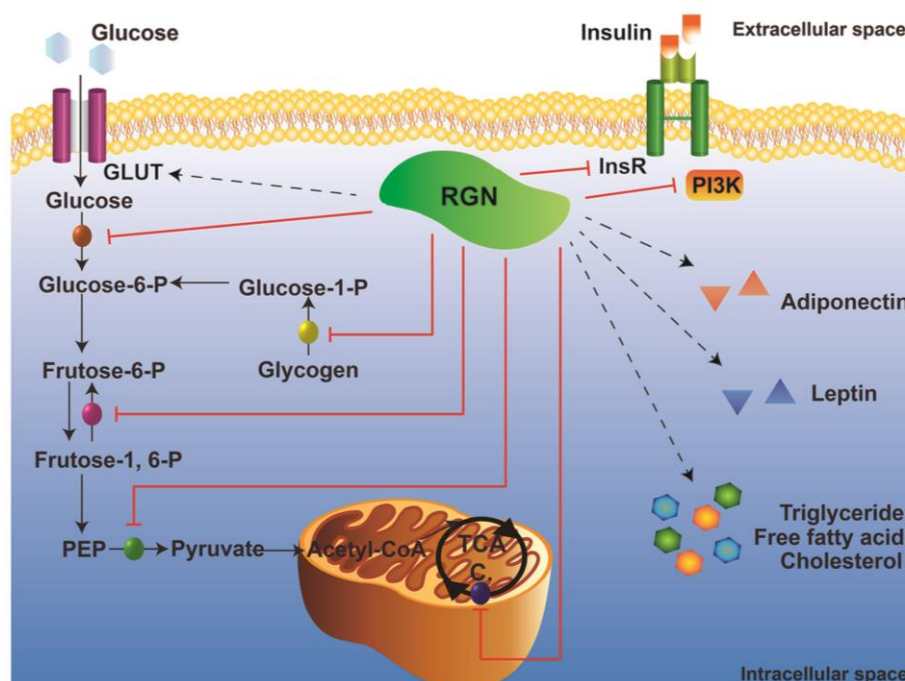


Figure 3.5. Schematic diagram of RGN actions controlling diverse metabolic pathways, namely glycolysis, gluconeogenesis, oxidative phosphorylation and lipogenesis, by regulating the expression or activity of regulators of these pathways. Also, RGN seems to be linked with the resistance to insulin, inhibiting insulin receptor (InsR) and the expression of phosphatidylinositol 3'-kinase (PI3K). Legend Head-arrows lines and bar-ended lines indicate activation and inhibition, respectively Dashed arrows indicate that up- or down-regulation depends on the dose and time of stimulation.

Tumor cells are recognized to have distinct energy requirements and actually the metabolic alterations are considered as a hallmark of cancer [53]. However, it was early in the 1920s that Warburg described the hyper-glycolytic phenotype of tumor cells, which consists in the increased usage of glucose to produce lactate instead of following oxidative phosphorylation [115]. Also, disorders in lipid metabolism are common events occurring in neoplastic cells, which affect several cellular processes, including cell growth, proliferation and differentiation. Some proteins involved in the metabolism of lipids, such as fatty acid synthase, have been viewed as possible targets for cancer treatment [116]. Actually, there is a substantial amount of data supporting that RGN may have a protective effect against the development of cancer metabolic phenotype by decreasing the activity of important metabolic regulators. RGN overexpression stimulated adipogenesis in bone marrow cells [117], and significantly increased triglyceride and free fatty acid production, which are further increased with glucose supplementation in H4-II-E cells [108]. In addition, with increasing age Tg-RGN animals showed a marked increase in serum triglyceride, HDL cholesterol, total cholesterol and free fatty acid concentrations, and also in triglyceride levels in adipose tissues [109, 118], whereas a diminished content of triglyceride, total cholesterol and free fatty acid was observed in the liver of old rats (Figure 3.5) [109]. These results indicated that Tg-RGN rats suffer from hyperlipidemia as result of changes observed in

lipid compounds in the liver and adipose tissues, which were underpinned by the suppression of leptin and adiponectin expression, both proteins involved in liver metabolism and in the control of hyperlipidemia [109]. Interestingly, RGN deficiency lead to the increase of triglyceride, cholesterol and neutral lipids and phospholipids, such phosphatidylethanolamine, cardiolipin, phosphatidylcholine, phosphatidylserine, and sphingomyelin levels and also peroxisome proliferator-activated receptor- γ in liver cells (Figure 3.4) [119-121]. Additionally, hepatocytes of RGN-KO mice had an increase in lipid droplets, an abnormally enlarged mitochondria with indistinct cristae, and enlarged lysosomes filled with electron-dense bodies, alterations that were worsened with aging [119].

Another important feature that has been described for RGN is its ability to regulate insulin function (Figure 3.4). In RGN transfected cells the effect of insulin in stimulating glucose consumption to produce triglyceride and free fatty acid was prevented and accompanied by suppressed expression of insulin receptor and diminished levels of Pi3K mRNA [108, 122]. Also in H4-II-E cultured cells with TNF- α and insulin, modulators of insulin resistance, RGN was shown to be differentially expressed [123]. These findings suggested that RGN may be linked with the resistance to insulin. In fact, glucose and insulin tolerance tests demonstrated that the deficiency of RGN in RGN-KO animals may lead to a decrease in glucose tolerance, through negatively regulating the secretion of insulin [124, 125]. Also, the static incubation of pancreatic islets of RGN-KO mice revealed a decrease of insulin secretion in response to glucose or potassium chloride [124]. These results suggested that RGN regulates insulin secretion by pancreatic cells and loss of RGN may contribute to the exacerbated glucose intolerance that naturally occurs with aging. On the other hand, several *in vitro* and *in vivo* studies suggested that also insulin regulates RGN expression as already explained, and that RGN mediates the effect of insulin [45, 126-128].

Altogether, these findings indicate that RGN exert a crucial role in the regulation of important metabolic pathways, and that alterations in its expression may be an event that favors the metabolic reprogramming of tumors cells fostering survival and invasion.

Expression pattern of RGN is deregulated in cancer cases

Age is considered a major risk factor for the development of neoplastic diseases [129] and as previously described RGN expression has been shown to decline with aging [22, 23] though this fact needs confirmation in humans. Considering the protective role of RGN against several hallmarks of cancer as discussed above, it is liable to assume that the deregulated expression of RGN with aging may contribute to the deterioration of cell function, favoring the onset and progression of cancer, and likely of other age-related diseases. Indeed, a downregulated expression of RGN has been shown in a set of animal and human cancers (Table 3.1), namely, liver, kidney, brain, breast, prostate and lung, and also in different cell lines representatives of some of these neoplastic tissues. The deregulated expression of RGN in cancer cell lines was recently reviewed, and it will not be discussed here [12]. Moreover, it is worth noting that also the RGN protein spliced variants show a differential expression

pattern between non-pathological and cancer tissues/cell lines [6, 18]. However, further research is warranted to disclose the physiological function of these alternative proteins, which predictability are devoid of the functional domain implicated in Ca^{2+} -binding [6].

In what concerns solid tumors, RGN expression was found to be decreased in rat and zebrafish liver tumors chemical-induced by diethylnitrosamine, 2-acetylaminofluorene, 3'-methyl-4-dimethylaminoazobenzene or diethylnitrosamine [24, 130, 131]. The diminished expression of RGN was detected since the early stages of liver carcinogenesis being already evident in pre-neoplastic lesions and diminishing continuously to the well-established hepatocellular carcinoma [131, 132]. It was also observed a marked reduction of RGN expression in the kidney of rats treated with ochratoxin A, usually used as renal carcinogen [133]. More recently, we demonstrated that Tg-RGN animals are protected against the development of 7,12-dimethylbenz[α]anthracene-induced mammary gland tumors displaying much lower tumor incidence and tumors with less aggressive phenotypes [15].

Besides the studies described above in animal models, it is also known that RGN expression is lost in human cancer cases comparatively with normal tissues, which includes hepatocellular carcinoma, kidney transitional cell carcinoma, brain malignant meningioma, lung non-small cell carcinoma, and breast and prostate cancers (Table 3.1) [6, 18-21]. In the case of breast and prostate cancer, RGN immunoreactivity was also negatively associated with the grade of infiltrating ductal carcinoma of breast cancer and the cellular differentiation of prostate adenocarcinoma [6], which indicates that loss of RGN expression is related with tumor onset, progression and aggressiveness.

Table 3.1. Neoplastic tissues displaying diminished expression of regucalcin in different species of vertebrates

Tissue	Species	Biomolecule	Tumor type	References
Liver	rat	mRNA	Hepatocellular carcinoma	[130]
	rat	mRNA	Pre-neoplastic lesion	[131]
			Foci	
	zebrafish	protein	Hepatocellular carcinoma Cholangiocellular carcinoma Mixed tumor	[24]
	human	mRNA/ protein	Hepatocellular carcinoma	[18-20, 134]
Kidney	rat	mRNA	-	[133]
	human	mRNA	Transitional cell carcinoma	[18]
Brain	human	mRNA	Meningioma	[18]
Lung	human	mRNA/ protein	Non-small cell carcinoma	[18, 21]
			Lung squamous carcinoma	
Breast	human	mRNA/ protein	Infiltrating ductal carcinoma	[6, 15]
Prostate	human	mRNA/ protein	Adenocarcinoma	[6]

Regucalcin has been identified as a secreted protein found in different biological fluids [12]. Zhou et al have found a correlation between serum levels of RGN and the differentiation and histopathological type of hepatocellular carcinoma [134]. Moreover, the reduced expression of RGN founded in hepatocellular carcinoma was parallel to the up-regulation of RGN in adjacent normal tissues [134]. However, it is entirely unknown whether this expression pattern of RGN depends on some kind of communication between neoplastic and non-neoplastic cells.

The described down-regulated expression of RGN in multiple cancer tissues strongly prompts the idea that RGN may be a useful biomarker or a possible therapeutic option to improve the management of this complex disease.

Conclusion

The expression levels of RGN are regulated by a panoply of factors of different nature, and are strongly implicated in the proliferative/apoptotic status of tissues, thus, are crucial for the maintenance of cell tissue homeostasis. Indeed, deregulated expression of RGN has been found in several human diseases, and characteristically cancer cases display loss of RGN expression, which is correlated with tumor onset and degree of differentiation and aggressiveness. On the other hand, the biological functions of RGN intersect several of the well-known hallmarks of cancer, namely, the control of cell survival/death, oxidative stress and metabolism. Actually, it is known that RGN actions protect cells from damage and concur to restrain cell proliferation and tumor development, being highly reasonable to consider RGN as a tumor suppressor protein. At this point, the possibility of manipulating RGN tissue levels positions itself as a quite exciting field of research. Future challenges may go through the development of methodologies to specifically delivery RGN to target tissues, alone or in combination with other cancer therapeutic drugs, as a strategy to prevent the development and progression of cancer.

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Chapter 4

Aim and outline of the thesis

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The development and progression of prostate cancer (PCa) depend on the cross-talk of several processes, which includes the metabolic reprogramming of tumor cells. Almost a century ago, Warburg's studies demonstrated that tumor cells utilize glycolysis as the energy source instead of rely on oxidative phosphorylation, and this feature is actually considered a hallmark of cancer. The growth of PCa cells at the initial stages of disease is androgen-dependent but with the progression to more aggressive stages, tumor cells acquire the ability to proliferate independently of the levels of circulating androgens. However, the metabolic alterations accompanying the progression to androgen-independent stages of PCa are poorly studied, and the role of androgens controlling the glycolytic metabolism is yet to be elucidated. On the other hand, the calcium (Ca^{2+})-binding protein Regucalcin (RGN) that has been identified as an androgen-target gene in other tissues other than prostate, was shown to be under-expressed in cases of human PCa comparatively to normal tissues. Also, it was suggested that RGN is involved in the control of tissue cell homeostasis, which includes the regulation of cell proliferation, apoptosis, oxidative stress and diverse metabolic pathways. These findings raised the question whether it is the loss of RGN that favors tumor development, which is further supported by the fact that RGN expression decreases with aging. However, the expression pattern of RGN in the prostate of adults and during aging, as well as, the factors that regulate its levels are unknown. Androgens and Ca^{2+} are both regulators of prostate physiology and PCa cell growth, and we hypothesized that they can regulate RGN expression levels. We also expect that RGN may be involved in the control of prostate physiology.

To shed light on these questions the present thesis aims to:

1. Establish the glycolytic profile of androgen-responsive and androgen-nonresponsive PCa cells, and unveil the role of androgens in the regulation of the glycolytic flux in PCa cells;
2. Determine the effect of androgens in the regulation of RGN expression *in vivo*, and explore the effect of RGN overexpression on cell proliferation, apoptosis, glycolytic metabolism and oxidative stress in rat prostate;
3. Study the relationship between Ca^{2+} and RGN in human prostate cell lines and rat prostate tissue;
4. Characterize the expression pattern of RGN in rat prostate along aging, and determine the impact of RGN overexpression in the prostate of aged rats in what concerns cell proliferation, apoptosis, and oxidative stress.

After the description of PCa generalities (Chapter 1), presentation of glycolytic metabolism in PCa (Chapter 2), the revision of the possible role of RGN as a tumor suppressor protein (Chapter 3), and the establishment of the main objectives of the thesis, Chapters 5-11 were organized as follows:

Chapter 5 compares the glycolytic profile of androgen-nonresponsive (PC3) and androgen-nonresponsive (LNCaP) PCa cell lines, and the normal prostate epithelial cell line (PNT1A) in what concerns glucose consumption, lactate production and the expression and activity of the diverse regulators of this metabolic pathway.

Chapter 6 describes the effect of 5 α -dihydrotestosterone (DHT) on the glycolytic flux in PCa cell lines, assessed by the alterations observed in metabolites production and glycolysis-regulators.

In Chapter 7, the *in vivo* effect of DHT on RGN expression in rat prostate is investigated. Also, the effect of RGN in regulating prostate cell proliferation and apoptosis is evaluated. The apoptotic index and the expression of key regulators of cell cycle and apoptotic cell death is compared between the prostate of 3 month-old (M) transgenic rats overexpressing RGN (Tg-RGN) and their wild-type (Wt) counterparts.

In Chapter 8, the effect of physiologic and non-physiologic concentrations of extracellular Ca²⁺ on the expression of RGN and proliferation of PCa (LNCaP) and normal (PNT1A) prostate cells is evaluated. Also, the role of RGN in modulating the expression of Ca²⁺-sensing receptor is explored in LNCaP and PNT1A prostate cells transfected with a small interfering RNA targeting RGN, and in the prostate of 3M Tg-RGN animals comparatively with their Wt counterparts.

Chapter 9 outlines the role of RGN in the regulation of glycolytic metabolism. Prostates of 3M Tg-RGN and Wt animals are used to determine the content of glucose and lactate metabolites, and study the expression and activity of several regulators of the glycolytic flux.

Chapter 10 establishes the expression pattern of RGN in the rat prostate along aging, using different experimental age groups: juvenile (20 days, 1 M and 2 M), young (3 M and 4 M), middle-aged (6 M, 8 M, 9 M and 12 M), and aged (24 M) rats. This chapter also explores the role of RGN in modulating oxidative stress, proliferation and apoptosis with aging. Lipid peroxidation and activity of antioxidant enzymes, and the expression of cell cycle and apoptotic regulators are measured in the prostates of 3 M and 9 M Tg-RGN and Wt animals.

Finally, Chapter 11 presents an integrative view of the main findings of this thesis and discusses their potential impact and the future perspectives in the context of prostate pathophysiology.

Chapter 5

Androgen-responsive and nonresponsive prostate cancer cells present a distinct glycolytic metabolism profile

This chapter was published in:

Vaz, C.V., Alves, M.G., Marques, R., Moreira, P.I., Oliveira, P.F., Maia, C.J., Socorro, S.
Androgen-responsive and nonresponsive prostate cancer cells present a distinct glycolytic metabolism profile. The International Journal of Biochemistry & Cell Biology. 2012; 44(11): p. 2077-84

Androgen-responsive and nonresponsive prostate cancer cells present a distinct glycolytic metabolism profile

Abstract

Prostate cancer (PCa) progresses from an early stage, confined to prostate, to a more aggressive metastasized cancer related with loss of androgen responsiveness. Although, it has been recognized that PCa cells have unique metabolic features, their glycolytic profile in androgen-dependent and androgen-independent stages of disease is much less known. Hence, the main purpose of this study was to compare glucose metabolism in androgen-responsive (LNCaP) and androgen-nonresponsive (PC3) PCa cells. Cell culture medium was collected and differences in glucose consumption and, lactate and alanine production were measured using Proton Nuclear Magnetic Resonance (^1H -NMR) spectra analysis. The mRNA and protein expression of glucose transporters (GLUT1 and GLUT3), Phosphofructokinase 1 (PFK1), lactate dehydrogenase (LDH) and monocarboxylate transporter (MCT4) were determined by real-time PCR and Western Blot, respectively. The obtained results demonstrate that androgen-responsive (LNCaP) and androgen-nonresponsive (PC3) cells consumed similar amounts of glucose, whereas PC3 cells present higher lactate production. This increase in lactate production was concomitant with higher levels of MCT4 protein, increased LDH activity and higher lactate/alanine ratio, also suggesting increased levels of oxidative stress in PC3 cells. However, protein levels of LDH, associated with lactate metabolism, and GLUT3, involved in glucose uptake, were decreased in PC3 comparatively with LNCaP. Androgen-responsive and nonresponsive PCa cells present distinct glycolytic metabolism profiles, which suggest that targeting LDH and MCT4 metabolic pathways may be an important step for the development of new diagnostic and therapeutic strategies in the different stages of PCa.

Keywords: androgen; LNCaP; metabolomics; NMR; PC3.

Introduction

Carcinogenesis has been defined as a progressive process that requires a sequence of genetic changes and a set of acquired phenotypic properties, which are maintained or lost based on cell-environment interactions. This concept may be associated with the metabolic reprogramming or the “Warburg phenomenon” proposed for cancer cells, which consist in increased glycolysis maintained even in the presence of high oxygen, and a reduced mitochondrial oxidative phosphorylation [1]. However, this must be evaluated for each particular type of tumor cells, which may have specific metabolic features to ensure survival and proliferation.

Prostate cancer (PCa) is characterized by a transition from an androgen-dependent to an androgen-independent phenotype and progresses from precursor lesions to a carcinoma, which is initially confined to the prostate but that can lead to a metastatic disease often

resulting in lethality [2, 3]. Thus, at an early stage most of the neoplastic mass is represented by androgen-dependent cells whose proapoptotic potential is regulated by androgen receptor (AR) [4, 5]. Progression of PCa is associated with tumor enrichment in androgen-independent propagating cells, as well as the emergence of new cell phenotypes, leading to a disease stage that frequently displays a fatal behavior [5]. Moreover, PCa has been considered to have unique metabolic features, related with high levels of alanine and lactate [6-8], and an increase in glucose consumption seems to be required for rapid proliferation of androgen-independent PCa cells [9]. One of the principal mechanisms of aerobic glycolysis is the conversion of glucose to pyruvate and lactate, achieved by upregulation of glucose transporter isoform 1 (GLUT1), hexokinase (HK), lactate dehydrogenase A (LDHA) and lactate-extruding enzyme monocarboxylate transporter 4 (MCT4) [10, 11].

Interestingly, and in contrast with PC3 cells, androgens seem to induce GLUT-1 expression in LNCaP cells [12, 13]. In addition, it has been established a relationship between androgens and the metabolic changes found in PCa. It was reported a decreased uptake of Fluorodeoxyglucose (FDG) in the prostate cancer tissues of PCa patients undergoing androgen ablation therapy [14], which suggests that glucose metabolism differs between early and advanced stages of PCa. Also *in vitro* studies showed that under androgen deprivation, a lower FDG uptake is observed in androgen-responsive LNCaP cells when compared with androgen-nonresponsive PC3 cells [15, 16]. However, the mechanisms underlying regulation of glycolytic activity in androgen-dependent and androgen-independent stages of PCa need to be deeply exploited.

The present study aims to make use of Proton Nuclear Magnetic Resonance (^1H -NMR) spectroscopy to establish the glycolytic phenotype of androgen-responsive (LNCaP) and androgen-nonresponsive (PC3) cells. The investigation of the metabolic phenotypes associated with distinct androgen-responsive conditions can be a valuable tool for the development of new diagnosis and therapeutic possibilities of PCa.

Material and methods

Cell lines and experimental design

The human prostate cancer cell lines LNCaP and PC3, and the immortalized noncancerous prostate epithelial cell line PNT1A, were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). LNCaP cell line was originated from a lymph node metastasis of a PCa, expresses AR and is androgen-responsive [17]. PC3 cells have origin in a bone metastasis of an undifferentiated grade IV adenocarcinoma of the prostate and are considered to be nonresponsive to androgens [18]. Therefore, LNCaP and PC3 cells have been routinely used as *in vitro* models of androgen-dependent and androgen-independent stages of PCa, respectively [19]. Approximately 6×10^6 LNCaP, PC3 and PNT1A cells were cultured in 15 mL of RPMI 1640 (Invitrogen) medium containing 11.11 mM of glucose, supplemented with 10% FBS (Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (Invitrogen), in an air

incubator equilibrated with 5% CO₂ at 37°C. At 90% confluence, achieved after five passages for each cell line, the medium was changed and maintained during 48h. Then extracellular medium was collected for ¹H-NMR analysis and cells were trypsinized, collected and frozen at -80°C, until RNA or protein extraction.

¹H-NMR

Culture medium (160 µL) collected from 90% confluent cells was used for ¹H-NMR analysis by means of standard methods [20]. ¹H-NMR spectra of the collected samples were acquired at 14.1 T, 25 °C, using a Varian 600 MHz spectrometer (Varian, Palo Alto, CA, USA) equipped with a 3 mm indirect detection probe with z-gradient. Solvent-suppressed ¹H-NMR spectra were acquired with 6 kHz sweep width, using 14 s delay for allowing total proton relaxation, 3 s water pre-saturation, 45° pulse angle, 3.5 s acquisition time, and at least 64 scans. The metabolite spin systems were assigned using the chemical shifts found in the literature and the following metabolites were determined: lactate, doublet located at 1.33 ppm; alanine, doublet at 1.45 ppm; and H1-α glucose, doublet at 5.22 ppm. 40 µL of a 10 mM sodium fumarate solution in 99.9% D₂O (Cambridge Isotope Laboratories, Cambridge, MA, USA) were added to each sample, as an internal reference (6.50 ppm) to quantify metabolites in solution. Data were processed and displayed offline using the curve-fitting routine supplied with the NUTSpro™ NMR spectral analysis program (Acorn, NMR Inc, Fremont, CA, USA).

Quantitative RT-PCR (qPCR)

Real-time qPCR was performed to analyze GLUT1, GLUT3, phosphofructokinase type 1 (PFK1), LDH and MCT4 mRNA expression. Total RNA was extracted from LNCaP, PC3 and PNT1A cells using TRI reagent (Sigma, Roedermark, Germany) according to the manufacturer's instructions. RNA concentration and absorbance ratios (A₂₆₀/A₂₈₀) were determined by spectrophotometry (Pharmacia Biotech, Ultrospec 3000, Denmark). cDNA synthesis was performed as previously described [21]. Briefly, 1 µg of total RNA was reverse transcribed in a final volume of 20 µL with 200 U of M-MLV RT, using 250 ng of random hexamer primers and 500 µM of each deoxynucleotide triphosphates. Specific primers were designed for the amplification of target genes (Table 5.1) and also beta-2-microglobulin (5'-3', Sense: ATGAGTATGCCTGCCGTGTG; antisense: CAAACCTCCATGATGCTGCTTAC) and glyceraldehyde 3-phosphate dehydrogenase (5'-3', sense: CGCCAGCCGAGCCACATC; antisense: CGCCCAATACGACCAAATCCG) used as internal controls to normalize gene expression. qPCR reactions were carried out in an iQ5 system (Bio-Rad, Hercules, USA) and efficiency of the amplification was determined for all primer sets using serial dilutions of cDNA (1, 1:10 and 1:100). PCR conditions and reagents concentrations were previously optimized and specificity of the amplicons was determined by melting curves. qPCR amplifications used 1 µL of synthesized cDNA in a 20 µL reaction containing 10 µL Maxima™ SYBR Green/Fluorescein qPCR Master Mix (Biorad) and 300 nM of sense and antisense primers for each gene. Reaction conditions comprised 5 min denaturation at 95°C, followed by 35 cycles at 95°C for 10

seconds, a specific annealing temperature for each gene (Table 5.1) for 30 seconds and 72°C for 10 seconds. Samples were run in triplicate in each PCR assay. Normalized expression values were calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta C_t}$ [22].

Table 5.1. PCR primer sequences, amplicon size, and annealing temperature.

Gene	Sequence (5'- 3')	AT (°C)	Amplificon Size (bp)
GLUT1	Sense: CAG GAG ATG AAG GAA GAG	47	144
	Antisense: TCG TGG AGT AAT AGA AGAC		
GLUT3	Sense: CGG CTT CCT CAT TAC CTT C	50	173
	Antisense: GGC ACG ACT TAG ACA TTG G		
PFK1	Sense: CCG CAT CAA GCA GTC AGC AG	58	62
	Antisense: AGC CAG GTA GCC ACA GTA GC		
LDH	Sense: ATT CAG CCC GAT TCC GTT AC	54	110
	Antisense: GAC ACC AGC AAC ATT CAT TCC		
MCT4	Sense: CCG TGT CAT TCC AGA GTG	54	103
	Antisense: CAG GTC CTT GAG CAT AGC		

Abbreviation: AT: annealing temperature; bp; base pairs.

Western Blot (WB)

Western Blot procedures were performed as previously described to determine GLUT1, GLUT3, PFK1, LDH and MCT4 protein expression [23]. Total proteins were extracted from prostate cancer cells and normal prostate cells using RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 8 and 1 mM EDTA) and protein concentration was determined by the Bradford micro-assay (Bio-Rad). 50 µg of each sample protein was resolved by SDS-PAGE on 12% gels and electrotransferred to a PVDF membrane (GE Healthcare, Buckinghamshire, UK). Membranes were incubated overnight at 4°C with rabbit anti-MCT4 (1:1000, sc-50329, Santa Cruz Biotechnology), anti-PFK1 (1:500, sc-67028, Santa Cruz Biotechnology), anti-GLUT1 (1:300, CBL242, Millipore), anti-GLUT3 (1:1000, Ab41525, Abcam) or anti-LDH (1:10000, Ab52488, Abcam) primary antibodies. A mouse monoclonal antibody anti-alpha tubulin (1:5000, T9026, Sigma) was used for protein loading control in all WB analysis. Goat anti-rabbit IgG-AP (1:5000, Santa Cruz Biotechnology) or goat anti-mouse IgG-AP (1:5000, Santa Cruz Biotechnology) were used as secondary antibodies. Membranes were incubated with ECF substrate (GE Healthcare) for 5min and immune-reactive proteins were detected with the Molecular Imager FX Pro plus Multimager (Bio-Rad). Band densities were obtained according to standard methods using the Quantity One Software (Bio-Rad) and normalized by division with the respective alpha-tubulin band density. Results are presented as fold variation relatively to the non-cancerous cells PNT1A.

LDH and PFK enzymatic activities

LDH activity was determined using a commercial assay kit (Promega, Madison, USA) and following the manufacturers' instructions. Briefly, enzymatic activity was calculated by

measuring the shift on the absorbance (492 nm) of samples resulted from the conversion of a tetrazolium salt (INT) into a red formazan product as the amount of formazan formed is directly proportional to the activity of LDH on the samples. The method was calibrated with the LDH Positive Control included in the assay kit. The attained activities were calculated using the molar absorptivity of formazan and expressed as fold variation versus the LDH activity of the LNCaP group.

PFK activity was determined by a method described by Sundaram et al [24]. Briefly, the assay is based on the coupling of fructose bisphosphate formation to the oxidation of NADH in the presence of aldolase, triosephosphate isomerase, and glycerolphosphate dehydrogenase as auxiliary enzymes. The oxidation of NADH was followed by the decrease in absorbance at 340 nm at 30°C. The attained activities were expressed as fold variation versus the PFK activity of the LNCaP group.

Statistical analysis

The statistical significance of differences among all experiments was assessed by two-way ANOVA, followed by Bonferroni post-test (GraphPad Software, San Diego, CA, USA). Significant differences were considered when $P < 0.05$. All experimental data are shown as mean $\pm$ SEM ($n=5$ for each condition).

Results

Androgen-responsive LNCaP cells produce less lactate than androgen-nonresponsive PC3 cells

Increased glucose consumption is typical in malignant cells [25] and high rates of glucose consumption are required for the rapid proliferation of androgen-nonresponsive prostate carcinoma cells [9]. Our results show that both, LNCaP and PC3 cells presented significantly higher glucose consumption than PNT1A cells, respectively 10 ± 2 and 10 ± 1 pmoles/cell (Figure 5.1A). After 48 h glucose consumption was similar in both cancerous cell lines. As expected lactate production is highly increased in both LNCaP and PC3 cells when compared to PNT1A (Figure 5.1B). However, androgen-responsive LNCaP cells produced significantly less lactate (66 ± 9 pmoles/cell) than androgen-nonresponsive PC3 cells (104 ± 9 pmoles/cell). The lactate produced from glucose, results from pyruvate conversion. Nevertheless, pyruvate may also be converted into alanine via transaminase reaction [26]. Thus, we also examined alanine production which was increased from 1.1 ± 0.2 pmoles/cell in PNT1A cells to 2.0 ± 0.3 pmoles/cell in LNCaP cells and 2.8 ± 0.5 pmoles/cell in PC3 cells (Figure 5.1C). Interestingly, the androgen-responsive cells produced significantly less alanine than androgen-nonresponsive cells. The lactate/alanine ratio which can be associated with cellular redox state [27] significantly increased in both, LNCaP and PC3, when compared with PNT1A cells (Figure 5.1D). In addition, the lactate/alanine ratio significantly increased from 3.8 ± 0.3 in LNCaP cells to 5.0 ± 0.4 in PC3 cells (Figure 5.1D). Overall, although the glucose consumption

was similar in both, LNCaP and PC3 cells, the fate of glucose metabolism to produce lactate or alanine is different according to the cell line.

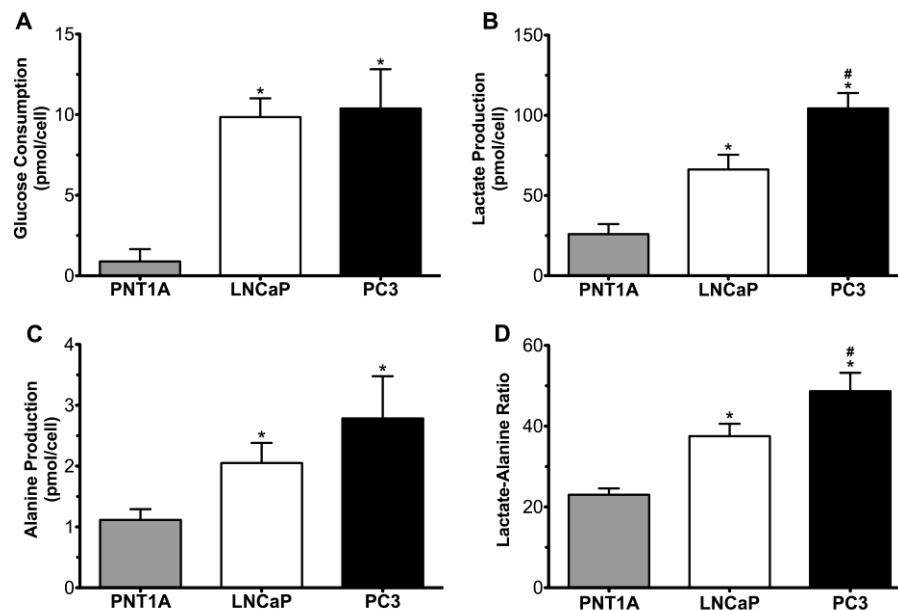


Figure 5.1. Glucose consumption and, lactate and alanine production in noncancerous prostate cells (PNT1A), and androgen-responsive (LNCaP) and androgen-nonresponsive (PC3) prostate cancer cells. (A) Glucose consumption, (B) Lactate production, (C) Alanine production, (D) Lactate-Alanine ratio. Results are expressed as mean $\pm$ SEM (n=5). *Significantly different when compared with PNT1A ($p < 0.05$); #significantly different when compared with LNCaP ($p < 0.05$).

Androgen-nonresponsive PC3 cells present a significant decrease on GLUT3 and PFK protein levels, and PFK enzymatic activity

There is evidence that glucose uptake is increased in prostate carcinoma cells [9] and it has been suggested that the enhanced glucose consumption in many tumors results from GLUTs overexpression. GLUT1 and GLUT3 are high affinity glucose transporters that are overexpressed in a set of tumors [28]. In order to determine differences in the glucose transport in androgen-responsive and nonresponsive cells, GLUT1 and GLUT3 mRNA and protein expression was determined by qPCR and Western Blot, respectively. Expression levels were determined upon normalization and comparison with those of noncancerous PNT1A cells.

GLUT1 mRNA expression is significantly decreased in androgen-nonresponsive cells comparatively with noncancerous PNT1A cells (0.53-fold reduction, Figure 5.2A), although GLUT1 protein levels were not significantly altered in both LNCaP and PC3 cells (Figure 5.2B and 5.2C). Concerning GLUT3 mRNA levels, a decreased expression was observed in both LNCaP and PC3 cancerous cells in comparison with PNT1A cells (0.56- and 0.75-fold reduction, respectively). No significant differences were observed between both cancer cell lines (Figure 5.2A). In terms of protein expression, a significant increase of GLUT3 expression was detected in androgen-responsive LNCaP cells relatively to noncancerous PNT1A prostate cells (1.48-fold variation, Figure 5.2A). No statistical differences were observed when comparing androgen-

nonresponsive cells with PNT1A cells. Nevertheless, PC3 cells presented a highly decreased GLUT3 protein expression when compared with LNCaP (1.48- to 0.75-fold variation to PNT1A, Figure 5.2B and 5.2C).

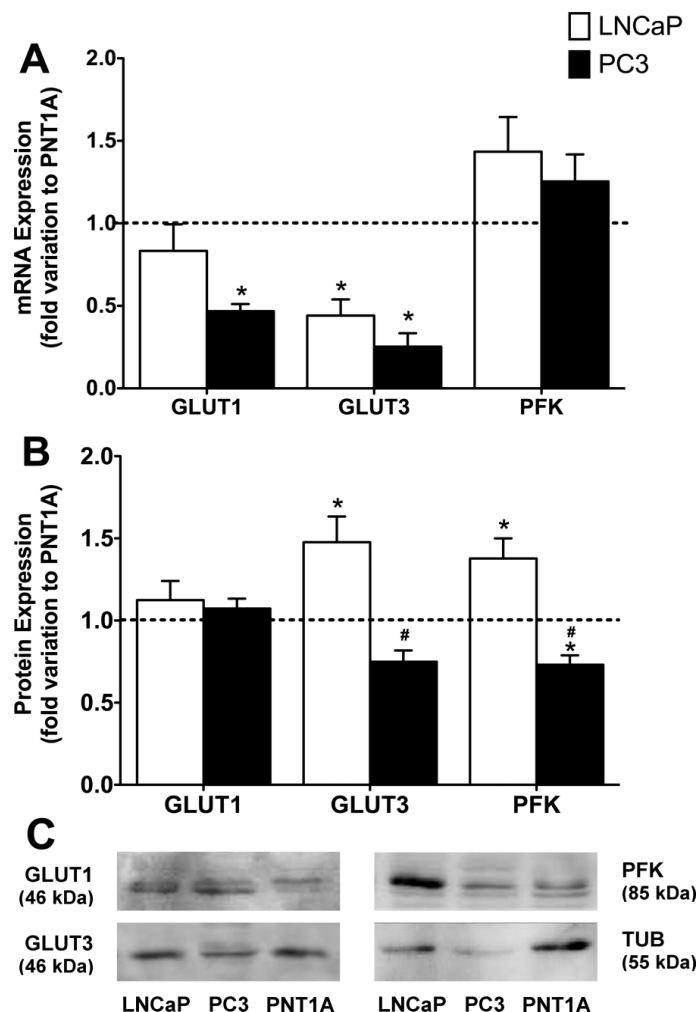


Figure 5.2. GLUT1, GLUT3 and PFK1 mRNA and protein expression in androgen-responsive (LNCaP) and androgen-nonresponsive (PC3) prostate cancer cells determined by means of qPCR and Western Blot, respectively. Pooled data of independent experiments indicate mRNA (A) and protein (B) fold variation relatively to those of noncancerous PNT1A cells; (C) Representative SDS-page protein bands. Results are expressed as mean $\pm$ SEM (n=5). *Significantly different when compared with PNT1A ($p < 0.05$); #Significantly different when compared with LNCaP ($p < 0.05$).

Other important controlling step of the cellular glycolytic flux is the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate by PFK1. Beyond the study of glucose transporters, we focused on PFK expression, which comprises the first irreversible reaction in glycolysis. Moreover, it has been suggested that PFK expression may be altered in tumor cells [29]. In terms of mRNA levels, no significant differences were observed among all studied prostate cell lines (Figure 5.2A). Protein levels were altered in cancerous cells comparatively to noncancerous cells, as PFK1 expression was increased in androgen-responsive LNCaP cells (1.38-fold variation to PNT1A) and decreased in androgen-nonresponsive PC3 cells (0.73-fold

variation to PNT1A, Figure 5.2B and 5.2C). In addition, PFK1 protein levels were decrease in androgen-nonresponsive PC3 cells in relation to androgen-responsive LNCaP cells. The significant decrease on PFK protein levels in androgen-nonresponsive PC3 cells was concomitant with a significant decrease in PFK enzymatic activity (0.73 ± 0.06 -fold variation to PNT1A, Figure 5.4). Thus, a distinct expression of both GLUT3 and PFK1 proteins was observed in androgen-responsive and -nonresponsive prostate cancer cell lines (Figure 5.2B) that was followed by PFK enzymatic activity (Figure 5.4).

Androgen-nonresponsive PC3 cells presented a significant increase in LDH enzymatic activity and in MCT4 protein levels

It has been reported that lactate may be involved in stimulation of cellular motility, angiogenesis and metastasis [30]. The LDH enzyme family is responsible for the interconversion of pyruvate to lactate [31], and MCT4 is a monocarboxylate transporter, which serves primarily as a lactate transporter. It has also been reported a correlation between MCT4 expression and a poor prognosis in PCa [32, 33]. Thus, in terms of lactate production and export, we analyzed the expression of LDH and MCT4 mRNA and protein by qPCR and western blot, respectively. Concerning mRNA levels, LDH is highly increased in cancerous cells, LNCaP and PC3, when compared with noncancerous cells (respectively 1.33- and 2.08-fold variation to PNT1A, Figure 5.3A). We also verified that LDH mRNA levels are significantly increased in androgen-nonresponsive PC3 cells when compared with LNCaP cells (Figure 5.3A). LDH protein levels demonstrated a significant increase in LNCaP, when comparing with noncancerous cells (2.12-fold variation to PNT1A) and no significant alterations were found comparing PC3 with PNT1A (Figure 5.3B and 5.3C). Furthermore, the LDH protein analysis in the two PCa cell lines demonstrated a reduction in androgen-nonresponsive PC3 cells, comparing with androgen-responsive cells, LNCaP (Figure 5.3B and 5.3C). LDH enzymatic activity was significantly increased in both LNCaP and PC3 cells (respectively 1.46 ± 0.2 and 2.08 ± 0.2 - fold variation to PNT1A, Figure 5.4), although androgen-nonresponsive PC3 cells presented a significant higher LDH activity when comparing with the androgen-responsive LNCaP cells.

MCT4 mRNA expression presented a 1.66 fold variation to PNT1A in LNCaP cells and 0,52 fold variation to PNT1A in PC3 cells thus, PC3 cells present decreased MCT4 mRNA expression levels when compared with LNCaP cells (Figure 5.3B). Protein quantification demonstrated a significant increase of MCT4 in both PCa cell lines, PC3 and LNCaP, when compared with noncancerous cells, PNT1A (1.26- and 1.82-fold variation, respectively). MCT4 protein has a higher expression in PC3 than in LNCaP (Figure 5.3B). Overall, we observed that, although LDH protein expression decreased, LDH enzymatic and MCT4 protein levels increased in androgen-nonresponsive cells, PC3 when comparing with androgen-responsive cells, LNCaP.

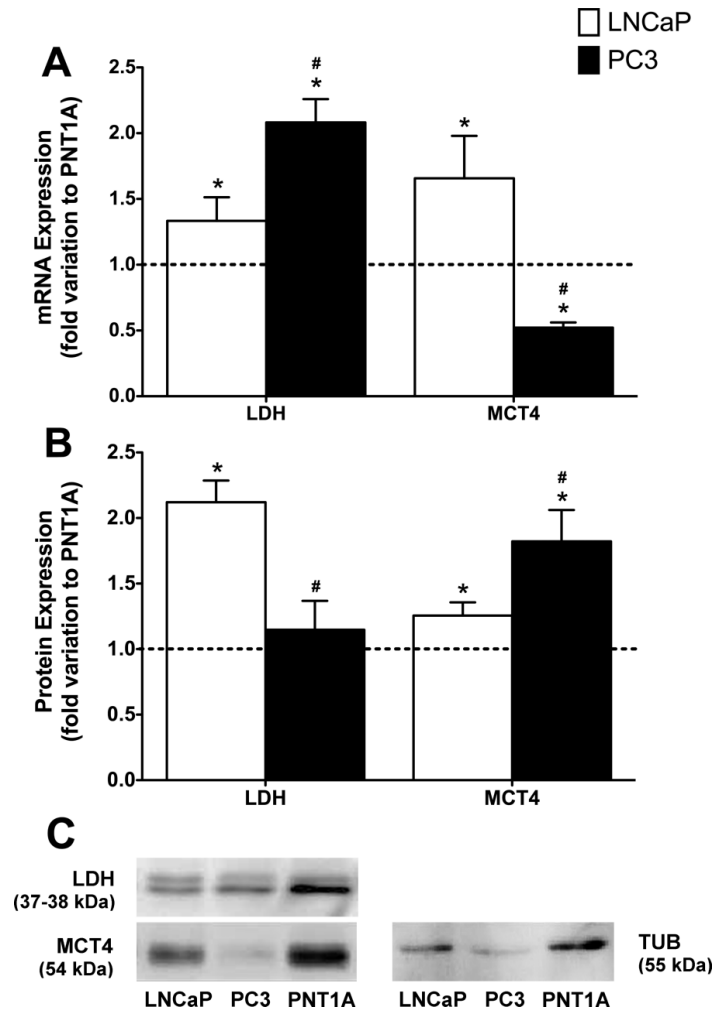


Figure 5.3. LDH and MCT4 mRNA and protein expression in androgen-responsive (LNCaP) and androgen-nonresponsive (PC3) prostate cancer cells determined by means of qPCR and Western Blot, respectively. Pooled data of independent experiments indicate mRNA (A) and protein (B) fold variation relatively to those of noncancerous PNT1A cells; (C) Representative SDS-page protein bands. Results are expressed as mean $\pm$ SEM (n=5). *Significantly different when compared with PNT1A ($p<0.05$); #Significantly different when compared with LNCaP ($p<0.05$).

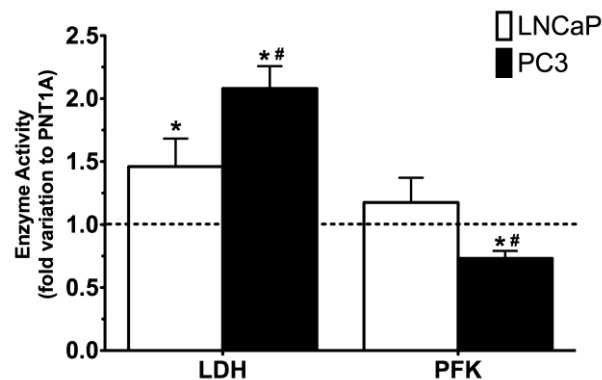


Figure 5.4. LDH and PFK enzymatic activities in androgen-responsive (LNCaP) and androgen-nonresponsive (PC3) prostate cancer cells represented as fold variation relatively to noncancerous PNT1A cells. Results are expressed as mean $\pm$ SEM (n=5). *Significantly different when compared with PNT1A ($p<0.05$); #Significantly different when compared with LNCaP ($p<0.05$)

Discussion

Energy metabolism in tumor cells has a special dependence upon glucose and it has been reported that the glycolytic capacity of cancer cells is enhanced even in the presence of a high oxygen concentration [33-35]. This characteristic is often associated with a metabolic strategy for tumor growth and survival and there are strong evidence that glycolysis is enhanced in tumor cells due to modulation of the expression of key enzymes and transporters involved in this metabolic pathway [36]. Although high glucose consumption has been described for PC3 cells [9], the distinctive glycolytic characteristics of androgen-responsive and nonresponsive PCa cells is poorly explored. The glycolytic process starts with glucose uptake and accordingly, glucose uptake from the extracellular media was highly increased in both, androgen-responsive LNCaP and androgen-nonresponsive PC3 cells (when compared to noncancerous prostate PNT1A cells). Others have also described that glucose uptake is increased in PCa for rapid cell proliferation, and that prostate androgen-nonresponsive DU145 cells may present a high rate of glucose consumption [9]. The suggestion that androgen-nonresponsive cells have higher rates of glucose consumption than androgen-responsive cells was not confirmed in our experiments with LNCaP and PC3 cells. Nevertheless, androgen-nonresponsive PC3 cells significantly produced more lactate than the androgen-responsive LNCaP. This is in agreement with other studies describing a higher lactate production by PC3 and DU145 cells when compared to LNCaP [37]. Also, high lactate production/concentration has been associated with more aggressive cancer stages and metastasis [38-40] and nonresponsive-androgen PC3 cells are considered an *in vitro* model of these stages of PCa [19]. Thus, although there is not a significant difference in glucose uptake between androgen-responsive and nonresponsive cells, lactate production is enhanced in PC3 cells suggesting that if there is a difference in the glycolytic pathway, it will be on metabolite transporters or glycolytic related enzymes. Concomitant with this proposal is the fact that although PC3 cells had no increase in LDH protein levels, the enzymatic activity of LDH was significantly increased when comparing with the androgen-responsive LNCaP cells.

There are clear evidences that GLUT1 and GLUT3 play important roles in cancer cells expansion and progression, being usually associated with glucose regulation and considered biomarkers of tumor differentiation and prognosis in many human cancers, namely, renal, breast, prostate, lung, colorectal and ovarian (reviewed by [28, 41, 42]). Thus, although there were no differences in glucose consumption, we analyzed GLUT1 and GLUT3 mRNA and protein expression. It has been reported that reduction of glucose transporters, namely GLUT1, is associated with the progression from normal to malignant prostate tissue [43] and that androgens induce GLUT1 expression in LNCaP cells [12, 13]. Our results also show that GLUT1 and GLUT3 mRNA expression is decreased in the androgen-nonresponsive PC3 cells. In LNCaP cells, although presenting a significant decrease on GLUT3 mRNA levels when compared with noncancerous cells, the corresponding protein levels were increased. This discrepancy between mRNA and protein levels, found also for other genes in this study, is something that has been described for several human cell lines (e.g. [44, 45] and can be

consequence of a myriad of factors associated with the great complexity of gene expression regulatory mechanisms, namely, mRNA retention in the nucleus, processing, stability and half-life time of mRNA molecule, control of protein translation efficiency, post-translational modifications, and protein degradation [46, 47]. Noteworthy a significant decrease of GLUT3 protein content was detected when comparing androgen-nonresponsive PC3 with the androgen-responsive LNCaP cells. These results suggest that GLUT3 may also be a biomarker and/or a control point in the transition to an androgen nonresponsiveness stage of PCa.

In the glycolytic process, there are several reactions catalyzed by different enzymes. Amongst those, the irreversible conversion of fructose-6-phosphate to fructose-1,6-bisphosphate (F-1,6-BP) by PFK1 is a main control step, often considered a rate-limiting step in glycolysis, and is associated with the energy status of the cell [48]. Although there were no differences in mRNA, PFK1 protein levels were significantly increased in androgen-responsive cells and decreased in nonresponsive cells in comparison with noncancerous cells. This different metabolic behaviour was already reported for different tumor cell lines with both an increase or a decrease in PFK1 activity [48]. We have showed that, PFK1 appears to be differently modulated in androgen-responsive and nonresponsive PCa cell lines, detecting decreased PFK protein levels in PC3 cells which were followed by a significant decrease in PFK enzymatic activity. After glucose enters the cells, and subsequently to its conversion to F-1,6-BP, it can originate pyruvate or lactate. A significant increase in lactate concentration has been demonstrated in PCa tissues [39, 40], which we also observed in the extracellular medium of both LNCaP and PC3 cells. Lactate production from substrates derived from glucose metabolism occurs via LDH. LDH is crucial for cell growth, particularly in those with enhanced glycolytic activity [49]. As expected, our results show a significant increase in LDH mRNA levels in both cancerous cell lines. In addition, there was a significant difference between the cancerous cell lines. LDH protein levels increase in androgen-responsive LNCaP cells while the androgen-nonresponsive PC3 cells display increased LDH mRNA levels but not LDH protein levels. These results show that androgen-nonresponsive PC3 cells, although producing more lactate, present an upregulation of LDH mRNA levels that is not followed by an increase in LDH protein levels but rather in its enzymatic activity. Although both PC3 and LNCaP cells presented a significant increase in LDH enzymatic activity relatively to PNT1A, when comparing both cancer cell lines, we found that the androgen-nonresponsive PC3 cells presented a significant higher LDH enzymatic activity. This suggests that the observed increase in lactate production can be consequence of both intracellular production by LDH and lactate transport to extracellular media.

The role of MCTs transporters maintaining hyper-glycolytic phenotypes has been described in other tumours beside prostate, namely, colon, central nervous system, breast, lung, gynaecologic tract and stomach [50]. In fact, concerning MCT4, the primarily lactate exporter expressed in cells with high glycolytic capacity [32, 51], a distinct pattern was present in different cancerous cell lines. Androgen-responsive LNCaP cells presented high mRNA and protein levels of MCT4 relatively to noncancerous cells while androgen-

nonresponsive cells displayed a decrease in MCT4 mRNA levels but an increase in protein expression. Increased levels of MCT4 protein have been associated with PCa progression and with a poor prognosis of disease [33, 52]. Our results are in concordance with those studies as the androgen-nonresponsive PC3 cells present a higher protein expression of MCT4. This is in agreement with the observed higher lactate export, following also a higher intracellular lactate production by LDH, which activity was found to be stimulated in these cells (Figure 5.5).

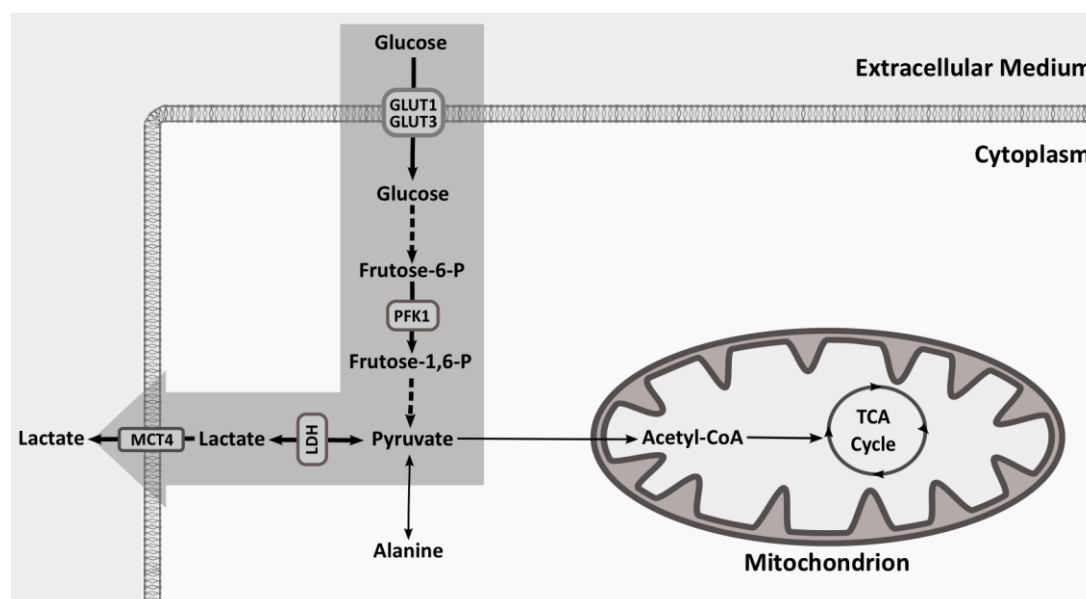


Figure 5.5. Schematic diagram of cancer cells metabolism. Tumor cells display enhanced glycolytic capacity following increased glucose uptake from the extracellular media which seems also to be associated with the regulation of expression of enzymes and transporters. After transport into the cells via glucose transporters (GLUT1 and GLUT3), glucose is metabolized to fructose-6-P and fructose-1,6-P, which in turn originates pyruvate. Pyruvate can be converted to lactate via lactate dehydrogenase (LDH) activity, or alternatively originate alanine. Lactate is transported to the extracellular milieu by monocarboxylate transporters (MCT4). Grey shaded arrow highlights the metabolic pathway identified in androgen-nonresponsive PC3 cells which exhibited increased lactate production concomitant with increased LDH activity and MCT4 protein levels.

Intracellular lactate results from the intermediate pyruvate formed from glucose. But pyruvate is at a crossroad between the interconversion of lactate and alanine. Also, intracellular alanine is in rapid equilibrium with pyruvate and thus it is assumed that the alanine turnover reflects the pyruvate concentration [53, 54]. Our results show that both, androgen-responsive and androgen-nonresponsive cells produce significantly more alanine than the noncancerous cells which is also concomitant with the expected enhancement of glycolytic activity that has been associated with PCa aggressiveness and metastasis [38]. Also, the lactate/alanine ratio is associated with the redox state of the cell because the conversion of pyruvate to lactate and alanine is coupled with NAD^+ and NADH reactions. Thus, lactate/alanine ratio reflects the NADH/NAD^+ equilibrium [55]. Our results show that lactate/alanine ratio is increased in both cancerous cell lines but the ratio is higher in androgen-nonresponsive PC3 cells. This phenomenon suggests a higher oxidative stress in

androgen-nonresponsive PC3 cells related to the glycolytic stimulation. This is particularly interesting since others have already suggested that antioxidant therapy prevents the progression from androgen-dependent to androgen-independent PCa [56]. The relationship between glycolysis stimulation and increased oxidative stress is well known. Proliferating cells, such as cancer cells, usually achieve high glycolytic rates by increasing the expression or activity of glycolysis-related enzymes such as PFK and LDH. Indeed, LDH overexpression is known to be sufficient to increase glycolytic activity of proliferating cells [49]. Moreover, NAD⁺ production is intimately linked to increased activity of LDH and is very unlikely that the extracellular glucose concentration can play a major role in cell stress conditions. Therefore the extracellular glucose concentrations detected in each cell line are only relevant by itself to overall cellular stress conditions if accompanied by alterations in enzymatic activities and/or protein expression.

The progression of PCa to a lethal state is related with a phenotype characterized by androgen unresponsiveness. Our work suggests that this progression may be related with important alterations in the glycolytic pathways, especially in glucose uptake by GLUTs, PFK1 expression and enzymatic activity, and finally lactate production and export by LDH and MCT4, respectively. The androgen-nonresponsive PC3 cells showed a marked increase in lactate production concomitant with increased LDH activity, MCT4 protein expression and higher oxidative stress levels than the androgen-responsive LNCaP. Noteworthy, the androgen-nonresponsive PC3 cells present lower GLUT3 and PFK protein expression and enzymatic activity than the androgen-responsive LNCaP suggesting that these cells might present other mechanisms responsible for the enhanced glycolytic activity. Enlightenment of the molecular mechanisms regulating glycolytic activity in the transition to androgen independent stages of PCa, is a key step for the design of new diagnosis and therapeutic options, since targeting cellular metabolism is tempting due to the unique metabolic features of androgen-responsive and nonresponsive cells.

Funding

This work was partially supported by the Pluriannual Programme of Portuguese Foundation for Science and Technology (FCT). PF Oliveira was financed by FCT under the program Ciência 2008. Cátia Vaz, Ricardo Marques and Marco Alves were funded by FCT fellowships (SFRH/BD/70316/2010, SFRH/BD/66875/2009 and SRFH/BPD/80451/2011, respectively).

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Chapter 6

Androgens enhance the glycolytic metabolism and lactate export in prostate cancer cells by modulating the expression of GLUT1, GLUT3, PFK, LDH and MCT4 genes

This chapter was published in:

Vaz, C.V., Marques, R., Alves, M.G., Oliveira, P.F., Cavaco, J.E., Maia, C.J., Socorro, S.
Androgens enhance the glycolytic metabolism and lactate export in prostate cancer cells by modulating the expression of GLUT1, GLUT3, PFK, LDH and MCT4 genes. Journal of cancer research and clinical oncology. 2015; DOI: 10.1007/s00432-015-1992-4

Androgens enhance the glycolytic metabolism and lactate export in prostate cancer cells by modulating the expression of GLUT1, GLUT3, PFK, LDH and MCT4 genes

Abstract

Purpose. The present study aims to investigate the role of androgens in controlling the glycolytic metabolism and lactate efflux in prostate cancer (PCa) cells.

Methods. Androgen-responsive LNCaP cells were treated with 5 α -dihydrotestosterone (DHT, 10 nM) for 12-48 h, and their glycolytic metabolism, lactate production and viability were analyzed. Intracellular and extracellular levels of glucose and lactate were determined spectrophotometrically, and the expression of glucose transporters (GLUT1/GLUT3), phosphofructokinase 1 (PFK1), lactate dehydrogenase (LDH) and monocarboxylate transporter (MCT4) was analyzed by real-time PCR and Western Blot. The enzymatic activity of LDH was determined by means of a colorimetric assay. Experiments were reproduced in androgen-nonresponsive DU145 and PC3 cells.

Results. Androgens stimulated glucose consumption in LNCaP cells by increasing the expression of GLUT3, GLUT1 and PFK, which was underpinned by increased cell viability. Accordingly, lactate production by LNCaP cells was enhanced upon DHT stimulation as evidenced by the increased levels of lactate found in cell culture medium. Although LDH enzymatic activity decreased in LNCaP cells treated with DHT, the expression of MCT4 was significantly increased with androgenic treatment, which sustains the increase on lactate export. Glucose consumption and the expression of GLUTs and PFK remained unchanged in DHT-treated DU145 and PC3 cells.

Conclusions. The results obtained establish androgens as modulators of glycolytic metabolism in PCa cells by stimulating glucose consumption, as well as, the production and export of lactate, which may represent a crucial issue driven prostate tumour development. These findings also highlight the importance of PCa therapies targeting AR and metabolism-related proteins.

Keywords: androgens; glucose; lactate; LNCaP; MCT4; prostate cancer.

Introduction

Metabolic changes and the capability to modify, or reprogram cellular metabolism are actually recognized as a hallmark of several human cancers [1]. In prostate, the neoplastic development has been associated with high alanine and lactate production, as well as, with increased glucose consumption [2-5]. Indeed, the dependency of cancer cells on abnormal rates of glucose consumption and lactate production is known since the Warburg's early studies in 1927 [6].

The enhanced glycolytic activity of tumours firstly relies on glucose uptake from the extracellular media, which occurs via specific glucose transporters (GLUTs) [7]. After entering the cell, glucose follows the glycolytic pathway and a set of catalytic reactions occur until its conversion to pyruvate. The phosphofructokinase 1 (PFK-1) is a key enzyme in this process since it catalyses the first irreversible reaction of glycolysis, the conversion of fructose-6-phosphate (F-6-P) to fructose-1,6-bis-phosphate (F-1,6-BP), being a rate-limiting step in glucose metabolism. The end-product of glycolysis, pyruvate, is metabolized by the lactate dehydrogenase (LDH) enzyme, which catalyzes the interconversion of pyruvate and lactate. The lactate produced by tumour cells is exported to the extracellular medium by specific monocarboxylate transporters (MCTs) [8] and contributes to the acidification of tumour microenvironment, which favours tumour invasion [9-11] and suppresses the anticancer immune defences [11, 12]. Furthermore, it has been shown that lactate may enter in stroma or cancer cells following oxidative phosphorylation or alternatively regenerating pyruvate that, in turn, may be used as energetic substrate by tumour cells [13, 14]. This process generates complementary metabolic pathways, which lead to survival and progression of cancer cells. Moreover, MCTs have been shown to be overexpressed in different human cancers and for this reason identified as attractive targets for cancer therapy [15].

Androgens are widely recognized as the main regulators of prostate cell proliferation and apoptosis, and are intimately associated with the development of prostate cancer (PCa) [16]. Androgenic actions are mediated through the intracellular androgen receptor (AR), which acts as a transcription factor regulating gene expression in target tissues [17]. In the last years, our and others studies have indicated androgens as important metabolic regulators in several tissues, including prostate [5, 18-22]. Androgens seem to stimulate the glycolytic flux in PCa cells by modulating the expression of glycolysis-related genes [18, 21], which is further supported by the fact that androgen-responsive and androgen-nonresponsive PCa cells display a distinct glycolytic profile [5]. However, the effect of androgens regulating lactate efflux by PCa cells has remained unknown.

The present study aimed to determine the effect of a non-aromatizable androgen on the glycolytic metabolism and production and export of lactate in androgen-responsive LNCaP cells. The gene expression of GLUT1, GLUT3, PFK-1, LDH and MCT4, and the intracellular and extracellular content of glucose and lactate were assessed. Experiments were comparatively carried out in androgen-nonresponsive DU145 and PC3 cells.

Material and methods

Experimental design

The human prostate cancer cell lines, LNCaP, DU145 and PC3, were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). PCa cells were cultured in RPMI 1640 medium (Invitrogen, New York, NY) supplemented with 10% FBS (Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (Invitrogen, Karlsruhe LMA, Germany), and

maintained at 37°C in an atmosphere equilibrated with 5% CO₂. At 60% confluence, the culture medium was replaced by phenol red-free medium containing 10% charcoal-stripped fetal bovine serum free of steroid hormones (Invitrogen) and cells were maintained for additional 24 h before being exposed to 10 nM of 5 α -dihydrotestosterone (DHT, Sigma-Aldrich, St Louis, MO, USA) for 12, 24, and 48 h. This experimental concentration of DHT (10 nM) has been shown to be effective in regulating the expression of several androgen-target genes in LNCaP cells, as demonstrated by previous studies of our research group [23, 24], and resembles the physiological concentrations [25]. DHT stimulation was repeated at 24 h in presence of 1 μ M of AR antagonist flutamide (Sigma-Aldrich) or 10 μ g/ml of protein synthesis inhibitor cycloheximide (Sigma-Aldrich). Inhibitors were added to culture medium 30 min before hormone stimulation. At different experimental time-points, cells were harvested and snap frozen in liquid nitrogen for extraction of RNA, protein and intracellular metabolites. For all experimental groups, the total number of cells per flask, as well as, cell viability using the Trypan Blue Exclusion Test were determined. Viability values higher than 85% were considered for experimental assays.

Culture medium of DHT-treated and untreated cells was collected for measurement of glucose consumption and lactate production.

Quantification of glucose and lactate

The concentration of glucose and lactate in the culture medium of DHT-treated and untreated cells was determined by means of spectrophotometric analysis using commercial kits (Spinreact, Girona, Spain). All measurements followed manufacturer's instructions and were normalized for the total number of cells in each experimental condition. The glucose consumption and lactate production by LNCaP, DU145 and PC3 cells in response to DHT was calculated.

A methanol/chloroform/water extraction was used to simultaneously extract polar and apolar metabolites from cells cultured or not in presence of DHT [26]. Briefly, cells were promptly quenched in liquid nitrogen followed by the addition of 1 ml of cold methanol and 500 μ l chloroform. After thawing on ice, samples were vortexed for 60 s and sonicated. 500 μ l chloroform and 500 μ l of ice-cold water were then added to each sample, which were vortexed and centrifuged at 10,000 g for 15 min at 4 °C. The upper layer containing water-soluble metabolites was collected, lyophilized and then diluted in sterile water for quantification of glucose and lactate concentrations using commercial kits (Spinreact).

Cell viability assay

LNCaP cells were grown in 96-well plates at a density of 1.5×10^3 cell per well in 100 μ l culture medium. Cell viability was determined by the colorimetric (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2M) tetrazolium (MTS) assay using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). The conversion of MTS compound to the colored formazan product was detected at 490 nm in a

Microplate Reader (Biochrom, Anthos 2020). The relative number of viable cells in each experimental condition was calculated by normalizing the absorbance to that of the corresponding control. All experiments were repeated at least three times, and each experiment was carried out in hexaplicated.

Quantitative RT-PCR (qPCR)

Real-time qPCR was carried out to evaluate the mRNA expression of GLUT1, GLUT3, PFK1, LDH and MCT4 in LNCaP cells in response to DHT treatment. Total RNA was extracted from LNCaP cells using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. The quantity and quality of extracted RNA were assessed by the spectrophotometric (NanoPhotometer, Implen, Munich, Germany) determination of absorbance ratio (A260/A280) and agarose gel electrophoresis, respectively. 1 µg of total RNA was reverse transcribed in a final volume of 20 µl using the First Strand cDNA synthesis Kit (Fermentas, Burlington, Canada), following the manufacturer's instructions. 1 µl of synthesized cDNA was used in each qPCR reaction. The optimization strategy for each primer set, the specificity and efficiency of the amplification, as well as primer details and cycling conditions were previously described [5]. β2-microglobulin was used as internal control to normalize gene expression. Normalized expression values were calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta C_t}$ [27].

Western Blot (WB)

Total proteins were extracted from LNCaP, DU145 and PC3 prostate cells using RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 8 and 1 mM EDTA), and protein concentration was determined by the Bradford micro-assay (Bio-Rad Laboratories, CA, USA). 50 µg of each sample protein were resolved in a 12% gel by SDS-PAGE and electrotransferred to a PVDF membrane (GE Healthcare, Buckinghamshire, UK). Membranes were incubated overnight at 4 °C with, rabbit anti-GLUT1 (1:300, CBL242, Millipore), rabbit anti-GLUT3 (1:2500, sc-30107, Santa Cruz Biotechnology), rabbit anti-PFK1 (1:500, sc-67028, Santa Cruz Biotechnology), rabbit anti-LDH (1:10000, Ab52488, Abcam, Cambridge, United Kingdom), or rabbit anti-MCT4 (1:1000, sc-50329, Santa Cruz Biotechnology, CA, USA) primary antibodies. Protein expression was normalized using a mouse anti-β-actin (1:1000, A5441, Sigma-Aldrich) antibody. Goat anti-rabbit IgG-AP (1:5000, NIF1317; GE Healthcare) or goat anti-mouse IgG + IgM-AP (1:5000, NIF1316; GE Healthcare) were used as secondary antibodies. Membranes were incubated with ECF substrate (GE Healthcare) for 5 min and immune-reactive proteins were visualized with the Molecular Imager FX Pro plus Multimager (Bio-Rad). Band densities were obtained using the Quantity One Software (Bio-Rad) and normalized to the corresponding β-actin density. Results are presented as fold variation relatively to the control untreated group.

LDH enzymatic activity

The enzymatic activity of LDH in LNCaP cells was determined using a commercial kit (Promega, Madison, USA) following the manufacturers' instructions. Briefly, enzymatic activity was calculated by measuring the shift on the absorbance (492 nm) of samples resulted from the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of produced formazan is directly proportional to the activity of LDH on each sample. The method was calibrated with the LDH Positive Control included in the kit. The attained activities were calculated using the molar absorptivity of formazan and expressed as fold variation relatively to the control untreated group.

Statistical analysis

The statistical significance of differences between experimental groups was assessed by the Student's t-test (GraphPad Software, San Diego, CA, USA). Significant differences were considered when $P < 0.05$. All experimental data are shown as mean $\pm$ SEM ($n = 6$ for each experimental condition).

Results

Glucose consumption and lactate production were regulated by androgens

In order to study whether androgens regulate the glycolytic metabolism in PCa cells, we determined the content of glucose and lactate in LNCaP cells and culture medium after exposure to 10 nM of DHT for 12, 24 and 48 h. DHT significantly increased glucose consumption relatively to the control at 24 h (19.28 ± 0.58 vs. 17.09 ± 0.73 pmol/cell) and 48 h (28.46 ± 0.19 vs. 26.45 ± 0.35 pmol/cell, Figure 6.1A). Also, lactate production was significantly increased in DHT-treated cells comparatively with untreated cells at 12, 24 and 48 h (6.73 ± 0.26 vs. 5.79 ± 0.28 pmol/cell; 15.86 ± 1.32 vs. 9.59 ± 0.73 pmol/cell and 14.33 ± 0.44 vs. 11.19 ± 1.1 pmol/cell, respectively, Figure 6.1B). No significant differences were observed in the intracellular concentration of glucose between DHT-treated and untreated cells at different time points (Figure 6.1C). The intracellular levels of lactate were significantly increased 12 h after treatment with DHT (0.10 ± 0.02 vs. 0.05 ± 0.01 pmol/cell in the control group, Figure 6.1E). At 24 and 48 h no differences were perceived on the intracellular content of lactate between experimental groups.

DU145 and PC3 cell lines, commonly used as *in vitro* models of androgen-insensitive stages of human PCa, were also treated with DHT and their glycolytic metabolism investigated. Interestingly, for 48 h of treatment DHT significantly increased the production of lactate in DU145 cells (66.74 ± 4.88 vs. 38.02 ± 3.75 pmol/cell, Figure 6.1B). An increase in intracellular lactate was observed both in DU145 and PC3 cells (0.033 ± 0.0008 vs. 0.020 ± 0.003 and 0.037 ± 0.003 vs. 0.026 ± 0.002 pmol/cell, respectively, Figure 6.1F). No

differences were observed in the consumption and intracellular content of glucose between experimental groups in both cell lines.

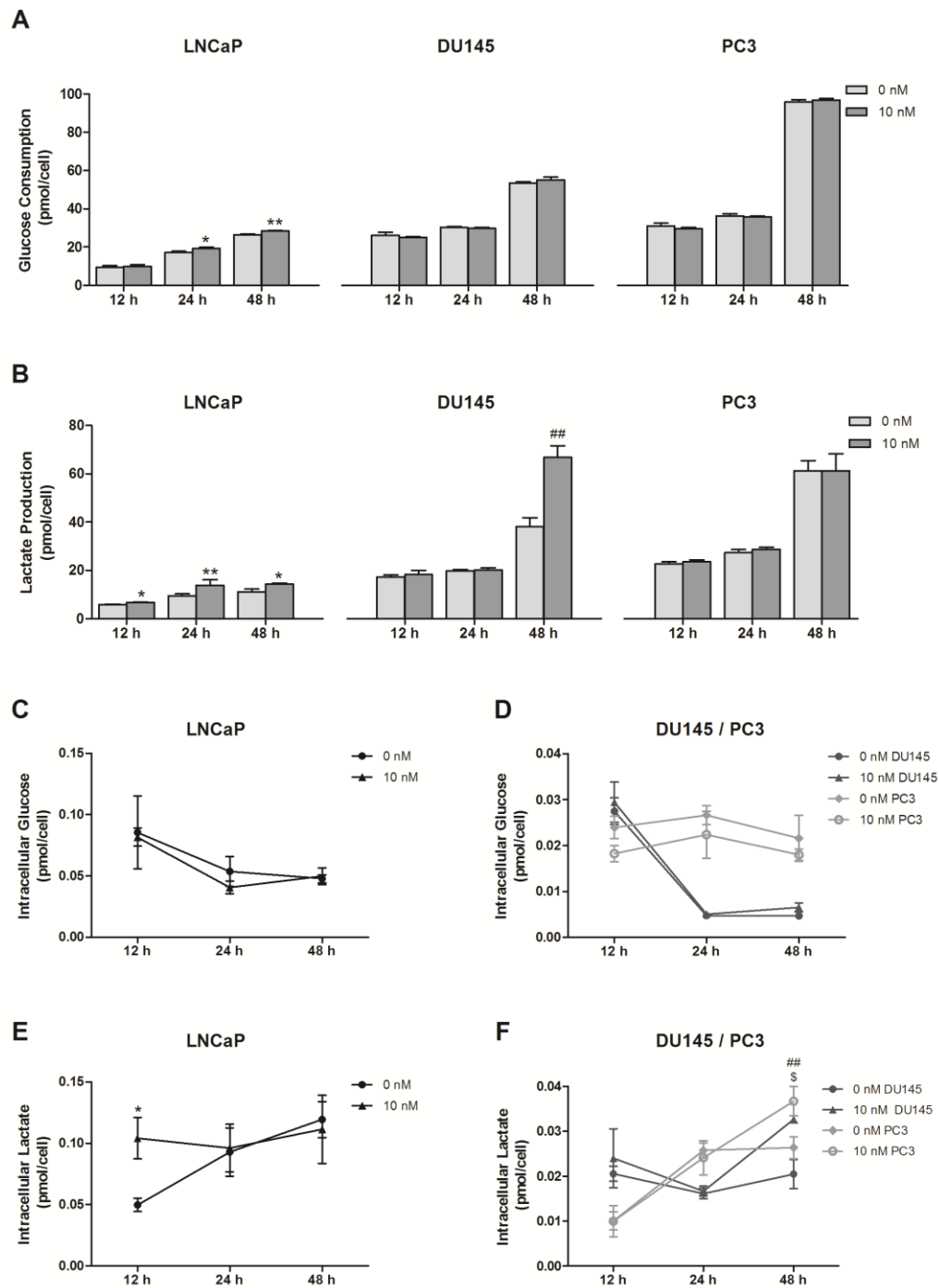


Figure 6.1. Glucose consumption (A), lactate production (B), and intracellular concentration of glucose (C and D) and lactate (E and F) in LNCaP, DU145 and PC3 prostate cancer cells in response to DHT treatment (10 nM) for 12 h, 24 h and 48 h. Error bars indicate mean $\pm$ SEM, n=6. * $P < 0.05$; ** $P < 0.01$ relatively to control group at each experimental time for LNCaP cells; ## $P < 0.01$ relatively to control group at each experimental time for DU145 cells; \$ $P < 0.05$ relatively to control group at each experimental time for PC3 cells.

DHT treatment increased the viability of LNCaP cells

To determine the effect of DHT on the viability of LNCaP cells, an MTS assay was performed. DHT significantly augmented the percentage of LNCaP viable cells in comparison with the control in about 25, 36 and 51 %, respectively, for 12, 24 and 48 h of treatment (Figure 6.2).

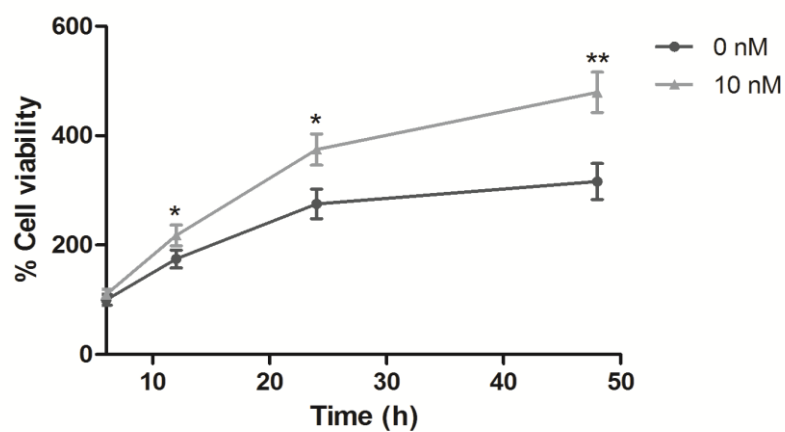


Figure 6.2. Effect of DHT (10 nM) on the viability of LNCaP cells determined by the MTS assay at 12, 24 and 48 h. Results are expressed as % of control. Error bars indicate mean $\pm$ SEM, n=6. * P <0.05; ** P <0.01 relatively to control group at each experimental time.

Androgens increased GLUT1, GLUT3 and PFK levels in LNCaP cells

The influence of androgens on the regulation of glucose transport was assessed through analyzing the expression of GLUT1 and GLUT3, which were both previously identified in LNCaP cells [5, 28]. The mRNA expression of GLUT1 was significantly decreased in LNCaP cells treated with 10 nM of DHT for 12 and 24 h (respectively, 0.70 ± 0.09 and 0.77 ± 0.05 fold variation relatively to control, Figure 6.3A). However, at 48 h of stimulation with DHT the protein expression of GLUT1 was increased comparatively with the control untreated group (1.19 ± 0.02 fold variation, $P < 0.05$, Figure 6.3C). Regarding GLUT3, its mRNA expression was decreased in response to DHT at 48 h (0.70 ± 0.05 fold variation relatively to control group, $P < 0.05$, Figure 6.3B), while the protein levels were increased comparatively with the control after 24 h of treatment with 10 nM DHT (1.42 ± 0.16 fold variation, $P < 0.05$, Figure 6.3D).

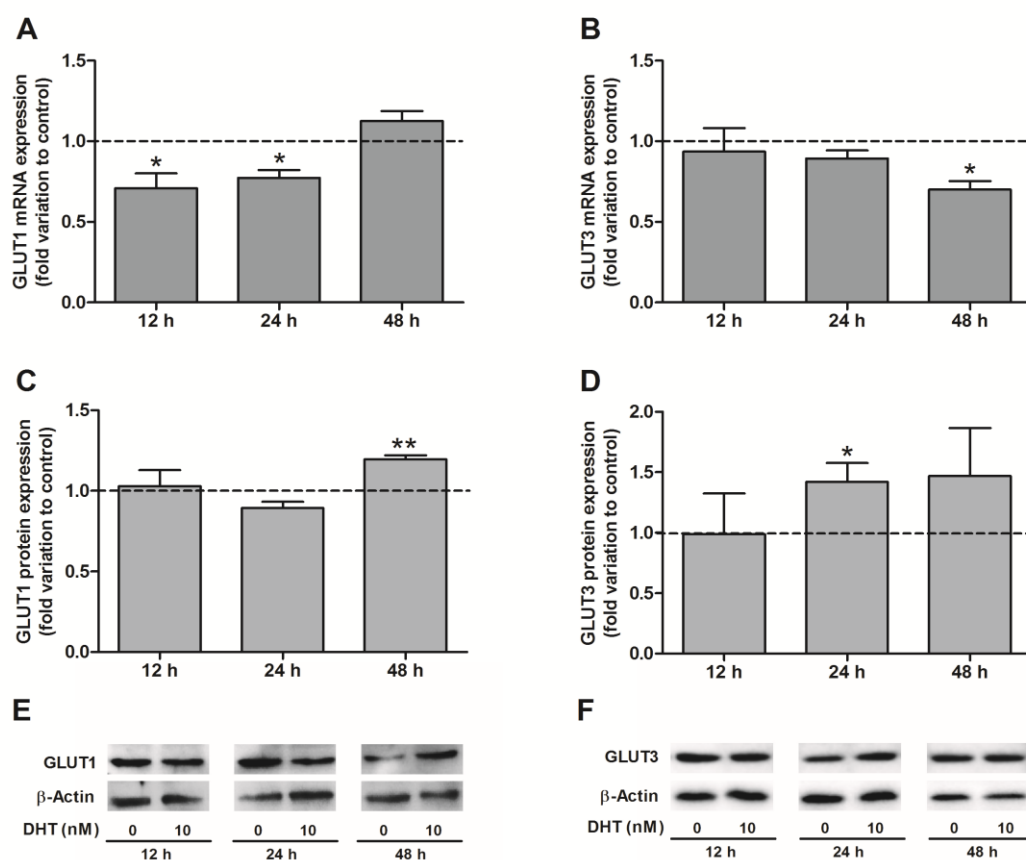


Figure 6.3. Effect of DHT (10 nM) on the expression of GLUT1 (A, C) and GLUT3 (B, D) in LNCaP prostate cancer cells treated for 12 h, 24 h or 48 h. mRNA expression was determined by qPCR after normalization with B2-microglobulin housekeeping gene. Protein expression was analyzed by WB and normalized with β-actin. All results are expressed as fold-variation relatively to the control untreated group (0 nM DHT) represented by the dashed line. Error bars indicate mean $\pm$ SEM, $n=6$. * $P < 0.05$; ** $P < 0.01$. Representative immunoblots are shown in panels E and F.

Phosphofructokinase is responsible for the first irreversible reaction in glycolysis, being also the most important control point in mammalian glycolytic pathways [29]. Treatment with 10 nM of DHT for 12 h reduced the mRNA levels of PFK in LNCaP cells (0.57 ± 0.09 fold variation relatively control, $P < 0.05$, Figure 6.4A). A significant increase on PFK protein expression was observed at 48 h (1.30 ± 0.11 fold variation comparatively with control, Figure 6.4B).

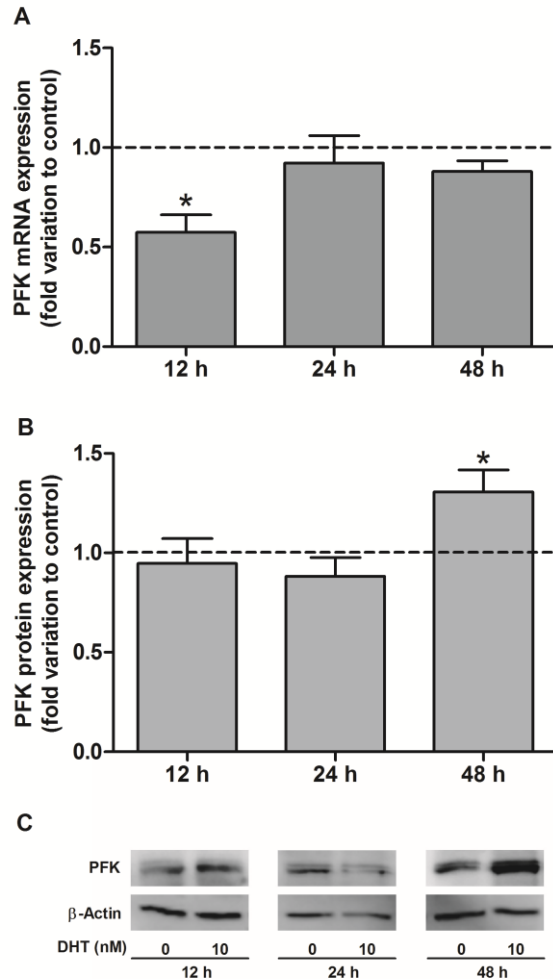


Figure 6.4. Expression of PFK in LNCaP prostate cancer cells treated with DHT (10 nM) for 12 h, 24 h or 48 h. (A) mRNA expression was determined by qPCR after normalization with B2-microglobulin housekeeping gene. (B) Protein expression was analyzed by WB and normalized with β-actin. All results are expressed as fold-variation relatively to the control untreated group (0 nM DHT) represented by the dashed line. Error bars indicate mean $\pm$ SEM, $n=6$. * $P < 0.05$. Representative immunoblots are shown in panel C.

DHT treatment decreased the enzymatic activity of LDH and increased the expression of MCT4 in LNCaP cells

The LDH enzyme catalyzes the reversible conversion of the final product of glycolysis pyruvate into lactate [30]. It was observed a significantly diminished expression of LDH mRNA in DHT-treated cells in comparison with the control group at 12 and 24 h of stimulation (respectively, 0.55 ± 0.07 and 0.64 ± 0.09 fold variation, Figure 6.5A). The protein expression of LDH remained unchanged at all experimental time points (Figure 6.5B). However, a significant reduction in the enzymatic activity of LDH was observed in LNCaP cells treated with DHT for 24 and 48 h (respectively, 0.76 ± 0.05 and 0.47 ± 0.14 fold variation comparatively with the control, Figure 6.5C).

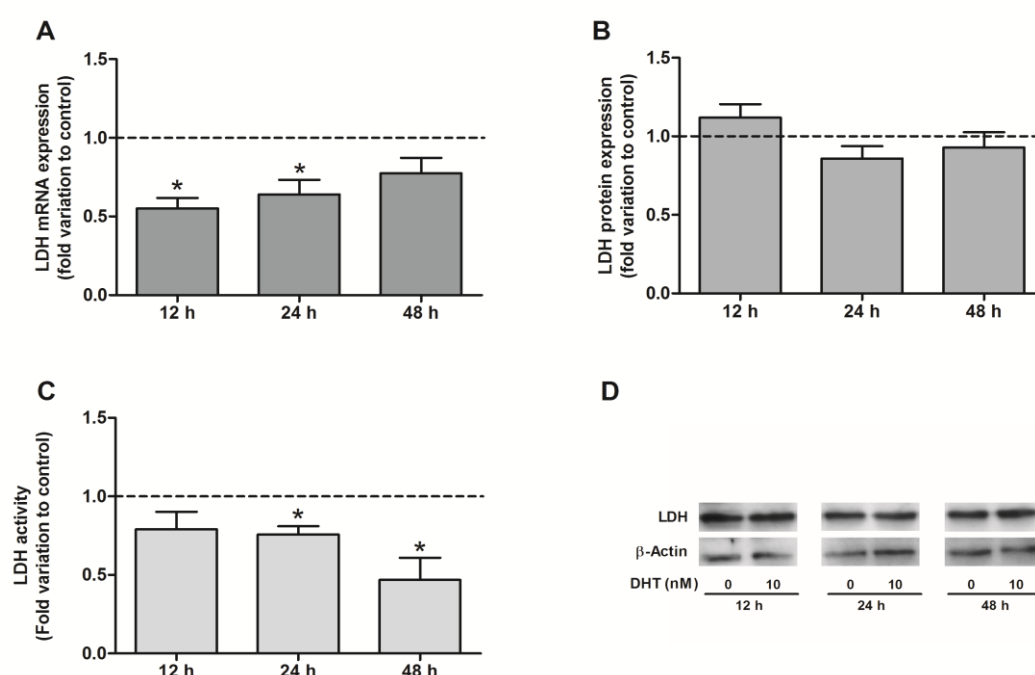


Figure 6.5. Effect of DHT (10 nM) on the mRNA (A) and protein (B) expression of LDH, and on enzymatic activity of LDH (C) in LNCaP prostate cancer cells treated for 12 h, 24 h or 48 h. mRNA expression was determined by qPCR after normalization with β 2-microglobulin housekeeping gene. Protein expression was analyzed by WB and normalized with β -actin. All results are expressed as fold-variation relatively to the control untreated group (0 nM DHT) represented by the dashed line. Error bars indicate mean $\pm$ SEM, n=6. * P <0.05. Representative immunoblots are shown in panel D.

The export of lactate by LNCaP cells under DHT stimulation was assessed by analyzing the mRNA and protein expression of MCT4, which has been indicated as the MCT family member mostly associated with lactate efflux in highly glycolytic cells and previously identified in LNCaP cells [5, 31, 32]. DHT treatment for 24 h significantly increased the mRNA expression of MCT4 (1.50 ± 0.20 fold variation comparatively with the control, Figure 6.6A). Also, the protein levels of MCT4 were enhanced in response to DHT treatment for 12, 24 and 48 h (respectively, 1.44 ± 0.18 , 1.21 ± 0.10 and 1.40 ± 0.11 fold variation relatively to untreated cells, P <0.05, Figure 6.6B).

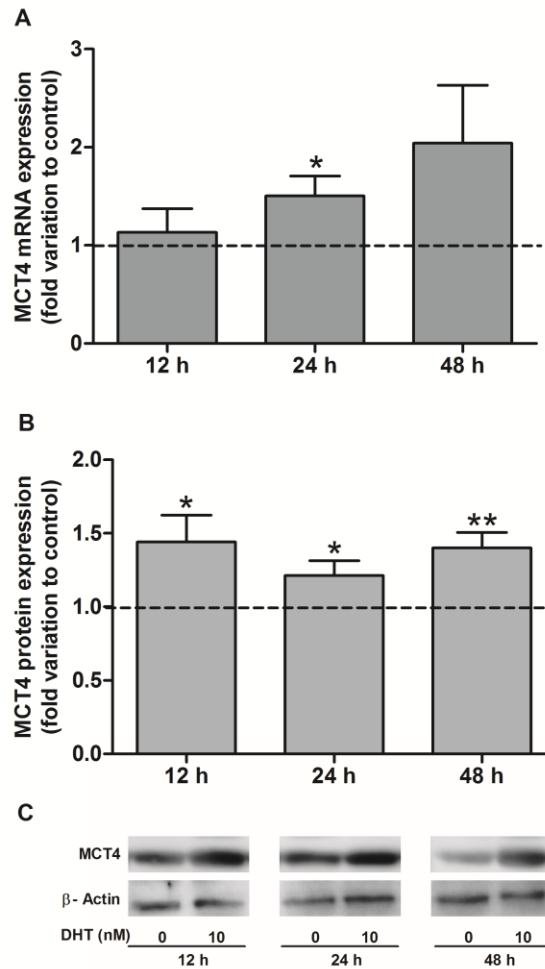


Figure 6.6. Expression of MCT4 in LNCaP prostate cancer cells treated with DHT (10 nM) for 12 h, 24 h or 48 h. (A) mRNA expression was determined by qPCR after normalization with B2-microglobulin housekeeping gene. Protein expression was analyzed by WB and normalized with β -actin. All results are expressed as fold-variation relatively to the control untreated group (0 nM DHT) represented by the dashed line. Error bars indicate mean $\pm$ SEM, $n=6$. * $P < 0.05$; ** $P < 0.01$. Representative immunoblots are shown in panel C.

It is well known that the effects of DHT are mediated through its nuclear receptor, the AR [17]. Therefore, to investigate the mechanisms underlying the regulation of LDH and MCT expression by DHT, hormonal-stimulation experiments were carried for 24 h in the presence of AR antagonist flutamide or protein synthesis inhibitor cycloheximide. Both flutamide and cycloheximide abrogated the effect of DHT down-regulating the expression of LDH mRNA (Figure 6.7A). Also, the stimulatory effect of DHT on MCT4 mRNA levels was reverted in the presence of flutamide and cycloheximide (Figure 6.7B).

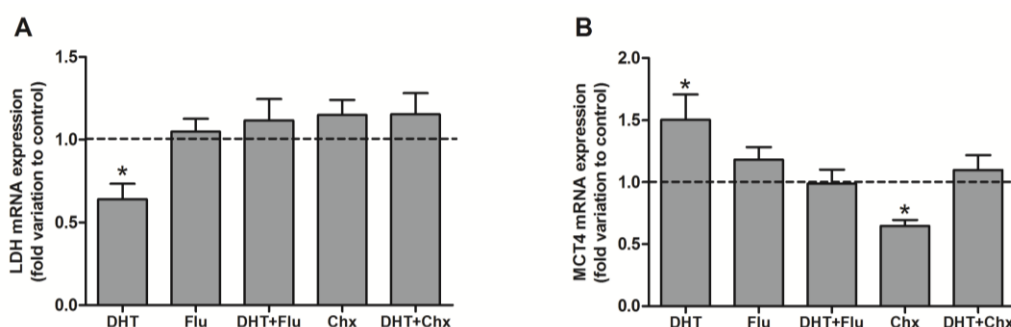


Figure 6.7. LDH (A) and MCT4 (B) mRNA expression in LNCaP prostate cancer cells treated with 10 nM DHT, 1 μ M flutamide (Flu), 1 mg/ml Cycloheximide (Chx), 10nM DHT plus 1 μ M flutamide, or 10 nM DHT plus 1mg/ml cycloheximide for 24 h. mRNA expression was determined by qPCR after normalization with β 2-microglobulin housekeeping gene. All results are expressed as fold-variation relatively to the control untreated group (0 nM DHT) represented by the dashed line. Error bars indicate mean $\pm$ SEM, n=6. * P < 0.05.

Androgens had no effect on the expression of GLUT1, GLUT3 and PFK in DU145 and PC3 cell lines while increasing LDH

In both DU145 and PC3 cells, the expression of GLUT1, GLUT3 and PFK remained unchanged in response to DHT treatment throughout the experimental time course (Figure 6.8). However, the protein expression of LDH was increased in DU145 and PC3 cells treated with DHT for 48 h (respectively, 1.29 ± 0.04 and 1.27 ± 0.05 fold variation relatively to untreated cells, P < 0.05, Figure 6.8D). Also, DHT significantly reduced MCT4 protein levels in PC3 cells at 48 h of treatment (0.73 ± 0.05 fold variation relatively to control, P < 0.05, Figure 6.8E).

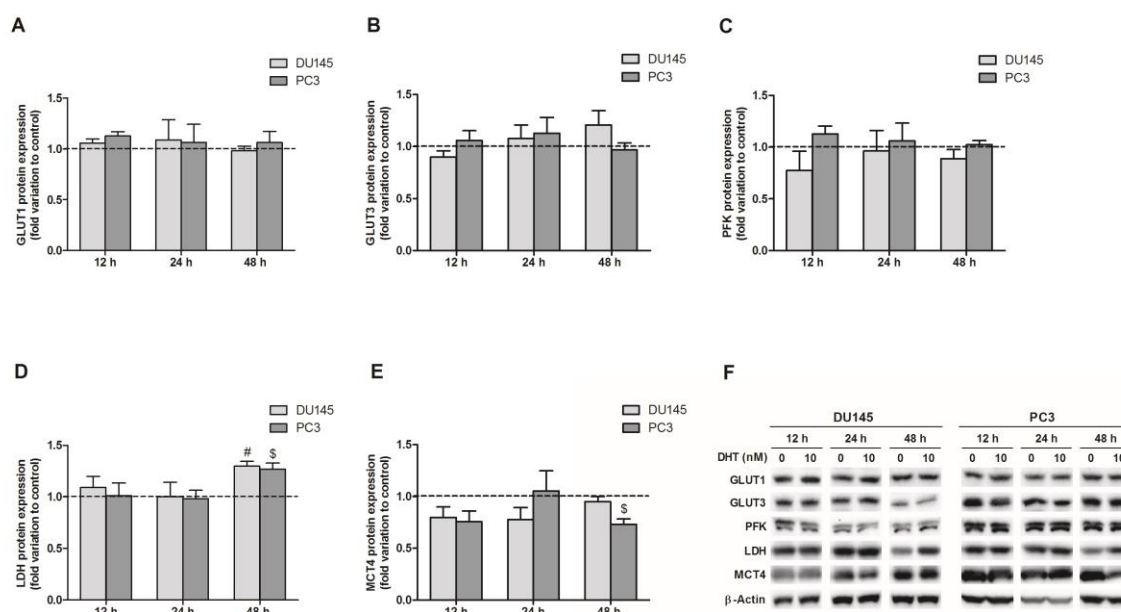


Figure 6.8. Expression of GLUT1 (A), GLUT3 (B), PFK (C), LDH (D) and MCT4 (E) in DU145 and PC3 prostate cancer cells treated with DHT (10 nM) for 12 h, 24 h or 48 h. Protein expression was analyzed by WB and normalized with β -actin. All results are expressed as fold-variation relatively to the control untreated group (0 nM DHT) represented by the dashed line. Error bars indicate mean $\pm$ SEM, n=6. # P < 0.05 for DU145 cells; \$ P < 0.05 for PC3 cells. Representative immunoblots are shown in panel F.

Discussion

Most tumour cells highly consume glucose through glycolysis with consequent production of lactate as a final source of ATP even in the presence of oxygen [33]. The lactate produced is exported into the extracellular space leading to acidification of tumour environment, and also it may be used as an alternative metabolic fuel by neighbouring cells [10, 14]. Collectively, these events confer a selective advantage that enhances survival and proliferation of cancer cells. Recent evidence has highlighted for the importance of androgens regulating the glycolytic metabolism in PCa cells [5, 18], but their effect on lactate handling needs clarification. In the present study we verified that DHT treatment enhanced lactate production by LNCaP cells at all experimental time points (Figure 6.1B), which followed a significantly increase of glucose consumption at 24 and 48 h of treatment (Figure 6.1A). The process of glycolysis starts with glucose uptake from the extracellular medium, a task achieved by hexose transporters present at cell membrane, the GLUTs [7]. Herein, it was shown that both GLUT1 and GLUT3 are under hormonal control in LNCaP cells (Figure 6.3). Although the GLUT1 and GLUT3 mRNA expression diminished with DHT stimulation, their protein levels were increased after 48 h or 24 h of DHT treatment, respectively. The non-concordant results between mRNA and protein levels, also found for other genes in this study, can be associated with the great complexity of gene expression regulatory mechanisms in eukaryotes, namely, the retention of mRNA in the nucleus, the processing, stability and half-life time of mRNA molecule, the control of protein translation efficiency, the post-translational modifications, and the protein degradation rate [34, 35]. Indeed, the stability of GLUT1 and GLUT3 (and also PFK) mRNAs in LNCaP cells was relatively high comparatively with the median estimated half-life for eukaryotic genes. At 12 h after the administration of transcription inhibitor actinomycin D (1µg/ml), still considerable mRNA levels of these genes exist (data not shown), which is concordant with the fact that, in general, the mRNA species of genes related to metabolism have high stability [36]. Nevertheless, the results obtained indicated that androgen-responsive LNCaP cells adjusted glucose uptake in response to DHT by modulating the expression of GLUT1 and GLUT3, and suggested the existence of a compensatory mechanism to ensure high glucose needs. The effect of androgens regulating the expression of GLUT1 in LNCaP cells was previously described [18, 37], but this is the first report showing the androgenic control of GLUT3 levels. This regulatory action of DHT over GLUT1 and GLUT3 expression was also established for other cell types of male reproductive tract [19, 20], and is further supported by the fact that LNCaP cells under androgen deprivation display a diminished uptake of fluorodeoxyglucose (FDG) [22]. Moreover, the GLUT12 that has been shown to be overexpressed in PCa [38], was also identified as an androgen target gene in MCF-7 cells [39, 40], which indicates a broad action of androgens modulating the expression levels of GLUTs. This also suggests that other family members besides GLUT1 and GLUT3 may have contributed to the enhanced glucose uptake observed in LNCaP cells in response to DHT. It is straightforward to expect that androgens support PCa

cell growth by accelerating the utilization of glucose, which is sustained by the increased viability observed in LNCaP cells treated with DHT (Figure 6.2).

No significant differences were found on the intracellular concentration of glucose between DHT-treated and untreated LNCaP cells, which suggests that the excess of glucose consumed under DHT stimulation is promptly metabolized. After two sequential reactions that convert glucose to glucose 6-phosphate and F-6-P, respectively, by the activity of hexokinase (HK) and phosphoglucose isomerase, the glycolytic process proceeds with the irreversible reaction catalyzed by PFK1. F-6-P is then converted to F-1,6-BP, which is considered a rate-limiting step in glycolysis [41]. Following the augmented consumption of glucose and the increased expression of GLUT1 in LNCaP cells, it was also observed an increase on protein levels of PFK 48 h after treatment with DHT (Figure 6.4B). These findings indicate that androgens stimulate the glycolytic process at multiple levels, from glucose uptake to its metabolization. Noteworthy, no changes were perceived on glucose consumption and GLUT1, GLUT3 and PFK protein expression in DU145 and PC3 cells under androgenic stimulation (Figure 6.1A and Figure 6.8). Moreover, since increased glucose consumption and lactate production in LNCaP cells treated with DHT for 24 h were not underpinned by increased expression levels of PFK, it is liable to assume that DHT may be also positively regulating other key glycolytic enzymes. Indeed, others have described the influence of androgens in upregulating the expression of HK2 and the cardiac isoform of PFK (PFKB2) in LNCaP cells [21]. Interestingly, our previous findings have shown that the expression of PFK1 is increased in LNCaP cells comparatively to the non-neoplastic cell line PNT1A [5]. Also, increased expression of PFK has been reported in cases of human PCa [42], which indicates that the inhibition of the enzyme that catalyzes one of the critical steps of glycolysis would be a promising strategy in PCa treatment. Recently, synthetic and/or natural pharmacological compounds that directly or indirectly inhibit PFK have been tested for treatment of leukemia, and breast and lung cancers [43], and their use in PCa is sought in a near future.

The end-product of glycolysis pyruvate is converted to lactate through the activity of LDH, and extruded to the extracellular medium mainly by MCT4. Although for 12 h stimulation with 10 nM DHT the glucose uptake by LNCaP cells did not change, the lactate concentration in the extracellular medium was enhanced. At 24 and 48 h of treatment the significant increase in glucose consumption by LNCaP cells was followed by an increase in lactate production, which suggests that glucose is metabolized and converted to lactate that then is exported to extracellular medium. However, our results showed that DHT downregulated the mRNA expression of LDH in LNCaP cells (Figure 6.5A), an effect completely reversed by the AR antagonist flutamide or the protein synthesis inhibitor cycloheximide (Figure 6.7A) indicating the involvement of a classical genomic mechanism of gene expression regulation through AR and the requirement of de novo protein synthesis. No differences were observed on LDH protein levels between LNCaP DHT-treated and untreated cells but the activity of LDH enzyme was significantly diminished upon DHT treatment for 24 and 48 h (Figure 6.5C). The diminution of LDH activity in LNCaP cells with prolonged exposure to DHT apparently is not in

accordance with the increased lactate production observed at all experimental time-points and may result from negative feedback exerted by the augmented intracellular lactate initially observed at 12 h of hormonal treatment. Anyway, the augment on lactate concentration in the extracellular medium is concordant with the increase of MCT4 protein levels at the same time-points (Figure 6.6), which demonstrated that the lactate produced was promptly extruded to the extracellular medium. Noteworthy, these results established for the first time the effect of DHT increasing the lactate export by LNCaP cells through the modulation of MCT4 expression both at mRNA and protein level. For 24 h of treatment DHT upregulated the MCT4 mRNA expression and, similarly with what was described for LDH, this effect was abolished in the presence of flutamide and cycloheximide indicating the involvement of AR and protein de novo synthesis in this process. Our findings are supported by a previous study demonstrating the regulation of MCT4 by testosterone in rat skeletal muscle [44]. Concerning lactate handling, is also important to note that androgen-nonresponsive DU145 and PC3 cells showed an opposite behaviour in relation to LNCaP under DHT stimulation. For 48 h of treatment, DHT promoted the increase in LDH expression (Figure 6.8) both in DU145 and PC3 cells, which was followed by augmented concentration of intracellular lactate (Figure 6.1F), an effect not seen in LNCaP cells. However, DU145 cells showed enhanced production of lactate (Figure 6.1B) despite their expression levels of MCT4 remained unaltered upon DHT treatment. Contrarily, as stated before, androgen-responsive LNCaP cells displayed augmented production of lactate in response to DHT underpinned by increased expression levels of MCT4. These results, further confirm that the increased expression of MCT4 and lactate export are dependent on AR while the observed increase in LDH expression in DU145 and PC3 cells may occur by AR-independent mechanisms. No other studies exist concerning the effect of androgens in the regulation of MCTs in prostate cells.

The export of lactate to the tumour environment has been identified as a condition that favours tumour development and invasion [11, 45], and the role of MCTs in maintaining the hyper-glycolytic phenotype of prostate tumours has been proposed [46]. Moreover, it was found that MCT4 expression is positively associated with the progression of PCa [5, 47], and its overexpression seems to be a marker of poor prognosis [48]. Therefore, at the initial phases of PCa, or in castrate-resistant stages displaying upregulation of AR signalling pathways, the inhibition of MCT4 may be an attractive strategy for treatment.

Herein, we demonstrated that DHT stimulated glucose uptake, glycolysis and lactate production, as well as, the export of lactate to the extracellular medium, due to enhanced expression of GLUTs, PFK and MCT4 in LNCaP cells. This reprogramming of cell metabolism in response to androgens favourably impacts on survival, proliferation and invasion ability of PCa cells. Although this study relied on an *in vitro* approach, it highlighted the importance of controlling glucose and lactate metabolism in PCa treatment. Moreover, the present findings are supported by clinical studies reporting the effect of androgens in the control of glucose consumption in prostate tumours. Larson et al. observed that the majority of metastatic PCa with overexpression of AR displayed higher glucose uptake [49]. Accordingly, it was shown

that androgen-ablation therapy suppressed the utilization of glucose in PCa cases [50]. Therefore, we may wonder that the use of combined therapies simultaneously targeting AR and glycolysis-related proteins may be an attractive strategy to improve outcomes in PCa treatment.

In conclusion, besides their direct influence over growth of prostate cells, androgens should be viewed as modulators of glycolytic metabolism and lactate export, which may represent a relevant aspect driven development and progression of PCa.

Funding

This work was partially supported by the Pluriannual Programme of Portuguese Foundation for Science and Technology (FCT) under Program COMPETE (PEst-OE/SAU/UI0709/2014). PF Oliveira was financed by FCT under the program Ciência 2008. Cátia Vaz, Ricardo Marques and Marco Alves were funded by FCT fellowships (SFRH/BD/70316/2010, SFRH/BD/66875/2009 and SRFH/BPD/80451/2011, respectively).

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Chapter 7

Regucalcin is an androgen-target gene in the rat prostate modulating cell-cycle and apoptotic pathways

This chapter was published in:

Vaz, C.V., Maia, C.J., Marques, R., Gomes, I.M., Correia, S., Alves, M.G., Cavaco, J.E., Oliveira, P.F., Socorro, S. Regucalcin is an androgen-target gene in the rat prostate modulating cell-cycle and apoptotic pathways. *Prostate*. 2014; 74(12): p. 1189-98.

Regucalcin is an androgen-target gene in the rat prostate modulating cell-cycle and apoptotic pathways

Abstract

Background. Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein underexpressed in prostate adenocarcinoma comparatively to non-neoplastic prostate or benign prostate hyperplasia cases. Moreover, RGN expression is negatively associated with the cellular differentiation of prostate adenocarcinoma, suggesting that loss of RGN may be associated with tumor onset and progression. However, the RGN actions over the control of prostate cell growth have not been investigated.

Methods. Androgens are implicated in the promotion of prostate cell proliferation, thus we studied the *in vivo* effect of androgens on RGN expression in rat prostate. The role of RGN modulating cell proliferation and apoptotic pathways in rat prostate was investigated using transgenic animals (Tg-RGN) overexpressing the protein.

Results. *In vivo* stimulation with 5 α -dihydrotestosterone (DHT) down-regulated RGN expression in rat prostate. Cell proliferation index and prostate weight were reduced in Tg-RGN which was concomitant with altered expression of cell-cycle regulators. Tg-RGN presented diminished expression of the oncogene H-ras and increased expression of cell-cycle inhibitor p21. Levels of anti-apoptotic Bcl-2, as well as the Bcl-2/Bax protein ratio were increased in prostates overexpressing RGN. Both caspase-3 expression and enzyme activity were decreased in the prostates of Tg-RGN.

Conclusions. Overexpression of RGN resulted in inhibition of cell proliferation and apoptotic pathways, which demonstrated its role maintaining prostate growth balance. Thus, deregulation of RGN expression may be an important event favouring the development of prostate cancer. Moreover, the DHT effect down-regulating RGN expression in rat prostate highlighted for the importance of this protein in prostatic physiology.

Keywords: androgen; apoptosis; proliferation; prostate; regucalcin.

Introduction

Regucalcin (RGN) was initially described as a Ca^{2+} -binding protein that regulates the activity of Ca^{2+} pumps in plasma membrane, endoplasmic reticulum and mitochondria, playing an important role in intracellular Ca^{2+} homeostasis [1]. Recently, it was demonstrated that RGN is widely distributed in male reproductive tract, including rodent and human prostate [2-4]. Moreover, our previous study have linked the RGN protein with human prostate cancer showing its diminished expression in prostate adenocarcinoma comparatively to non-neoplastic prostate or benign prostate hyperplasia cases [2]. Also RGN immunoreactivity was negatively associated with the cellular differentiation of primary prostate tumors [2], suggesting that loss of RGN may be a relevant aspect in tumor onset and progression.

On the other hand, prostate carcinogenesis is intimately dependent of both androgens and Ca^{2+} signalling mechanisms [5, 6] and we also demonstrated by *in vitro* stimulation that RGN is an androgen-target gene in LNCaP prostate cancer cells [2]. Altogether, our prior findings highlighted for the importance of RGN in prostate pathophysiology, but the mechanisms underlying its actions need to be clarified. The present work aims to study the role of RGN regulating prostate cell growth. As a starting point we sought to confirm that androgens regulate the *in vivo* expression of RGN in rat prostate. Making use of a transgenic animal model overexpressing RGN the role of RGN modulating cell proliferation and apoptotic pathways in rat prostate was investigated.

Material and methods

Animals and tissue collection

Wild-type male rats (*Rattus norvegicus*) of Wistar and Sprague Dawley strains were obtained from Charles River (Barcelona, Spain). Sprague Dawley transgenic rats overexpressing RGN (Tg-RGN) were originally generated by Yamaguchi M by oocyte transgene pronuclear injection [7] and were purchased from Japan SLC (Hamamatsu, Japan) that commercializes the strain. Wistar rats were used for the hormonal stimulation experiment while wild-type animals of Sprague Dawley strain served as controls in the analysis using Tg-RGN. In all cases three month old adult rats were used.

Animals were housed under a 12 h light, 12 h dark cycle, with food and water available *ad libitum* and handled in compliance with the NIH guidelines and the European Union rules for the care and handling of laboratory animals (Directive 2010\63\EU) during the course of all experiments. All rats were euthanized under anesthesia (Clorketam 1000, Vetoquinol, Lure, France) and whole prostates were removed and weighted. Right or left dorsolateral lobe of the prostate, including ventral, dorsal, and lateral prostate [8] were either frozen in liquid nitrogen for RNA/protein extraction or fixed in 4% paraformaldehyde for histological processing.

Hormonal stimulation

Wistar male rats (n=16) were orchietomized under anesthesia (Clorketam 1000, Vetoquinol, Lure, France) and, five days after surgery, received daily intraperitoneal injections (for 5 days) of either 5 α -dihydrotestosterone (DHT (Sigma-Aldrich, St.Louis, USA), 500 $\mu\text{g}/\text{Kg}.\text{day}$) or vehicle alone (physiologic serum/ethanol 30%). Another group of intact animals was treated daily with vehicle alone (n=8). Sprague Dawley wild-type and Tg-RGN rats were orchietomized and treated with DHT (Sigma-Aldrich) following the same experimental procedures and hormone dose (n=8).

RNA isolation and cDNA synthesis

Total RNA was extracted from rat prostate tissues using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. In order to assess the quantity and integrity of total RNA, its optical density was determined (Pharmacia Biotech, Ultrospec 3000, Denmark) and RNA extracts were analyzed by agarose gel electrophoresis.

cDNA was synthesized from 1 µg of total RNA which was denatured for 5 min at 65 °C with 500 µM deoxynucleotide triphosphates (GE Healthcare, Buckinghamshire, UK) and 250 ng of random primers (Invitrogen, Karlsruhe LMA, Germany). Mixtures were incubated for 2 min at 37 °C with reverse transcriptase buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂ and 0.1 M DTT), 40 U of RNaseOUT (Invitrogen) and 10 mM DTT (Invitrogen). 200 U of M-MLV RT (Invitrogen) was added and incubations proceeded during 10 min at 25 °C, followed by 50 min at 37 °C. The reaction was stopped by heating at 70 °C for 15 min and synthesized cDNA was stored at -20 °C until further use.

Quantitative RT-PCR (qPCR)

Analysis of the expression of RGN, AbZIP and cell-cycle and apoptosis related genes (Table 7.1) in rat prostate was performed by qPCR using indicated specific primers and cycling conditions. B-Actin (sense: 5' ATGGTGGGTATGATGCAG3'; antisense: 5' CAATGCCGTGTTCAATGG3'), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sense: 5' GTTCAACGGCACAGTCAA3'; antisense: 5' CTCAGCACCCAGCATCACC3') and cyclophilin A (CypA) (sense: 5' CAAGACTGAGTGGCTGGATGG3'; antisense: 5' GCCCGCAAGTCAAAGAAATTAGAG3') were used as internal controls for normalization of expression of interest target genes. Reactions were carried out in an iQ5 system (Bio-Rad, Hercules, USA) and efficiency of the amplifications was determined for all primer sets using serial dilutions of cDNA (1, 1:10 and 1:100). PCR conditions and reagents concentrations were previously optimized and specificity of the amplicons was determined by melting curves. Each reaction consisted of Maxima™ SYBR Green/Fluorescein qPCR Master Mix (Fermentas, Burlington, Canada), sense and anti-sense primers (500 nM for RGN, H-ras, CypA; 300 nM for all other genes) and 1 µl of cDNA in a final volume of 20 µl. Reaction conditions comprised 5 min denaturation at 95°C, followed by 35 cycles at 95 °C for 10 seconds, a specific annealing temperature for each gene (Table I) for 30 seconds and 72 °C for 10 seconds. Samples were run in triplicate in each PCR assay. Normalized expression values were calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta Ct}$ [9].

Table 7.1. Oligonucleotides sequences, amplicon size and annealing temperature of qPCR reactions.

Gene	Sequence (5'- 3')	AT (°C)	Amplificon Size (bp)
RGN	Sense: GGA GGA GGC ATC AAA GTG	60	137
	Antisense: CAA TGG TGG CAA CAT AGC		
AbZIP	Sense: TCT ATG AAG TTG TCT ATG AGG	58	170
	Antisense: AGG CAG GAT GTG TCT ATG		
p53	Sense: CTG CCC ACC ACA GCG ACA GG	60	471
	Antisense: AGG AGC CAG GCC GTC ACC AT		
P21	Sense: GTT CCT TGC CAC TTC TTA C	53	103
	Antisense: ACT GCT TCA CTG TCA TCC		
Chk2	Sense: ATG AAG GAA GAT GGT CTA AGC	53	168
	Antisense: TGG TGG AGG AAC TGG ATG		
Cdk1	Sense: GGT TGA CAT CTG GAG GAT AG	53	115
	Antisense: GCC ACA CTT CGT TGT TAG G		
Myc	Sense: AAG AAC AAG ATG ATG AGG AAG	53	143
	Antisense: GTG CTG GTG AGT AGA GAC		
H-ras	Sense: AGA CCC GGC AGG GTG TGG AG	60	278
	Antisense: CCG GGA CGG GCA CAA AGG AC		
Bax	Sense: CGC GTG GTT GCC CTC TTC TAC TTT	60	124
	Antisense: CAA GCA GCC GCT CAC GGA GGA		
Bcl-2	Sense: GGG CTA CGA GTG GGA TAC	53	63
	Antisense: AGG CTG GAA GGA GAA GAT G		
Caspase-9	Sense: TGC AGG GTA CGC CTT GTG CG	60	130
	Antisense: CCT GAT CCC GCC GAG ACC CA		
Caspase-3	Sense: AGG CCT GCC GAG GTA CAG AGC	60	255
	Antisense: CCG TGG CCA CCT TCC GCT TA		

Abbreviation: AT: annealing temperature; bp; base pairs.

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded prostate tissue sections (5 µm) were deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was carried out in a heated citrate bath (10 mM, pH 6) for 30 min. Slides were allowed to cool, washed for 5 min in water and rinsed in phosphate buffered saline (PBS, 0.01 M, pH 7.4) with Tween 20. Sections were permeabilized with 0.1% Triton X-100 for 15 min and endogenous peroxidases were inactivated by a 3% hydrogen peroxide solution. Non-specific protein binding was eliminated by incubation with PBS containing 1% (w/v) BSA and 0.3 M glycine for 30 min at room temperature (RT). Sections were washed with PBS and incubated overnight at 4° C with a rabbit anti-RGN antibody (1:50, SML-ROI001-EX, COSMOBIO CO., LTD., Tokyo, Japan), followed by 1 h incubation with a biotinylated goat anti-rabbit IgG (1:20, Sigma-Aldrich), at RT. After incubation for 30 min with ExtrAvidin Peroxidase (Sigma-Aldrich) diluted 1:400 in PBA and incubation with DAB (Dako, Lisbon, Portugal), immunoreactivity of RGN was detected. Sections were slightly counterstained with Harris' hematoxylin (Merck, Darmstad, Germany), dehydrated, cleared, and mounted with Entellan® neu mounting media (Merck).

Specificity of the staining was assessed by the omission of primary antibody. The preparations were observed in the Axio Imager A1 microscope (Carl Zeiss, Gottingen, Germany).

Western blot (WB)

Total proteins were extracted from rat prostates using RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 8 and 1 mM EDTA) supplemented with protease inhibitors cocktail. Protein concentration was determined by the Bradford assay (Bio-Rad) and approximately 75 µg of protein extracts were resolved by SDS-PAGE on 12% gels and electrotransferred to a PVDF membrane (GE Healthcare). Membranes were incubated overnight at 4 °C with mouse anti-RGN (1:1000, ab81721, Abcam, Cambridge, United Kingdom), rabbit anti-Bax (1:4000, no. 2772, Cell Signaling Technology, MA, USA), rabbit anti-cleaved caspase-9 (1:1000, no. 9507, Cell Signaling Technology), mouse anti-cleaved caspase-8 (1:1000, no. 9746, Cell Signaling Technology) or rabbit anti-Bcl-2 (1:1000, no. 2876, Cell Signaling Technology). A mouse anti-β-actin (1:1000, A5441, Sigma-Aldrich) was used for normalization of protein expression. The membranes were then incubated for 1 h with Goat anti-rabbit IgG-AP (1:5000, Santa Cruz Biotechnology, CA, USA) or goat anti-mouse IgG-AP (1:5000, Santa Cruz Biotechnology), used as secondary antibodies. At the end, membranes were incubated with ECF substrate (GE Healthcare) for 3 min, and visualized on the Molecular Imager FX Pro plus Multimager (Bio-Rad).

Caspase-3 activity assay

The activity of caspase-3 was assessed determining the cleavage of a colorimetric substrate. Briefly, proteins (50 µg) were incubated with a reaction buffer (25 mM HEPES, pH 7.5, 0.1% CHAPS, 10% sucrose and 10 mM DTT) and 100 µM of caspase-3 substrate (Ac-DEVD-pNA) for 2 h at 37° C. The caspase-3-like activity was determined after cleavage of the labeled substrate by detection of the chromophore p-nitroaniline, measured spectrophotometrically at 405 nm. The method was calibrated with known concentrations of p-nitroanilide.

Ki67 fluorescent immunohistochemistry

Formalin-fixed, paraffin-embedded prostate tissue sections (5 µm) were deparaffinized with xylene and rehydrated through a series of graded ethanol. Antigen retrieval was carried out in a heated citrate bath (10 mM, pH 6) for 30 min. Slides were allowed to cool, washed for 5 min in water and rinsed in PBS (0.01 M, pH 7.4) with Tween 20. Following additional PBS washes for 5 min, sections were permeabilized with 0.1% Triton X-100 for 15 min. Non-specific protein binding was eliminated by incubation with PBS containing 1% (w/v) BSA and 0.3 M glycine for 1h at RT. Sections were then washed with PBS and incubated overnight at 4° C with rabbit anti-Ki67 (1:50, no. 16667, Abcam). Incubation with the Alexa fluor 546 goat anti-rabbit IgG secondary antibody (1:500, Invitrogen) was performed for 1h at RT. Specificity of immunostaining was assessed by omission of primary antibody. For cell nuclei staining, sections were incubated with Hoechst 33342 (15 µg/ml, Invitrogen) for 5 min, washed with

PBS for 10 min and mounted in Dako fluorescent mounting medium (Dako). The preparations were observed in the Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, NY, USA). The proliferation index was estimated by counting the number of Ki67-positive cells and Hoechst stained nuclei in twenty randomly selected 40x magnification fields for each section. The ratio between the number of Ki67 stained cells and total number of nuclei was calculated.

Statistical analysis

The statistical significance of differences in mRNA and protein expression among experimental groups was assessed by Student's t-test or by ANOVA, followed by the Tukey test. Significant differences were considered when $P < 0.05(*)$, $P < 0.01(**)$ or $P < 0.001(***)$. All experimental data are shown as mean $\pm$ SEM.

Results

Effect of DHT on regucalcin expression in rat prostate

In order to confirm RGN as an androgen-responsive gene in rat prostate *in vivo*, we investigated the effect of DHT on RGN expression using the following experimental groups: intact animals, orchietomized (ORCHX), and ORCHX receiving DHT treatment (500µg/Kg.day) for 5 days. As expected, a reduction of prostate weight (approximately 85%, Figure 7.1C) was observed in ORCHX animals relatively to intact controls demonstrating the success of surgical procedures. DHT treatment of ORCHX rats restored the expression of AbZip, a known androgen responsive gene [10], to the levels of intact animals (Figure 7.1D), which indicated the effectiveness of hormonal treatment.

The responsiveness of RGN mRNA and protein expression to DHT stimulation was analyzed by qPCR and WB, respectively (Figure 7.1A and Figure 7.1B). A significant increase of RGN expression (2.6-fold for mRNA and 1.4-fold for protein, $P < 0.05$) was observed in ORCHX animals in comparison with intact controls. DHT treatment of ORCHX animals reduced both RGN mRNA and protein expression to levels similar to those observed in intact animals (Figure 7.1A and Figure 7.1B).

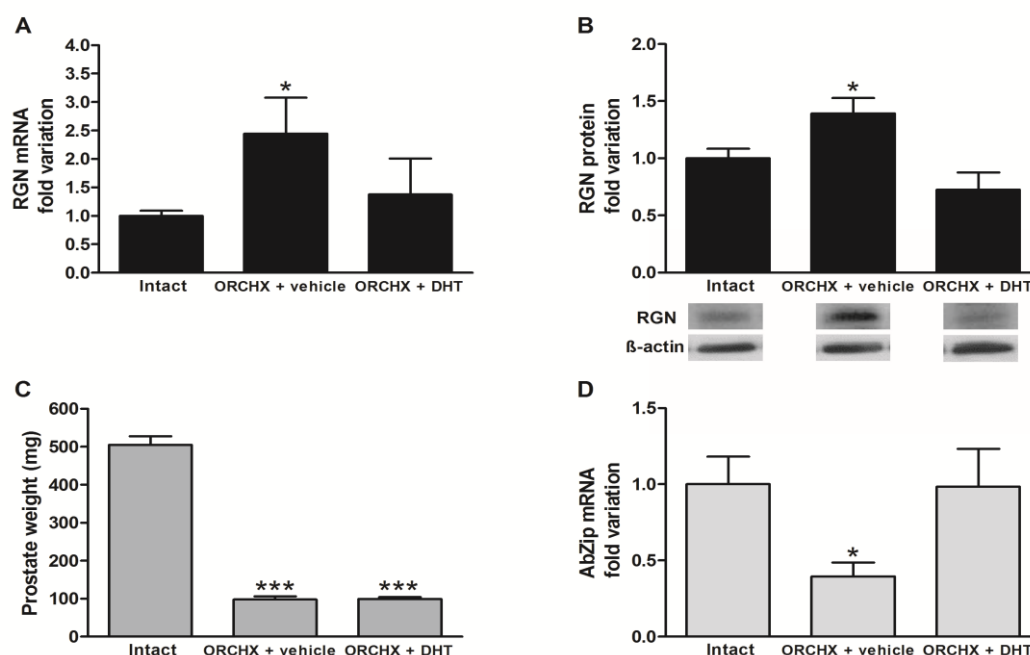


Figure 7.1. Effect of DHT on RGN expression in rat dorsolateral prostate. Three months old ORCHX animals received i.p. injections of vehicle (ORCHX+vehicle) or DHT (ORCHX+DHT, 500 µg/Kg.day) for 5 days. Intact group, are not surgically intervened animals receiving vehicle only. (A) mRNA expression of RGN determined by qPCR after normalization with β -actin and GAPDH housekeeping genes. (B) RGN protein relative expression determined by WB analysis upon normalization with β -actin; representative immunoblots are shown. (C) Variation of prostate weight among experimental groups. (D) qPCR results for mRNA expression of Abzip, an androgen-responsive-gene [10] used as experimental control. Results are expressed as fold-variation relatively to intact animals. Error bars indicate mean $\pm$ SEM ($n \geq 8$). * $P < 0.05$; *** $P < 0.001$.

Proliferation index in the rat prostate under overexpression of regucalcin

As a starting point, the RGN overexpression in the prostates of transgenic rats was demonstrated by means of qPCR, WB and IHC analysis. A 2-fold increased expression of RGN mRNA ($P < 0.05$, Figure 7.2A), as well as augmented protein expression ($P < 0.01$, Figure 7.2B), were found in the prostates of Tg-RGN when compared with their wild-type counterparts. IHC results also showed enhanced staining of RGN in the prostates of Tg-RGN animals (Figure 7.2C).

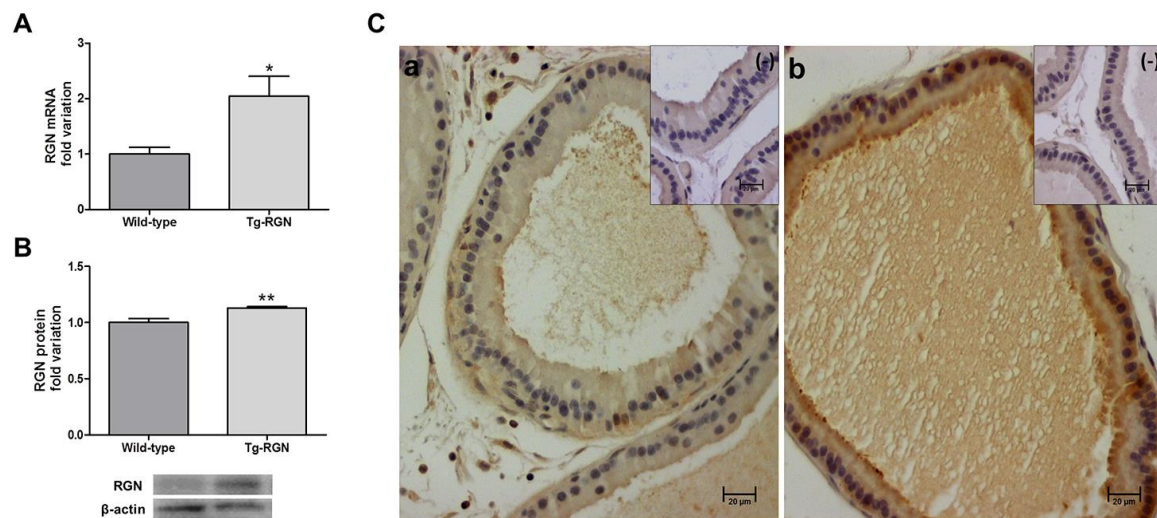


Figure 7.2. Overexpression of RGN in the dorsolateral prostate of 3 month old Tg-RGN in comparison with wild-type animals. (A) mRNA expression levels determined by qPCR after normalization with CypA and GAPDH housekeeping genes. (B) Protein expression levels determined by WB analysis upon normalization with β -actin. Results are expressed as fold-variation relatively to the wild-type group. Error bars indicate mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$. (C) Representative images of RGN immunohistochemical staining in prostates of wild-type (a) and Tg-RGN animals (b). Negative controls obtained by omission of the primary antibody are provided as insert panels (-).

Ki67 fluorescent immunohistochemistry was used to assess the cell proliferation index in the prostates of Tg-RGN comparatively with wild-type controls. The number of Ki67-stained cells relatively to total cells is 64% decreased in prostates of Tg-RGN when compared with control animals ($P < 0.01$, Figure 7.3A). Also, a diminution in prostate weight was observed in Tg-RGN relatively to wild-type animals ($P < 0.05$, Figure 7.3C).

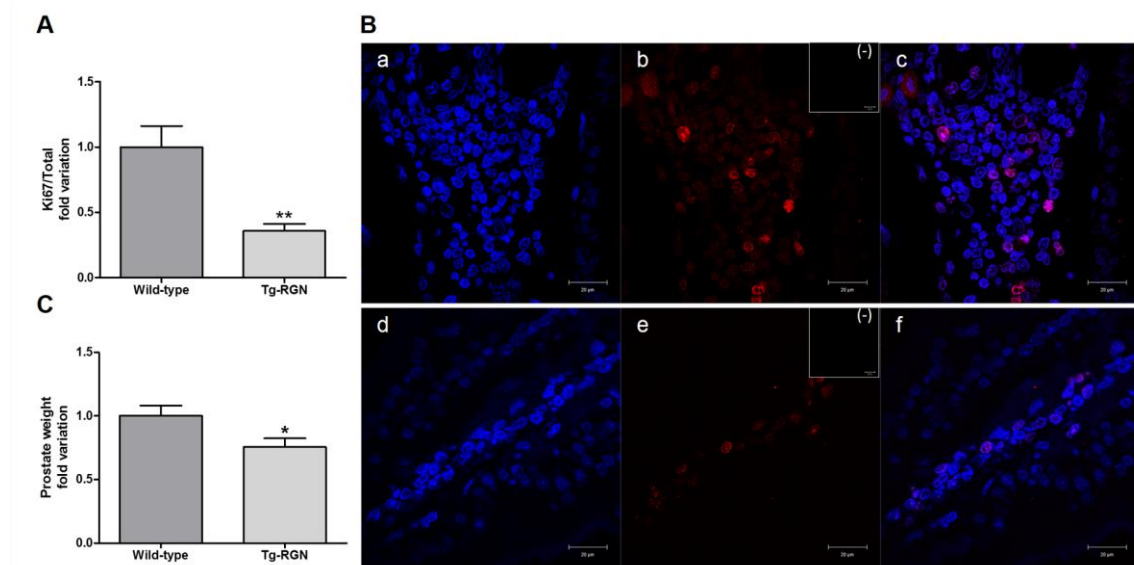


Figure 7.3. Proliferation in the dorsolateral prostate of 3 month old Tg-RGN in comparison with wild-type animals determined by Ki67 fluorescent immunohistochemistry. (A) Percentage of Ki67 positive cells relatively to total cells. (B) Representative images of Hoechst stained nuclei, Ki67 immunofluorescence and corresponding merged images in wild-type (a, b and c) and Tg-RGN animals (d, e and f). Negative controls for Ki67 obtained by omission of the primary antibody are provided as insert panels (-). (C) Prostate weight in Tg-RGN and wild-type animals. Results are expressed as fold-variation relatively to the wild-type group. Error bars indicate mean $\pm$ SEM ($n \geq 4$). * $P < 0.05$; ** $P < 0.01$.

Differences in prostate cells proliferation between Tg-RGN and wild-type are maintained under conditions of DHT-stimulation after orchietomy ($P < 0.05$, Figure 7.4).

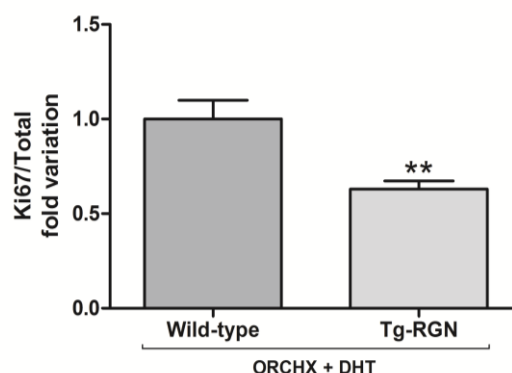


Figure 7.4. Proliferation in the dorsolateral prostate of 3 month old Tg-RGN and wild-type animals after orchietomy and DHT replacement (ORCHX+DHT) for 5 days (500 $\mu\text{g/Kg.day}$). Proliferation was determined by Ki67 fluorescent immunohistochemistry and the Ki67/Total ratio correspond to the percentage of Ki67 positive cells relatively to total cells. Results are expressed as fold-variation relatively to the wild-type group. Error bars indicate mean $\pm$ SEM ($n \geq 5$). ** $P < 0.01$.

Cell-cycle and apoptosis regulators in the prostate of rats overexpressing regucalcin

To determine the influence of RGN on cell-cycle and apoptosis pathways we studied the expression of target regulators of these processes in Tg-RGN animals in comparison with their wild-type counterparts.

Tg-RGN rats presented diminished mRNA expression of the oncogene, H-ras, and the tumor suppressor gene, p53, in comparison with the wild-type group (0.7- and 0.5-fold respectively, $P < 0.05$ and $P < 0.01$, Figure 7.5A). The expression of p21, a cell-cycle inhibitor, was increased in Tg-RGN comparatively to wild-type controls (1.23-fold, $P < 0.05$, Figure 7.5A). No significant differences were found on the mRNA expression of other cell-cycle regulators, namely, Chk2 and Cdk1. Also the expression of the oncogene Myc has remained unchanged between Tg-RGN and wild-type animals (Figure 7.5A).

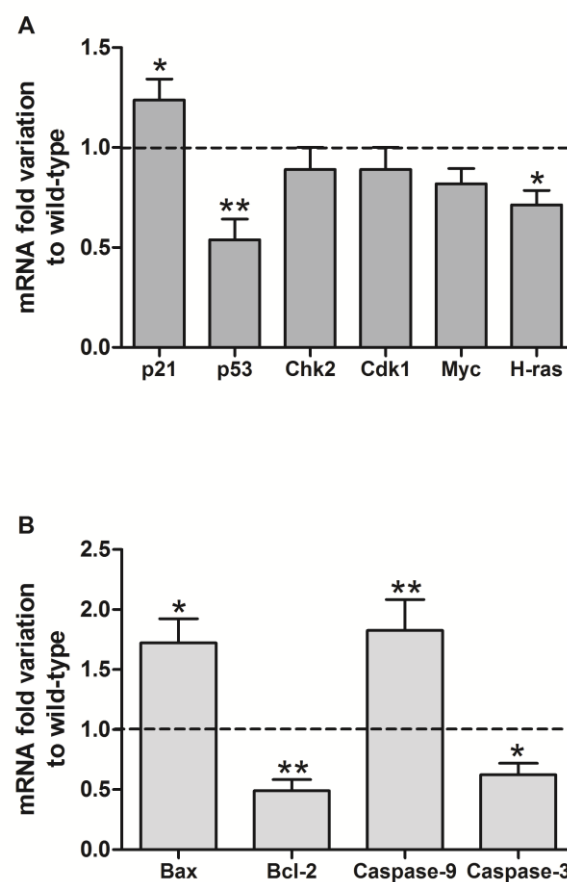


Figure 7.5. mRNA expression of cell-cycle (A) and apoptosis (B) regulators in the dorsolateral prostate of Tg-RGN in comparison with wild-type counterparts. In both groups, animals were 3 months old ($n \geq 7$). Expression was determined by qPCR after normalization with CypA and GAPDH housekeeping genes. Results are expressed as fold-variation relatively to the wild-type group, represented by the dashed line. Error bars indicate mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$.

Concerning apoptosis target genes, we verified that mRNA and protein levels of pro-apoptotic factor Bax are increased in the prostate of Tg-RGN relatively to wild-type animals (1.7- and 1.2-fold variation, respectively, $P < 0.05$; Figure 7.5B and Figure 7.6A). Although a decrease on mRNA levels was observed (0.5-fold, $P < 0.01$, Figure 7.5B), the levels of anti-

apoptotic Bcl-2 protein were increased in rat prostate overexpressing RGN (2,5-fold variation, $P < 0.05$, Figure 7.6A). Moreover, calculation of Bcl-2/Bax protein ratio (Figure 7.6B) demonstrated an increase in this parameter by 2.9-fold in Tg-RGN animals ($P < 0.05$). In terms of expression of caspases, it was observed a reduction of caspase-8 protein in Tg-RGN animals comparatively to their wild-type counterparts (0.8-fold variation, $P < 0.05$, Figure 7.6A). Although caspase-9 mRNA was increased in Tg-RGN (1.8-fold variation to wild-type, $P < 0.01$, Figure 7.5B), no differences were detected at protein level (Figure 7.6A).

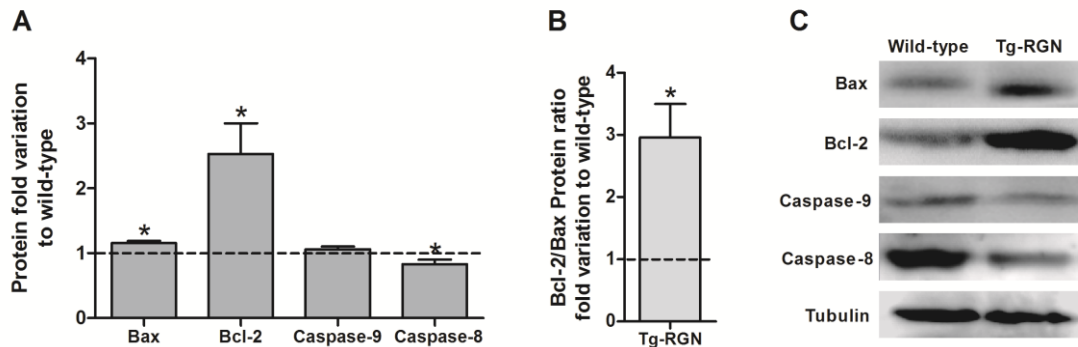


Figure 7.6. Protein levels of apoptosis regulators (A) and Bcl-2/Bax protein ratio (B) in the dorsolateral prostate of Tg-RGN determined by WB analysis after normalization with β -actin. Animals were 3 months old ($n \geq 7$). (C) Representative immunoblots displaying differences between Tg-RGN and wild-type animals are shown. Results are expressed as fold-variation relatively to the wild-type group, represented by the dashed line. Error bars indicate mean $\pm$ SEM. * $P < 0.05$.

Finally, we demonstrated that caspase-3 activity is reduced in about 40% in Tg-RGN ($P < 0.05$, Figure 7.7), which is concomitant with a diminution of mRNA transcript levels (0.6-fold, Figure 7.5B).

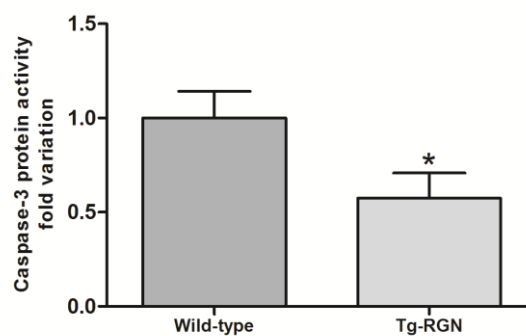


Figure 7.7. Caspase-3 activity in the dorsolateral prostate of Tg-RGN in comparison with wild-type animals. Results are expressed as fold-variation relatively to wild-type group. Error bars indicate mean $\pm$ SEM ($n \geq 7$). * $P < 0.05$.

Discussion

Androgens and Ca^{2+} are recognized as having a crucial role in prostate development and maintenance, controlling target points of diverse signalling pathways involved in both physiological and pathophysiological conditions (reviewed by [5, 6]). Our recent studies have identified the Ca^{2+} -binding protein RGN as an androgen-target gene in male reproductive tract, namely in seminiferous tubules cultured *ex vivo* [3] and in prostate cell lines [2]. In this study, we investigated the androgenic regulation of RGN expression *in vivo* by DHT stimulation after orchiectomy. ORCHX animals displayed a significant increase of RGN mRNA and protein expression, which was reverted to levels of intact animals by DHT treatment (Figures 7.1A and 7.1B). These results are concordant with those previously described in LNCaP prostate cancer cells [2] where DHT treatment induced a reduction in RGN expression, and confirmed that also *in vivo* androgens down-regulate RGN levels in prostate cells.

DHT is the main androgen responsible for the differentiation and survival of the prostate gland, as well as for the maintenance of differentiated functions in the mature tissue [11]. Androgenic effects are mainly exerted through the activation of the classical intracellular androgen receptor, which acts as transcription factor regulating the transcription of target genes [12] and, in consequence, influencing the cell protein network. Most of the androgen-sensitive signaling networks have been coupled to proliferation of prostate cells [13] and differential levels of androgens have been associated with prostate growth and emergence of benign prostate hyperplasia or prostate cancer [14]. The fact that DHT regulates RGN levels in prostate tissues strongly points this protein as an important target in the regulation of prostatic physiology.

Interestingly, our research group demonstrated that human cases of prostate cancer display a diminished expression of RGN protein, which was correlated with tumor differentiation [2]. Loss of RGN expression also has been associated with other cancer cases, such as breast cancer [2] and hepatocellular carcinoma (HCC) [15, 16], supporting the idea that RGN's diminished expression may be a selective event for malignant transformation and cancer progression.

Cancer onset and development is intimately associated with a disequilibrium in tissue homeostasis due to deregulation of cell death and proliferation pathways [17] and it has been suggested that RGN may play a role in the control of cell proliferation and apoptosis [18, 19]. Thus, we decided to investigate the influence of RGN on cell-cycle and apoptosis pathways in prostatic tissues under conditions of protein overexpression *in vivo*.

Although Tg-RGN and wild-type controls do not display differences in prostate histology (Figure 7.2C), Ki67 immunofluorescence results demonstrated a sharp reduction of proliferation index in the prostate of Tg-RGN animals comparatively to wild-type controls (Figure 7.3A), which is supported by the observed diminished weight of prostates of Tg-RGN animals (Figure 7.3C). Moreover, the reduced proliferation in Tg-RGN was accompanied by altered expression of cell-cycle target regulators. Our results demonstrated that the expression of cell-cycle regulators, Myc, Chk2 and Cdk1, remained unchanged between the

wild-type and Tg-RGN groups (Figure 7.5A), as was similarly described in rat cloned hepatoma or kidney cells transfected with RGN [20, 21]. However, considering the tumor suppressor gene p53, although it has been shown to be increased in hepatoma cells overexpressing RGN [22], a significant diminished expression was found in the prostate of Tg-RGN relatively to wild-type group (Figure 7.5A). Nevertheless, the expression of its transcription target p21, which is an inhibitor of CDKs and responsible for cell-cycle arrest, was increased in Tg-RGN animals (Figure 7.5A), in agreement with the observed diminished proliferation of prostate cells under conditions of RGN overexpression (Figure 7.3). Yamaguchi & Daimon [21] also reported an increased expression of p21 in response to RGN overexpression in cancerous liver cells, which was concomitant with suppression of cell proliferation. We also showed that the expression of the oncogene H-ras is diminished in the prostate of Tg-RGN animals relatively to wild-type controls (Figure 7.5A), which is in concordance with observations in hepatoma cells cultured *in vitro* [22].

The present study also showed that prostate cell proliferation after orchiectomy and androgen-replacement is restricted under conditions of RGN overexpression (Figure 7.4). Our findings indicate that RGN is involved in the suppression of prostate cell proliferation by modulating the expression of tumor suppressor genes and/or oncogenes, such as p21 and H-ras, which could be a relevant mechanism in the control of prostate cancer initiation and/or progression. Previous work of our research group and others, showing a diminished expression of RGN in cancer cases comparatively to non-cancerous tissues [2, 15, 16] greatly support the significance of those observations, indicating that loss of RGN expression may be contributing for tumor development.

Apoptosis can be induced by internal and external stimuli involving intrinsic and extrinsic pathways that converge to the activation of specific proteases, caspases [23]. Caspase-3 is one of the latest activated caspases, designated as effector caspases, which then cleave important cellular substrates that irreversibly lead to apoptosis and thus, activation of caspase-3 is considered an end-point of apoptosis [23]. Caspase-9 and caspase-8 are both initiator caspases that lead to caspase-3 activation and apoptosis, respectively, by the intrinsic and extrinsic pathways [23, 24]. Analysis of caspase-3 activity showed that it diminishes with RGN overexpression, as evident by the 40% reduction observed in the prostate of Tg-RGN relatively to their wild-type counterparts (Figure 7.7). Although caspase-9 mRNA expression was increased in Tg-RGN group, this result was not confirmed at protein levels, which seem to be maintained in both Tg-RGN and wild-type animals (Figure 7.6A). Therefore, diminished activity of caspase-3 may be consequence of decreased protein levels of caspase-8 (Figure 7.6A). Also the Bcl-2/Bax ratio, which is associated with apoptosis commitment [24], is increased in Tg-RGN (Figure 7.6B). This result, as well as the diminished caspase-3 activity, suggests a decrease of apoptosis in the prostate of Tg-RGN animals. Other studies also demonstrated that RGN overexpression has a preventive effect on cell death and apoptosis induced by noxious stimuli with enhanced expression of the anti-apoptotic Bcl-2 protein [19, 25, 26].

Altogether the data obtained in the present study, showing that RGN is involved in suppression of both cell proliferation and apoptosis, and that its levels are controlled by androgens, indicate an important role for RGN in prostate cell physiology maintaining the appropriate tissue homeostasis and, thus, controlling prostate growth. Other examples of proteins controlling both biological processes are available in the literature, which depends of their expression levels and/or localization in distinct cellular compartments [27, 28]. Curiously, RGN is a protein detected in the cytoplasm and also in the nucleus [2] and further research is required to disclose the factors that determine its sub-cellular localization, as well as the consequences over the control of cell-cycle and apoptosis.

In conclusion, the present study establishes RGN as an important target in prostatic physiology, which could play a crucial role preventing tumor development by suppression of cell-cycle. The down-regulatory effect of DHT on RGN expression also suggests that the androgenic effects promoting prostate cell growth and proliferation may be linked to the decrease of RGN levels in prostatic cells. On the other hand, RGN overexpression seems also to suppress apoptosis, which underscores its significance maintaining the cell homeostasis and controlling prostate growth and tumorigenesis.

Funding

This work was partially supported by Portuguese Foundation for Science and Technology (FCT) under Program COMPETE (PEst-C/SAU/UI0709/2011). Cátia V. Vaz (SFRH/BD/70316/2010), Ricardo Marques (SFRH/BD/66875/2009), Sara Correia (SFRH/BD/60945/2009) and Marco G. Alves (SFRH/BPD/80451/2011) are recipient of fellowships from FCT. Pedro F. Oliveira was financed by FCT under the program Ciência 2008 and PTDC/QUI-BIQ/121446/2010.

Acknowledgements

The authors thank Catarina Ferreira for technical assistance in tissue processing and histological sections preparation.

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Chapter 8

Effect of extracellular calcium on regucalcin expression and cell viability in neoplastic and non-neoplastic human prostate cells

This chapter was published in:

Vaz, C.V., Rodrigues, D.B., Socorro, S., Maia, C.J. Effect of extracellular calcium on regucalcin expression and cell viability in neoplastic and non-neoplastic human prostate cells. *Biochimica et biophysica acta*. 2015; doi: 10.1016/j.bbamcr.2015.07.006.

Effect of extracellular calcium on regucalcin expression and cell viability in neoplastic and non-neoplastic human prostate cells

Abstract

Extracellular calcium (Ca^{2+}_o) and its receptor, the Ca^{2+} -sensing receptor (CaSR), play an important role in prostate physiology, and it has been shown that the deregulation of Ca^{2+} homeostasis and the overexpression of CaSR are involved in prostate cancer (PCa). Regucalcin (RGN), a Ca^{2+} -binding protein that plays a relevant role in intracellular Ca^{2+} homeostasis, was identified as an under-expressed protein in human PCa. Moreover, RGN was associated with suppression of cell proliferation, suggesting that the loss of RGN may favour development and progression of PCa. This work aims to unveil the role of Ca^{2+}_o on RGN expression and viability of non-neoplastic (PNT1A) and neoplastic (LNCaP) prostate cell lines. It was demonstrated that Ca^{2+}_o up-regulates RGN expression in both cell lines, but important differences were found between cells for dose- and time-responses to Ca^{2+}_o treatment. It was also shown that high $[\text{Ca}^{2+}]_o$ triggers different effects on cell proliferation of neoplastic and non-neoplastic PCa cells, which seems to be related with RGN expression levels. This suggests the involvement of RGN in the regulation of cell proliferation in response to Ca^{2+}_o treatment. Also, the effect of Ca^{2+}_o on CaSR expression seems to be dependent of RGN expression, which is strengthened by the fact that RGN-knockdown in PNT1A cells increases the CaSR expression, whereas transgenic rats overexpressing RGN exhibit low levels of CaSR. Overall, our results highlighted the importance of RGN as a regulatory protein in Ca^{2+} -dependent signaling pathways and its deregulation of RGN expression by Ca^{2+}_o may contribute for onset and progression of PCa.

Keywords: calcium; CaSR; LNCaP; PNT1A; prostate cancer; regucalcin.

Introduction

Prostate cancer (PCa) is the most common male cancer concerning the number of new cases diagnosed, and represents one of the major causes of cancer death in men worldwide [1, 2]. Apart from androgens, it is well known the role of calcium (Ca^{2+}) in the physiology of prostate, and the deregulation of Ca^{2+} homeostasis has been intimately associated with development and progression of PCa [3]. Over recent years, a growing body of evidence has indicated that extracellular Ca^{2+} (Ca^{2+}_o), mainly through the activity of extracellular calcium-sensing receptor (CaSR), regulates cell proliferation, differentiation, and apoptosis [4]. In addition, several studies have pointed out that the activation of CaSR is associated with the onset of PCa metastasis [5, 6], which strongly highlights the importance of Ca^{2+} signaling and homeostasis in the pathophysiology of prostate gland.

Regucalcin (RGN) is a Ca^{2+} -binding protein that plays an important role in maintaining intracellular Ca^{2+} homeostasis by regulating the activity of Ca^{2+} pumps in plasma membrane

and endoplasmic reticulum, and the uptake of Ca^{2+} by mitochondria [7, 8]. Also, it was reported that overexpression of RGN suppresses the expression of CaSR [9]. Noteworthy RGN was described as an underexpressed protein in different types of human cancer cell lines and tissues, namely, in the hepatocellular carcinoma (HCC), and in breast and prostate cancers [10-13]. Our previous work also demonstrated that loss of RGN expression in human PCa cases is negatively correlated with the cellular differentiation of adenocarcinoma [12]. Moreover, it was recently shown that RGN decreases cell proliferation in rat prostate through regulating the expression of cell cycle regulators [14], which supports that reduced levels of RGN may be implicated in development and progression of prostate tumors. Regarding the regulation of RGN expression, it was demonstrated that DHT down-regulates RGN levels in rat prostate and in human androgen-sensitive LNCaP PCa cells. However, the myriad of factors that maintain RGN expression levels in human prostate cells is not entirely known. Considering the importance of Ca^{2+} in prostate pathophysiology, and the role of RGN in Ca^{2+} homeostasis and cell proliferation, this work aims to determine the effect of $[\text{Ca}^{2+}]_o$ on the expression of RGN, and on the viability of human non-neoplastic and neoplastic prostate cells. In addition, the expression of CaSR in human non-neoplastic and neoplastic prostate cells, as well as, in the prostate of transgenic animals overexpressing RGN (Tg-RGN), was also investigated.

Materials and methods

Cell lines and experimental layout

The human PCa cell lines, LNCaP and PC3, and the immortalized non-neoplastic human prostate epithelial cell line, PNT1A, were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). All prostate cell lines were cultured in DMEM 21068 (Gibco, Paisley, UK), supplemented with 1.8 mM CaCl_2 , (VWR, Leuven, Belgium) 10% FBS (Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (Invitrogen) at 37 °C in an incubator equilibrated with 5% CO_2 .

Cells were grown up to 60% confluence, and 24 h before stimulation, culture medium was changed to a Ca^{2+} free medium, DMEM 21068 (Gibco) containing 10% FBS (Biochrom) and 1% penicillin/streptomycin (Invitrogen). Cultured cells were maintained for additional 24 h and then exposed to different concentrations of CaCl_2 (0, 1, 1.8, 3, 5 and 10 mM) for 0, 1.5, 3, 6, 12, 24, 48 and/or 72 h. During the time-course experiment for different Ca^{2+} stimuli, cells were harvested and stored at -80 °C until RNA and protein extraction, or cell viability assays were performed.

For RGN gene knockdown in PNT1A and LNCaP cells, several transfection conditions were previously optimized. Cells were seeded in six plate multiwells and at 40% confluence were transfected with 10 nM of a small interfering RNA (siRNA) targeting the RGN (s17374) (Ambion, USA) and 5 μL of lipofectamine 2000 (Invitrogen, USA) for 24 h in Opti-MEM medium (Invitrogen), following manufacturer's instructions. As control for RGN specific targeting, a Scramble siRNA sequence (AM4635) (Ambion, USA) was used. The medium was replaced to

DMEM 21068 (Gibco) supplemented with 1.8 mM CaCl₂ (VWR), 10% FBS (Biochrom) and 1% penicillin/streptomycin (Invitrogen), and the RGN gene knockdown was confirmed by Western blot after 36 h of incubation with the siRNA.

Animals

Wild-type male rats (*Rattus norvegicus*) of Sprague Dawley strain were obtained from Charles River (Barcelona, Spain). Sprague Dawley transgenic rats overexpressing RGN (Tg-RGN) were originally generated by Yamaguchi M by oocyte transgene pronuclear injection [15] and were purchased from Japan SLC (Hamamatsu, Japan) that commercializes the strain. All rats were euthanized under anesthesia (Clorketam 1000, Vetoquinol, Lure, France) and whole prostates were removed and frozen in liquid nitrogen for protein extraction.

Total RNA extraction and cDNA synthesis

Total RNA from human prostate cell lines was extracted using the TRI reagent (Sigma-Aldrich, Saint Louis, Missouri, USA) according to the manufacturer's instructions. The quantity and quality of extracted RNA were assessed by spectrophotometry (Pharmacia Biotech, Ultrospec 3000, Cambridge, England) and agarose gel electrophoresis, respectively. For cDNA synthesis, 1 µg of total RNA was denatured for 5 min at 70 °C together with 250 ng of random hexamer primers (Invitrogen), and reverse transcription was carried out at 37°C for 60 min in a 20 µl reaction containing reverse transcriptase buffer, 1 µl of dNTP Mix (10 mM each; GE Healthcare, Buckinghamshire, UK) and 200 U of M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). The reaction was stopped at 75 °C for 15 min and synthesized cDNA was stored at -20 °C until further use.

Quantitative RT-PCR (qPCR)

Quantitative Real-Time (qPCR) was carried out to evaluate the mRNA expression of RGN in human prostate cell lines (PNT1A and LNCaP), in response to Ca²⁺ stimuli. Specific primers for human RGN (Sense: 5'-GCAAGTACAGCGAGTGACC-3'; antisense: 5'-TTCCCATCATTGAAGCGATTG-3') amplified a fragment of 177 bp. Human beta-2-microglobulin (B2M) (Sense: 5'-ATGAGTATGCCTGCCGTGTG-3'; antisense: 5'-CAAACCTCCATGATGCTGCTTAC-3') and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sense: 5'-CGCCAGCCGAGCCACATC-3'; antisense: 5'-CGCCCAATACGACCAAATCCG-3') primers were used as internal controls to normalize RGN expression. qPCR reactions were carried out in IQ5 system (Bio-Rad, Hercules, USA) and the efficiency of amplifications was determined for all primer sets using serial dilutions of cDNA (1, 1:10 and 1:100). PCR conditions and reagents concentrations were previously optimized and the specificity of the amplicons was determined by melting curves analysis. For qPCR reactions, 1 µl of synthesized cDNA was used in a 20 µl reaction containing 10 µl Maxima™ SYBR Green/Fluorescein qPCR Master Mix (Fermentas, Burlington, Canada) and sense and antisense primers (200 nM for RGN and 300 nM for B₂M and GAPDH). Reaction conditions comprised 5 min denaturation at 95 °C, followed by

35 cycles of 95 °C for 10 seconds, a specific annealing temperature of 60 °C for 30 seconds and 72 °C for 10 seconds. Samples were run in triplicate in each PCR assay. Normalized expression values were calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta C_t}$ [16].

Western Blot (WB)

Total proteins were extracted from human prostate cell lines (PNT1A and LNCaP) and rat prostate using RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 8 and 1 mM EDTA) supplemented with protease inhibitors cocktail. Protein concentration was determined by the Bradford assay (Bio-Rad) and approximately 60 µg of cell and tissue protein extracts was resolved by SDS-PAGE on 12 % gels and electrotransferred to a PVDF membrane (GE Healthcare). Membranes were incubated overnight at 4 °C with mouse anti-RGN (1:1000, ab81721, Abcam, Cambridge, United Kingdom) or mouse anti-calcium sensing receptor clone HL1499 (1:1000, C0493, Sigma-Aldrich) primary antibodies. A mouse anti-β-actin antibody (1:5000, A5441, Sigma-Aldrich) was used for normalization of protein expression. Membranes were incubated for 1 h with goat anti-mouse IgG + IgM-AP (1:5000, NIF1316; GE Healthcare) used as secondary antibody. Finally, membranes were incubated with ECF substrate (GE Healthcare) for 3 min, and visualized on the Molecular Imager FX Pro plus Multimager (Bio-Rad). Band densities were obtained according to standard methods using the Quantity One Software (Bio-Rad) and normalized by division with the respective β-actin band density.

Immunofluorescence microscopy

PNT1A and LNCaP cells were fixed with 4 % paraformaldehyde for 10 min and permeabilized with 0.1 % Triton X-100 during 5 min. A blocking step was performed by incubating cells with 20 % FBS in phosphate buffer saline (PBS) containing 0.1 % tween®-20 (PBST) for 1 h at room temperature, and then, cells were incubated with anti-RGN antibody (1:50, ab81721, Abcam) for 1 h at room temperature. The Alexa fluor 488 and 546 conjugated goat anti-mouse IgG (Invitrogen) were used as secondary antibodies. The specificity of the staining was accessed by omission of the primary antibody. Cell nuclei were stained with Hoechst 33342 (10 mg/mL, Invitrogen) for 10 minutes. Lamellae were washed and mounted onto microscope slides with Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany).

Cell viability assay

PNT1A and LNCaP cells were seeded into 96-well plates at a density of 1.5×10^3 cell per well in 100 µl of cell medium culture, and exposed to 0, 1, 1.8, 3, 5 and 10 mM of CaCl₂ for 0, 6, 12, 24, 48 and 72h, as described above. Cell viability was determined by a colorimetric (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfo-phenyl)-2M) tetrazolium (MTS) assay using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega,

Madison, WI, USA). The conversion of MTS compound to the colored formazan product was detected by measurement of absorbance at 490 nm in a Microplate Reader (Biochrom, Anthos 2020). The relative number of viable cells in each experimental condition was calculated by normalizing the absorbance to that of the corresponding control. All experiments were repeated at least three times, and each experiment was carried out in hexaplicated.

Statistical analysis

The statistical significance of differences in mRNA and protein expression and cell viability among experimental groups was assessed by Student's t-test or by ANOVA followed by the Bonferroni test. Significant differences were considered when $P < 0.05$ (*,#), $P < 0.01$ (**, ##) or $P < 0.001$ (***, ###). All experimental data are shown as mean $\pm$ SEM. The Graphpad Prism 5.0 program (GraphPad Software, San Diego, CA, USA) was used for this analysis.

Results

Regucalcin expression is significantly diminished in human prostate cancer cell lines

The mRNA and protein expression of RGN in neoplastic (LNCaP and PC3) and non-neoplastic (PNT1A) human prostate cells, was compared by means of qPCR and Western blot analysis (Figure 8.1). The expression of RGN mRNA (Figure 8.1A) and protein (Figure 8.1B) was significantly decreased in both PCa cell lines comparatively with PNT1A cells (LNCaP cells, 0.24- and 0.79-fold variation for mRNA and protein, respectively; PC3 cells, 0.46- and 0.83-fold variation for mRNA and protein, respectively).

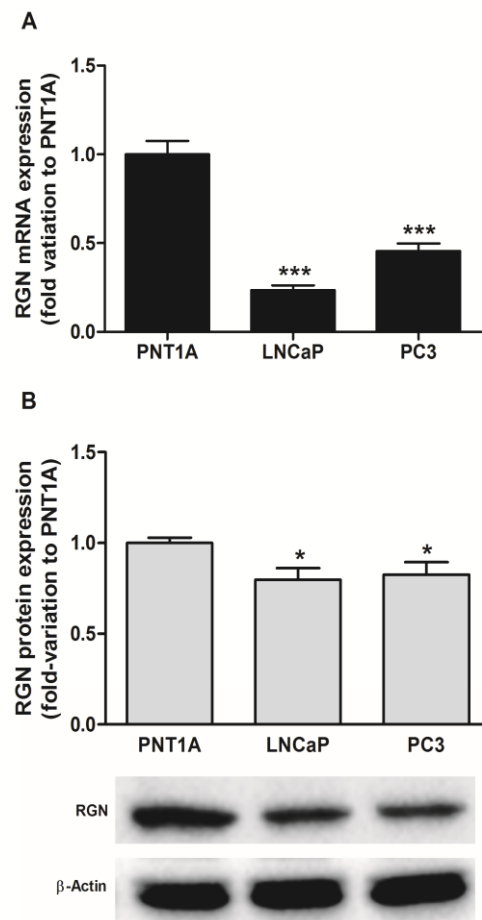


Figure 8.1. RGN mRNA (A) and protein (B) expression in non-neoplastic (PNT1A) and neoplastic (LNCaP and PC3) human prostate cell lines. mRNA expression was determined by qPCR after normalization with β_2M and GAPDH housekeeping genes. Protein expression was analyzed by Western Blot and normalized with β -actin. All results are expressed as fold-variation relatively to PNT1A. Error bars indicate mean $\pm$ SEM, n=5. * $P < 0.05$; *** $P < 0.001$. Representative immunoblots are shown in bottom panel.

Extracellular calcium exerts a biphasic effect on regucalcin expression

Considering the role of RGN in Ca^{2+} homeostasis, we have hypothesized that Ca^{2+}_o regulates RGN gene expression, and that this effect may differ between neoplastic and non-neoplastic prostate cells. Taking into account that RGN is an androgen-responsive gene [12, 14, 17], we

have used the androgen-sensitive LNCaP and the non-neoplastic PNT1A cell lines to evaluate the responsiveness of RGN expression to Ca^{2+}_o .

qPCR results demonstrated that Ca^{2+}_o exerts a biphasic effect on RGN mRNA expression in both cell lines. In PNT1A cells, it was observed an up-regulation of RGN after 1.5 h of stimulation with 1, 1.8 and 3 mM Ca^{2+}_o (respectively, 1.48-, 1.6- and 1.71-fold variation relatively to PNT1A untreated cells; Figure 8.2A).

Regarding LNCaP cells, a significant up-regulation was only visible after 3 h of treatment with 1.8 mM Ca^{2+}_o (1.7-fold variation relatively to LNCaP untreated cells; Figure 8.2B). Thereafter, the levels of RGN were reduced along the experimental time-course, and a significant down-regulation of RGN mRNA was observed in both cell lines after 12 h of stimulation with $[\text{Ca}^{2+}]_o$ of 1, 1.8, 3, 5 and 10 mM (respectively, 0.40-, 0.36-, 0.39-, 0.71- and 0.53-fold variation in PNT1A cells relatively to control untreated group; and 0.68-, 0.70-, 0.64-, 0.70- and 0.59-fold variation in LNCaP cells relatively to control untreated group).

Considering 1.8 mM as the physiological $[\text{Ca}^{2+}]_o$, the effects of low (1 mM) and high (5 and 10 mM) doses of Ca^{2+}_o were also compared to 1.8 mM Ca^{2+}_o . In PNT1A cells, the results showed a diminished expression of RGN after 1.5 h of stimulation with 5 and 10 mM (respectively, 43% and 48% of reduction in PNT1A cells relatively to 1.8 mM Ca^{2+}_o). However, a significant increase in RGN levels was observed at 3 and 12 h after treatment with 5 mM Ca^{2+}_o (respectively, 36 and 49 % of reduction in PNT1A cells relatively to 1.8 mM Ca^{2+}_o). The treatment with 10 mM Ca^{2+}_o decreased the levels of RGN in PNT1A cells at 3 h (30 %), but increased at 12 h (32 %) relatively to 1.8 mM Ca^{2+}_o (Figure 8.2A).

In LNCaP cells, a significant reduction in RGN levels was observed with 3 mM Ca^{2+}_o at 3 and 6 h (respectively, 41 and 51% reduction relatively to 1.8 mM Ca^{2+}_o). Also, a significant reduction in RGN expression was observed after treatment of LNCaP cells with 5 and 10 mM Ca^{2+}_o (respectively, 39 and 50 % at 1.5 h; 49 and 69 % at 3 h; and 62 and 50 % at 6 h relatively to 1.8 mM Ca^{2+}_o). At 12 h of stimulation, no significant differences were perceived for the effect of 1, 3, 5 and 10 mM Ca^{2+}_o comparatively with the 1.8 mM Ca^{2+}_o . These results suggest a time and dose-dependent effect of Ca^{2+}_o on RGN expression in human prostate cells. Subsequently, the RGN protein expression in response to Ca^{2+} -stimulation was investigated by means of Western blot and immunofluorescence analysis. Ca^{2+}_o treatment for 1.5 h and 3 h was the experimental time-points selected for protein analysis, respectively, in PNT1A and LNCaP cells, as it corresponded to the increased expression of RGN mRNA relatively to untreated control group. Dose-response analysis for different $[\text{Ca}^{2+}]_o$ demonstrated that PNT1A cells treated with 1, 1.8 and 3 mM Ca^{2+}_o for 1.5 h show increased expression of RGN protein (respectively, 2.11-, 1.96- and 1.59-fold variation relatively to PNT1A untreated cells; Figure 8.2C), whereas no changes were observed in the experimental group treated with high $[\text{Ca}^{2+}]_o$.

In accordance with the results of mRNA expression, a significant reduction on RGN protein levels after treatment with 5 and 10 mM Ca^{2+}_o (respectively, 36 and 57 % in PNT1A cells relatively to 1.8 mM Ca^{2+}_o , Figure 8.2C) was also observed.

Also, LNCaP cells treated with 1 and 1.8 mM of Ca^{2+}_o for 3 h, displayed increased RGN protein expression (respectively, 1.26- and 1.45-fold variation relatively to LNCaP untreated cells; Figure 8.2D), whereas the expression of RGN is diminished in cells stimulated with 10 mM of Ca^{2+}_o (0.62-fold variation relatively to LNCaP untreated cells; Figure 8.2D). Comparing the effect of high doses (3, 5 and 10 mM Ca^{2+}_o) with the physiological dose, a significantly decreased protein expression of RGN was observed in LNCaP cells (respectively, 21, 41 and 57 % of reduction relatively to 1.8 mM Ca^{2+}_o , Figure 8.2D).

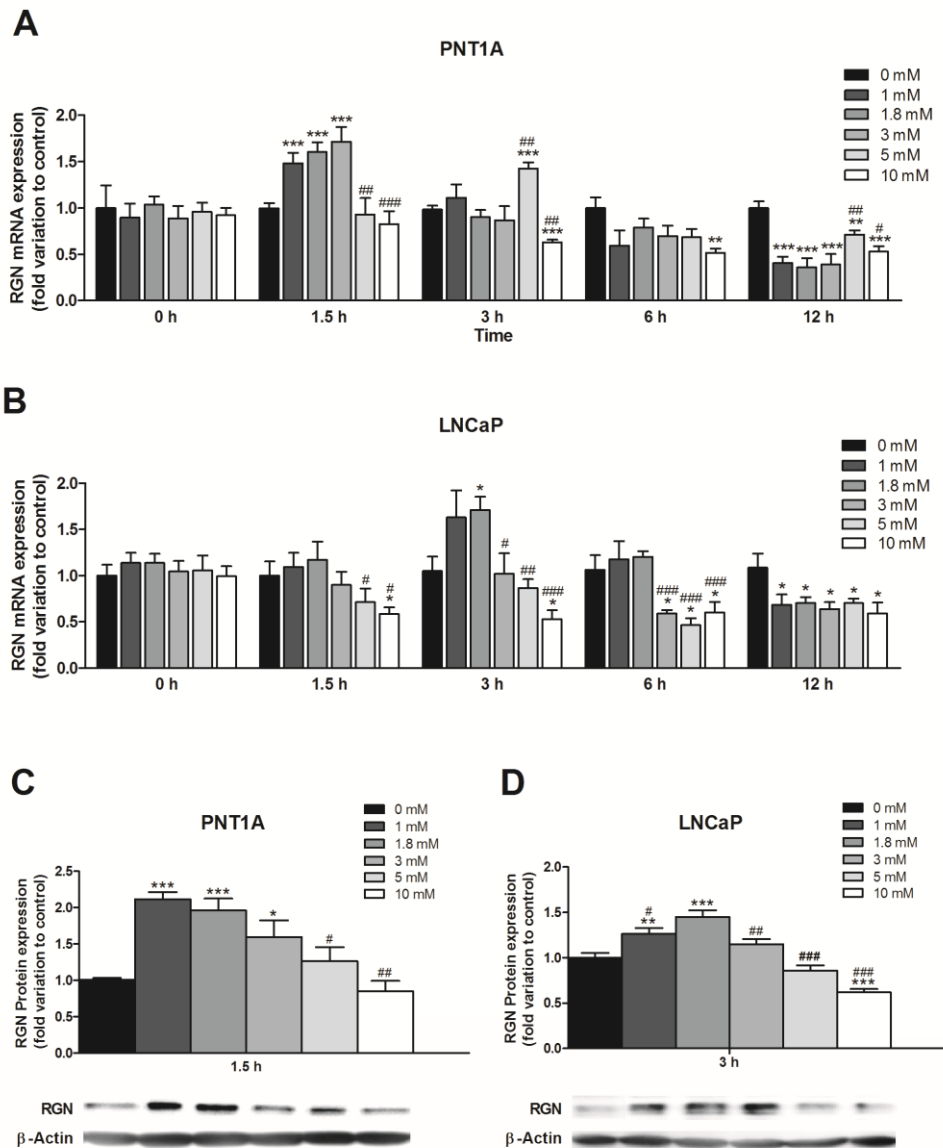


Figure 8.2. Effect of extracellular Ca^{2+} (Ca^{2+}_o) on RGN expression in PNT1A and LNCaP cells. mRNA expression of RGN (A, B) was determined by qPCR after normalization with $\beta_2\text{M}$ and GAPDH housekeeping genes. RGN protein expression (C, D) was analyzed by Western blot and normalized with β -actin. All results are expressed as fold-variation relatively to the control untreated group (0 mM Ca^{2+}_o). Error bars indicate mean $\pm$ SEM, $n=6$. *, # $P < 0.05$; **, ## $P < 0.01$; ***, ### $P < 0.001$ relatively to untreated cells or 1.8 mM Ca^{2+}_o , respectively. Representative immunoblots are shown in bottom panel.

Comparing the effect of Ca^{2+}_o between non-neoplastic and neoplastic human prostate cell lines, it is possible to infer that Ca^{2+}_o -induced up-regulation of RGN protein expression is more pronounced in PNT1A cells than in LNCaP cells. Immunofluorescence results (Figure 8.3) also showed enhanced staining of RGN in the cytoplasm of PNT1A and LNCaP cells treated with 1.8 mM Ca^{2+}_o for 1.5 h and 3 h, respectively. This enhancement also was evident in the nucleus of PNT1A cells.

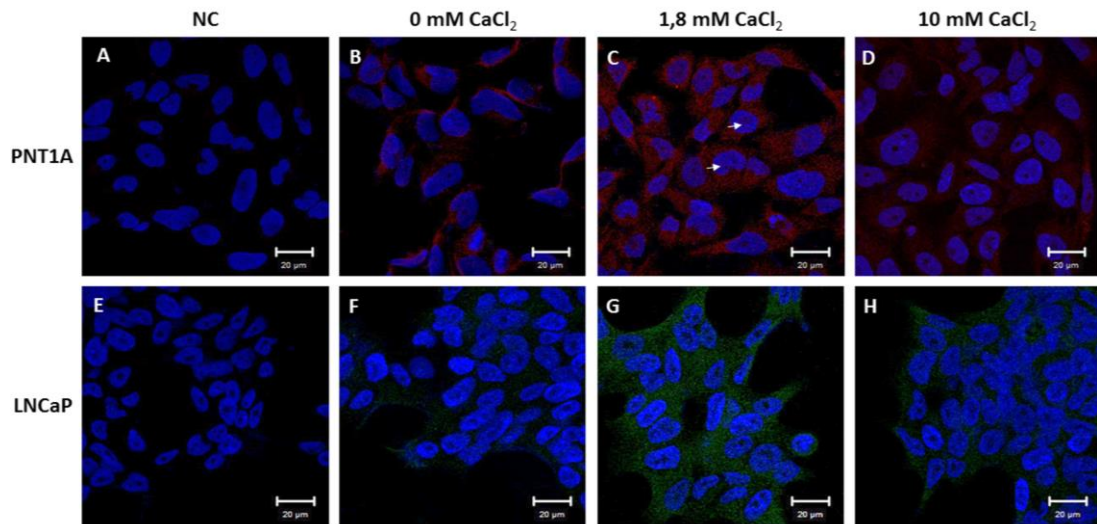


Figure 8.3. Representative confocal microscopy images showing the expression and subcellular localization of RGN in PNT1A (red) and LNCaP (green) cells treated with 0, 1.8 and 10 mM of Ca^{2+}_o for 1.5 h and 3 h, respectively. Nuclei are stained with Hoechst 33342 (blue). Negative controls (NC) for RGN were obtained by omission of the primary antibody. Co-localization of RGN and nucleus is shown by the inserted arrows.

Cell viability of prostate cells is dependent on extracellular calcium concentration

Considering that Ca^{2+}_o can affect cell growth rate, we evaluated the effect of several $[\text{Ca}^{2+}]_o$ on the viability of PNT1A (Figure 8.4A) and LNCaP (Figure 8.4B) cells for 72 h by means of MTS assay. PNT1A cells stimulated with 3, 5 and 10 mM of Ca^{2+}_o displayed diminished cell viability comparatively with the control untreated group (** $P < 0.01$ or *** $P < 0.001$) throughout the experimental time-course. For 48 h of stimulation with 1.8 mM Ca^{2+}_o , the percentage of viable PNT1A cells was enhanced when compared to control group, increasing even further thereafter. On the other hand, the viability of LNCaP cells after 48 h of treatment was stimulated with 1.8 mM or higher $[\text{Ca}^{2+}]_o$ relatively to untreated cells or cells treated with 1 mM of Ca^{2+}_o (*** $P < 0.001$).

Comparing the effect of high and low $[\text{Ca}^{2+}]_o$ on cell viability, it was observed a diminished viability in both cell lines when compared to the physiological dose of Ca^{2+}_o (1.8 mM, Figure 8.4).

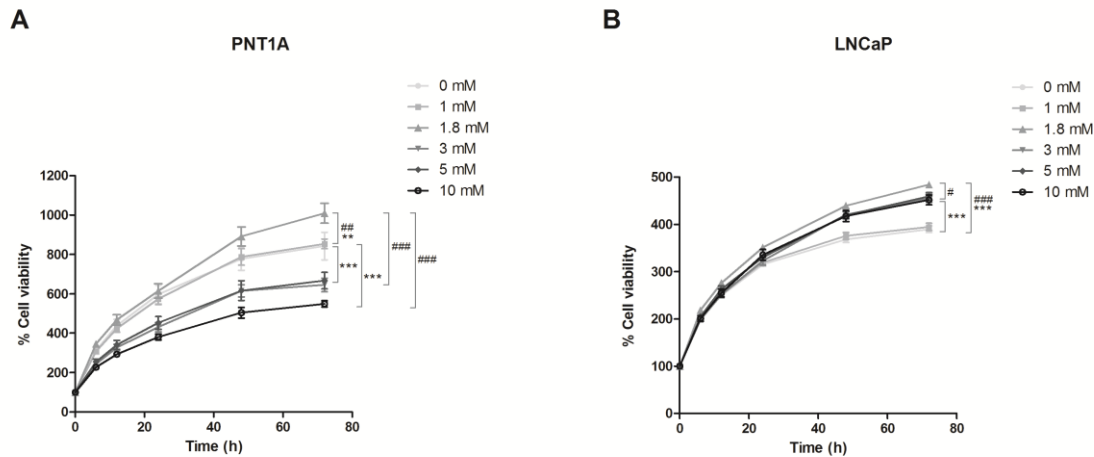


Figure 8.4. Effect of extracellular Ca^{2+} on the viability of PNT1A and LNCaP cells determined by the MTS assay. Error bars indicate mean $\pm$ SEM, $n=6$. # $P<0.05$ relatively to 1.8 mM Ca^{2+}_0 ; **, ## $P<0.01$ and ***, ### $P<0.001$ relatively to untreated cells or 1.8 mM Ca^{2+}_0 , respectively.

Extracellular calcium regulates the expression of calcium-sensing receptor

Even small changes in $[\text{Ca}^{2+}]_0$ are sensed by the G-protein coupled receptor CaSR, which upon ligand binding translates this information to intracellular signaling pathways [4]. So, we first analyzed the expression of CaSR protein in PNT1A and LNCaP cells by means of Western blot analysis. The obtained results demonstrated that LNCaP cells express higher levels of CaSR relatively to the non-neoplastic PNT1A cells (2.08-fold variation; Figure 8.5A). Considering the effect of $[\text{Ca}^{2+}]_0$ on the expression of RGN and viability of PNT1A and LNCaP cells, together with other reports indicating that up-regulation of RGN expression may influence the expression of CaSR [9], we decided to evaluate the effect of Ca^{2+}_0 on the expression of CaSR in these human prostate cell lines. The protein levels of CaSR in response to 1.8 mM Ca^{2+}_0 in PNT1A and LNCaP cells were determined by Western blot analysis. The results obtained showed that CaSR protein expression significantly decreased in PNT1A cells treated with 1.8 mM of Ca^{2+}_0 for 3 h and 6 h when compared with the control group (0.7-fold variation relatively to PNT1A untreated cells; Figure 8.5B). In what concerns LNCaP cells, no significant differences were observed on CaSR protein expression between the experimental groups (Figure 8.5C).

RGN down-regulates the expression of CaSR

In order to investigate the role of RGN on the regulation of CaSR, we analyzed the expression of CaSR protein in PNT1A and LNCaP cells after RGN knockdown. In addition, the expression of CaSR was compared between the prostate of Tg-RGN animals and their wild-type counterparts. As expected, the levels of RGN in PNT1A and LNCaP cells decreased after transfection with siRNA targeting RGN (respectively, 0.27 and 0.56-fold variation relatively to control cells, * $P<0.05$; Figure 8.5D and 8.5E). Western blot results demonstrated that RGN knockdown induces the expression of CaSR in PNT1A cells (1.21-fold variation relatively to control cells, * $P<0.05$; Figure 8.5D), but no differences were observed in LNCaP cells (Figure

8.5E). Accordingly, transgenic overexpression of RGN down-regulates CaSR levels. The prostates of Tg-RGN rats displayed diminished expression of CaSR protein relatively to the wild-type group (0.82-fold variation, * $P < 0.05$; Figure 8.5F). Taken together, these results indicate that RGN may control the expression levels of CaSR in prostate cells.

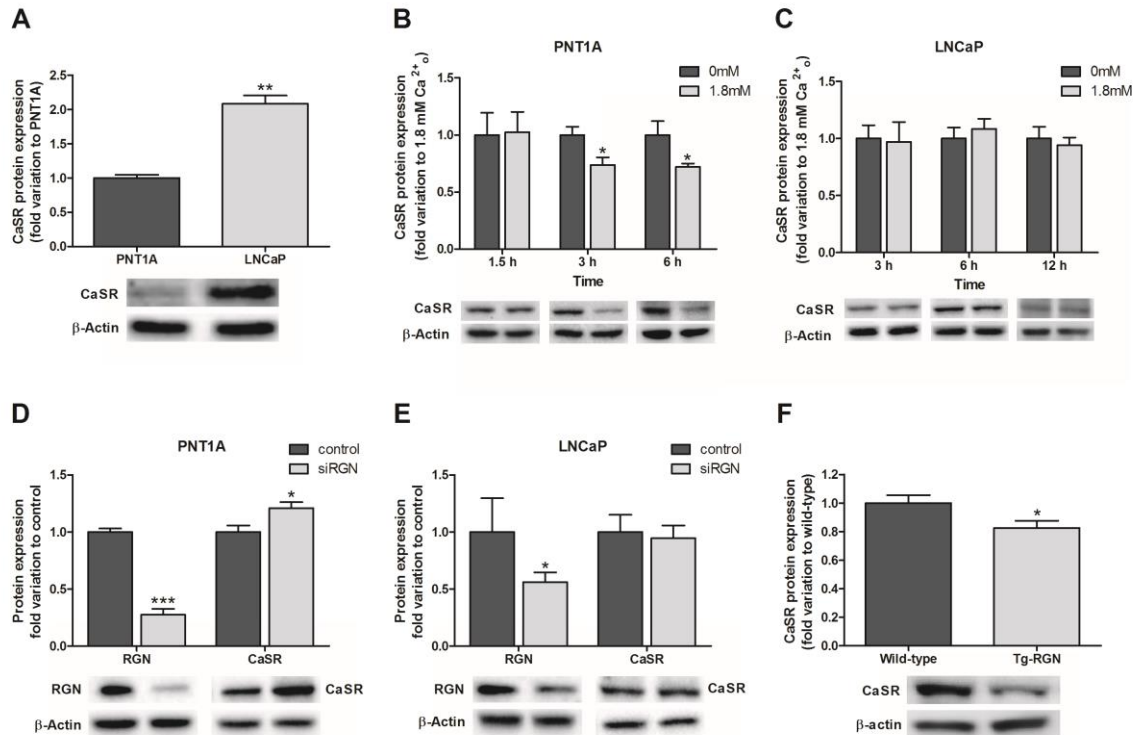


Figure 8.5. CaSR protein expression in human prostate cell lines (PNT1A and LNCaP) (A), and effect of 1.8 mM of extracellular Ca^{2+} on CaSR expression in PNT1A (B) and LNCaP (C) cells. RGN gene knockdown and its effect on CaSR expression in PNT1A (D) and LNCaP (E) cells, and CaSR protein expression in the prostate of transgenic rat overexpressing RGN (Tg-RGN) comparatively with their wild type counterparts (F). Results are expressed as fold-variation relatively to the control groups. Protein expression was analyzed by Western blot and normalized with β -actin. Representative immunoblots are shown. Error bars indicate mean $\pm$ SEM, $n=6$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Discussion

The onset and progression of PCa are intimately dependent on the regulation of different signaling pathways controlled by androgens and Ca^{2+} [3, 18], which also includes the influence of Ca^{2+} through the activity of CaSR. Although the mechanisms underlying Ca^{2+} actions in PCa are not completely elucidated, several studies have linked the CaSR, and also the RGN protein, with prostate carcinogenesis.

It has been demonstrated that loss of RGN expression is associated with progression of PCa [12] and that RGN attenuates proliferation of prostate cells [14], suggesting that this Ca^{2+} -binding protein may have a protective role against the development of prostate tumors. In this way, it is absolutely crucial to characterize the factors that control RGN expression in prostate cells under normal and pathological conditions. This work first intended to ascertain

the effect of Ca^{2+}_o on the expression of RGN in neoplastic and non-neoplastic prostate cell lines. As a starting point, the expression of RGN was analyzed in non-neoplastic, PNT1A cells, and in androgen-responsive and androgen non-responsive neoplastic cell lines, LNCaP and PC3, respectively. LNCaP and PC3 cells displayed lower levels of RGN in comparison with PNT1A cells, which is in agreement with other studies demonstrating that RGN is underexpressed in human PCa cases [12]. Also, the reduced levels of RGN in LNCaP cells are concordant with the previously described down-regulatory effect of DHT on RGN expression in PCa cells [12]. Nevertheless, other factors must be involved in the diminution of RGN levels in PCa, since androgen-insensitive PC3 cells also displayed reduced expression of RGN. Taking into account the present findings, and our previous data indicating RGN as an androgen-responsive gene [14, 19, 20], PNT1A and LNCaP cells were selected to investigate the role of Ca^{2+}_o in regulating the expression of RGN. The results obtained demonstrated that Ca^{2+}_o induced time- and dose-dependent effects on the expression of RGN. A physiological $[\text{Ca}^{2+}]_o$ (1.8 mM) promptly increased RGN mRNA and protein levels in PNT1A and LNCaP cells for 1.5 h and 3 h of stimulus, respectively. However, when cells were stimulated with higher $[\text{Ca}^{2+}]_o$ (3 mM and 5 mM) the effect on up-regulation of RGN expression was only observed in PNT1A cells, which indicates that the regulation of RGN expression by Ca^{2+}_o differs between non-neoplastic and neoplastic prostate cells. Others have described the regulation of RGN by Ca^{2+}_o in rat and mice liver, with RGN mRNA being markedly increased at 30 and 60 min after intraperitoneal administration of CaCl_2 [21, 22], or between 30 and 180 min following a single oral administration of CaCl_2 solution [23]. The stimulatory effect on RGN concentration was also described in the rat kidney cortex 60 to 120 min after of a single intraperitoneal administration of a CaCl_2 solution [24].

Interestingly, when PNT1A and LNCaP cells were treated for longer periods (6 to 12 h), the levels of RGN mRNA and protein were down-regulated relatively to untreated cells, suggesting that RGN may trigger a negative feedback in order to decrease its cell levels and avoid an over-stimulation by RGN. Considering that RGN has been involved in the regulation of Ca^{2+} -dependent intracellular signaling pathways [25], and the importance of Ca^{2+}_o in prostate cells, it is liable to speculate that the up-regulation of RGN expression in response to Ca^{2+}_o may play an important role in prostate physiology. In addition, it also should be highlighted the Ca^{2+}_o effects relatively to 1.8 mM Ca^{2+}_o , which is considered the physiological $[\text{Ca}^{2+}]_o$. The present results showed that extreme concentrations of Ca^{2+}_o , namely high levels, decrease the expression of RGN in both cell lines relatively to physiological $[\text{Ca}^{2+}]_o$. Therefore, it is liable to speculate that changes in serum $[\text{Ca}^{2+}]_o$ may decrease the expression of RGN, and consequently, favor the proliferation of prostate cells and tumor development and progression. Indeed, although there are contradictory results, some epidemiologic studies have found that higher levels of Ca^{2+}_o are associated with PCa risk [26, 27].

Our research group previously demonstrated that prostates from Tg-RGN rats present reduced cell proliferation underpinned by decreased expression of the oncogene H-Ras and increased levels of the cell cycle inhibitor p21 [14]. Although it has not been demonstrated

the role of RGN as a transcription regulator, the immunofluorescence results obtained herein showed increased accumulation of RGN in the nucleus, namely in PNT1A cells, suggesting that RGN may play a role in regulating the nuclear function. These results are corroborated by other studies, which also demonstrated the presence of RGN in the nucleus of several human cell types including prostate cells, and suggested that it may regulate gene expression [9, 12, 28].

Considering the available reports describing the effect of Ca^{2+}_o on cell proliferation [4], the present study also evaluated how distinct $[\text{Ca}^{2+}]_o$ influence the proliferation of PNT1A and LNCaP cells. Remarkably, the stimulation with high $[\text{Ca}^{2+}]_o$ (3, 5 and 10 mM) triggered different responses in non-neoplastic and neoplastic cell lines. In PNT1A cells, high $[\text{Ca}^{2+}]_o$ decreased cell viability comparatively with the control but the opposite effect was observed in LNCaP cells. Noteworthy, the perceived effects on cell proliferation followed concomitant responses on the expression of RGN, since it was always visible, except with 10 mM Ca^{2+}_o , an up-regulation of RGN expression in PNT1A cells, whereas in LNCaP cells this effect was only visible with physiological dose. Our results also showed that both cell lines presented decreased proliferation when treated with low or high concentrations of Ca^{2+}_o relatively to 1.8 mM Ca^{2+}_o . However, the diminished proliferation in response to high doses of Ca^{2+}_o is less evident in LNCaP cells, suggesting that cancer cells may become more sensitive to proliferation than normal cells in the presence of high concentrations of Ca^{2+}_o . Altogether, and even considering many other mechanisms involved in the action of Ca^{2+}_o , our results suggest that the up-regulation of RGN in response to physiological $[\text{Ca}^{2+}]_o$ may play an important role in protecting cells from proliferation. These observations are supported by previous findings demonstrating that overexpression of RGN suppresses cell proliferation in NRK52E and hepatoma H4-II-E cells, and in rat prostate by decreasing the expression of cell cycle regulators [14, 29-32]. Nevertheless, other studies are required to fully clarify the impact of RGN levels on the regulation of cell proliferation in neoplastic and non-neoplastic prostate cell lines.

It is well described that one of the mechanisms of Ca^{2+}_o action is through its cognate receptor, the CaSR [4]. It has been reported that Ca^{2+} and the CaSR are important players in tumorigenesis, and in PCa, the CaSR is even over-expressed [6]. Therefore, we compared the expression of CaSR between PNT1A and LNCaP cells. Our results showed that LNCaP cells display higher levels of CaSR than PNT1A cells, which is in accordance with previous findings describing a positive correlation between activation of CaSR and development of PCa [6]. We investigated the putative role of RGN controlling CaSR expression in PNT1A and LNCaP cells after treatment with 1.8 mM Ca^{2+}_o . Interestingly, a decreased expression of CaSR protein was only observed in PNT1A cells after 3h of treatment, but no significant changes were found in LNCaP cells up to 12h of treatment. Considering the high levels of RGN at 1.5 h after treatment with physiological $[\text{Ca}^{2+}]_o$ in PNT1A cells, these results suggest that the down-regulation of CaSR may occur due to the higher levels of RGN induced by this $[\text{Ca}^{2+}]_o$. Inversely, the reduced levels of RGN in LNCaP cells associated with their lower capacity to

increase RGN expression in response to $[Ca^{2+}]_0$ may explain the absence of effects on the expression of CaSR in LNCaP cells treated with 1.8 mM Ca^{2+}_0 . In an effort to support the hypothesis that RGN may be involved in the regulation of CaSR in prostate cells, we analyzed the expression of CaSR protein in PNT1A and LNCaP cells after RGN knockdown, and, also, in the prostate of Tg-RGN animals. The RGN gene knockdown induced the CaSR expression in PNT1A but not in LNCaP cells, suggesting that the normal role of RGN in down-regulating CaSR may be lost in cancer cells. The role of RGN in regulation of CaSR levels in normal prostate is strengthened by the fact that Tg-RGN animals displayed lower levels of CaSR in comparison with their wild type counterparts. These results are in accordance with the previously described in kidney cells, where RGN had a suppressive effect on the expression of proteins involved in intracellular Ca^{2+} -signaling pathways, including the CaSR [9]. Taken together, our results suggest that RGN may not only have an important role in the control of cell proliferation but also in desensitizing the signaling pathways triggered by CaSR. This is strongly supported by the histopathological findings in human PCa samples, which showed over-expression of CaSR and loss of RGN [12, 33].

In sum, it was first demonstrated that RGN expression is regulated by Ca^{2+}_0 , in non-neoplastic and neoplastic PCa cells. However, important differences on dose- and time-responses to $[Ca^{2+}]_0$ were observed between PNT1A and LNCaP cells, with PNT1A cells displaying more pronounced effects in the up-regulation of RGN by Ca^{2+}_0 . In addition, a physiological $[Ca^{2+}]_0$ down-regulated CaSR expression in non-neoplastic cells, an effect not observed in PCa cells, which plausibly could be linked to the differential response in RGN expression upon treatment Ca^{2+}_0 . These results strengthen the role of RGN in the modulation of intracellular Ca^{2+} -signaling pathways, and indicate that abnormalities in the $[Ca^{2+}]_0$ may deregulate RGN expression levels contributing for PCa onset and progression.

Funding

This work was partially supported by the Pluriannual Programme of Portuguese Foundation for Science and Technology (FCT) under Program COMPETE (PEst-OE/SAU/UI0709/2014). Cátia Vaz is funded by FCT fellowship (SFRH/BD/70316/2010).

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Chapter 9

Suppressed glycolytic metabolism in the prostate of transgenic rats overexpressing calcium-binding protein regucalcin underpins reduced cell proliferation

This chapter was accepted for publication after minor revisions:

Vaz, C.V., Marques, R.; Cardoso, H.J., Maia, C.J., Socorro, S. Suppressed glycolytic metabolism in the prostate of transgenic rats overexpressing calcium-binding protein regucalcin underpins reduced cell proliferation. 2015

Suppressed glycolytic metabolism in the prostate of transgenic rats overexpressing calcium-binding protein regucalcin underpins reduced cell proliferation

Abstract

Regucalcin (RGN) is a calcium-binding protein underexpressed in human prostate cancer cases, and it has been associated with the suppression of cell proliferation and the regulation of several metabolic pathways. On the other hand, it is known that the metabolic reprogramming with augmented glycolytic metabolism and enhanced proliferative capability is a characteristic of prostate cancer cells. The present study investigated the influence of RGN on the glycolytic metabolism of rat prostate by comparing transgenic adult animals overexpressing RGN (Tg-RGN) with their wild-type counterparts. Glucose consumption was significantly decreased in the prostate of Tg-RGN animals relatively to wild-type, and accompanied by the diminished expression of glucose transporter 3 and glycolytic enzyme phosphofructokinase. Also, prostates of Tg-RGN animals displayed lower lactate levels, which resulted from the diminished expression/activity of lactate dehydrogenase. The expression of the monocarboxylate transporter 4 responsible for the export of lactate to the extracellular space was also diminished with RGN overexpression. These results showed the effect of RGN in inhibiting the glycolytic metabolism in rat prostate, which was underpinned by a reduced cell proliferation index. The present findings also suggest that the loss of RGN may predispose to a hyper glycolytic profile and fostered proliferation of prostate cells.

Keywords: glucose; lactate; MCT4; proliferation; prostate; RGN.

Introduction

Regucalcin (RGN) is a protein with calcium (Ca^{2+})-binding ability, which regulates Ca^{2+} -pumping activity at plasma membrane, endoplasmic reticulum and mitochondria [1, 2]. Despite its known function in the maintenance of intracellular Ca^{2+} homeostasis [1-3], RGN seems to play an important role in tissue cell homeostasis by regulating the equilibrium between cell survival/proliferation and death [3-7]. Several *in vitro* and *in vivo* approaches have shown that RGN overexpression suppresses cell proliferation [4, 5, 8] and a reduced cell proliferation index was reported in the prostate of rats overexpressing RGN [4]. On the other hand, a down-regulated expression of RGN has been found in several types of human cancer and cell lines, including prostate cancer [5, 9, 10]. Moreover, we have found that the immunoreactivity of RGN in human prostate cancer cases was negatively associated with the cellular differentiation of prostate adenocarcinoma [10], linking this protein to the onset and progression of prostatic tumors.

Also, RGN seems to be a protein with multiple roles and facets [3], and its association with the control of cell metabolism also has been reported. A substantial body of evidence indicates that RGN influences diverse metabolic pathways such as glycolysis, oxidative phosphorylation, glycogenolysis and gluconeogenesis [11, 12]. The metabolic adaptation is widely recognized as an hallmark in cancer cell transformation and our research group demonstrated that alterations in the glycolytic metabolism, including high glucose consumption and lactate production, are associated with the progression of prostate cancer [13], and directly regulated by androgens [14]. Moreover, RGN was identified as an androgen target gene in human and rat prostate [4, 10]. This raises the curiosity about the influence of RGN on the glycolytic metabolism in prostate tissue. In the present study, we investigate glucose consumption and lactate production, as well as, the expression and activity of glycolytic metabolism-associated genes in the prostate of transgenic rats overexpressing RGN (Tg-RGN) and their wild-type counterparts. The proliferation index in the prostates of both groups was also evaluated.

Materials and methods

Animals

Sprague Dawley wild-type male rats (*Rattus norvegicus*) were obtained from Charles River (Barcelona, Spain), and Tg-RGN rats of Sprague Dawley strain were purchased from Japan SLC (Hamamatsu, Japan) that commercializes the strain. Tg-RGN animals were originally generated by Yamaguchi M by oocyte transgene pronuclear injection [15], and the overexpression of RGN in the prostate of these rats was previously demonstrated both at the mRNA and protein level [4]. Animals were handled in compliance with the NIH guidelines and the European Union rules for the care and handling of laboratory animals (Directive number 2010/63/EU). Three-month old animals were euthanized under anesthesia (Clorketam 1000, Vetoquinol, Lure, France), blood was collected for measurement of serum glucose and liver, brain, testis and whole prostates were removed. All tissues were used for metabolites extraction to quantify glucose and lactate content. Right or left dorsolateral lobe of the prostate, including ventral, dorsal, and lateral prostate, were frozen in liquid nitrogen for RNA/protein or metabolites extraction.

Glucose and lactate assays

Glucose concentration in the serum of Tg-RGN and wild-type animals was determined using a commercial Kit (Spinreact, Girona, Spain).

For determination of glucose and/or lactate concentration in the prostate, liver, brain and testis of wild-type and Tg-RGN rats, collected tissues were ground in a liquid nitrogen-cooled mortar and a methanol/chloroform/water extraction was used to simultaneously extract polar and apolar metabolites [16]. Briefly, 1 ml of cold-methanol and 500 μ l chloroform were added to the powdered samples. After thawing on ice, samples were vortexed for 60 s and

sonicated. 500 µl chloroform and 500 µl of ice-cold water were then added to each sample, which were vortexed and centrifuged at 10,000 g for 15 min at 4 °C. The upper layer containing water-soluble metabolites was collected, lyophilized and then diluted in sterile water for quantification of glucose and lactate concentrations using commercial kits (Spinreact).

Quantitative RT- PCR (qPCR)

qPCR was performed to evaluate the mRNA expression of glucose transporters (GLUTs, namely, GLUT1 and GLUT3), phosphofructokinase 1 (PFK1) and monocarboxylate transporter (MCT4) in the prostate of wild-type and Tg-RGN rats. Total RNA was extracted from rat prostates using TRI reagent (Sigma-Aldrich, St.Louis, USA) according to the manufacturer's instructions. The quantity and integrity of total RNA were evaluated as previously described [4], and 1 µg RNA was reverse transcribed in a final volume of 20 µl using the First Strand cDNA synthesis Kit (NZYtech, Lisboa, Portugal), following the manufacturer's instructions.

Primer sequences and cycling conditions used in qPCR amplification of metabolism-related genes are provided in Table 9.1. Rat B-Actin (sense: 5' ATGGTGGGTATGATGCAG3'; antisense: 5' CAATGCCGTGTTCAATGG3') and cyclophylin A (CypA) (sense: 5' CAAGACTGAGTGGCTGGATGG3'; antisense: 5' GCCCCAAGTCAAAG AAATTAGAG3') housekeeping genes were used as internal controls for normalization of gene expression. qPCR reactions were carried out in the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA) and the efficiency of amplification was determined for all primer sets using serial dilutions of cDNA (1, 1:10 and 1:100). PCR conditions and reagents concentrations were previously optimized and the specificity of the amplicons was determined by melting curve analysis. 1 µl of synthesized cDNA was used in each qPCR reaction of 20 µl containing 10 µl Maxima™ SYBR Green/Fluorescein qPCR Master Mix (Fermentas, Burlington, Canada) and sense and antisense primers (300 nM). Reaction conditions comprised 5 min denaturation at 95 °C, followed by 35 cycles of 95 °C for 10 seconds, a specific annealing temperature for each primer for 30 seconds and 72 °C for 10 seconds. Samples were run in triplicate in each qPCR assay. Normalized expression values were calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta Ct}$ [17].

Table 9.1. Oligonucleotide sequences and qPCR cycling condition

Gene	Sequence (5'-3')	Amplicon size (bp)	AT (°C)
GLUT1	Sense: CAGCCACTCTCCTATCTC Antisense: AGCCATTGTTCAATGG3'	155	50
GLUT3	Sense: GGTGTTTCGCTGTTACTGTTG Antisense: CTCGCTTGGTAGGTCTTCC	138	56
PFK1	Sense: CGCACCTTGAGCATAGAC Antisense: AGAGCAGCACAGTAGACC	172	56
MCT4	Sense: ACACTTAGGAGACAACAC Antisense: GGCAATATAGGAGACTGG	131	50

Abbreviation: AT: annealing temperature; bp: base pairs.

Western Blot (WB)

Western Blot procedure was used to evaluate the protein expression of GLUT1, GLUT3, PFK, lactate dehydrogenase (LDH) and MCT4 in the prostate of wild-type and Tg-RGN rats. Total proteins were extracted from rat prostates using RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 8 and 1 mM EDTA), and protein concentration was determined by the Bradford micro-assay (Bio-Rad Laboratories). 75 µg of each protein sample were resolved in a 12% gel by SDS-PAGE and electrotransferred to a PVDF membrane (GE Healthcare, Buckinghamshire, UK). Membranes were incubated overnight at 4 °C with, rabbit anti-GLUT1 (1:300, CBL242, Millipore, CA, USA), rabbit anti-GLUT3 (1:2500, sc-30107, Santa Cruz Biotechnology, CA, USA), rabbit anti-PFK1 (1:500, sc-67028, Santa Cruz Biotechnology), rabbit anti-LDH (1:10000, Ab52488, Abcam, Cambridge, United Kingdom), or rabbit anti-MCT4 (1:1000, sc-50329, Santa Cruz Biotechnology) primary antibodies. Protein expression was normalized using a mouse anti-β-actin (1:1000, A5441, Sigma-Aldrich) antibody. Goat anti-rabbit IgG-AP (1:5000, NIF1317; GE Healthcare) or goat anti-mouse IgG + IgM-AP (1:5000, NIF1316; GE Healthcare) were used as secondary antibodies. Membranes were incubated with ECF substrate (GE Healthcare) for 5 min and immunoreactive proteins were visualized with the Molecular Imager FX Pro plus Multimager (Bio-Rad). Band densities were obtained using the Quantity One Software (Bio-Rad) and normalized to the corresponding β-actin density. Results are presented as fold-variation relatively to the wild-type group.

LDH and PFK enzymatic activities

Lactate dehydrogenase activity was determined using a commercial assay kit (Spinreact) following the manufacturers' instructions. LDH catalyzes the reduction of pyruvate to lactate with the oxidation of NADH to NAD⁺, and, thus, the concentration of LDH in the sample is proportional to the rate of decrease in NADH concentration that was measured spectrophotometrically at 340 nm using a xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). PFK activity was determined as previously described [13]. Results were expressed as fold-variation relatively to the wild-type group.

Ki67 fluorescent immunohistochemistry

Fluorescent immunohistochemistry of the proliferation marker Ki67 was used to estimate the proliferation index in the prostate of Tg-RGN animals vs wild-type as previously described [4]. Briefly, prostate tissue sections (5 µm) were overnight incubated at 4° C with the rabbit anti-Ki67 (1:50, no. 16667, Abcam) antibody. Alexa fluor 546 goat anti-rabbit IgG (1:500, Invitrogen) was used as secondary antibody (1:500, Invitrogen) and the specificity of immunostaining was assessed by omission of primary antibody. Cell nuclei were stained with Hoechst 33342 (15 µg/ml, Invitrogen) for 5 min. Finally, sections were mounted in Dako fluorescent mounting medium (Dako) and the preparations were observed in the Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, NY, USA). The proliferation index was estimated by counting the number of Ki67-positive cells and Hoechst stained nuclei in twenty

randomly selected 40x magnification fields for each section ($n \geq 5$). The ratio between the number of Ki67 stained cells and total number of nuclei was calculated.

Statistical analysis

The statistical significance of differences between experimental groups was assessed by the Student's t-test (GraphPad Software, San Diego, CA, USA). Significant differences were considered when $P < 0.05$. All experimental data are shown as mean $\pm$ SEM ($n \geq 6$ for each experimental condition).

Results

Glucose and lactate concentration were reduced in the prostate of Tg-RGN rats

In order to investigate the role of RGN modulating the glycolytic profile in rat prostate, the content of glucose and lactate in prostatic tissues in Tg-RGN animals comparatively with the wild-type control group were determined. The obtained results demonstrated that the prostates of Tg-RGN rats displayed diminished concentration of glucose and lactate in comparison with their wild-type counterparts (19.29 ± 6.12 vs 48.97 ± 6.11 nmol/g and 197.0 ± 33.77 vs. 407.6 ± 80.12 nmol/g, respectively, $P < 0.05$, Figure 9.1a and 9.1b). Serum glucose levels were also determined, and no significant differences were observed between Tg-RGN and wild-type animals (Figure 9.1c).

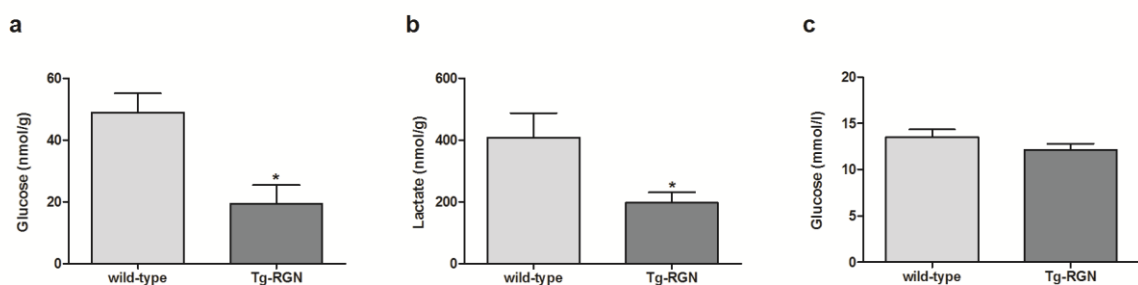


Figure 9.1. Concentration of glucose (a) and lactate (b) in the prostate of transgenic rats overexpressing RGN (Tg-RGN) comparatively with their wild-type counterparts. c Serum glucose levels in wild-type and Tg-RGN. Animals were 3 months old ($n \geq 6$). Error bars indicate mean $\pm$ SEM * $P < 0.05$ relatively to the wild-type group

However, the diminished glucose content in tissues of Tg-RGN animals was also observed in the liver and brain (respectively, 3372.7 ± 57.39 vs. 4317.6 ± 154.2 nmol/g in wild-type group and 547.8 ± 87.24 vs. 979.0 ± 166.3 nmol/g in wild-type group, Figure 9.2), while no differences were found in the testis of transgenic and control groups.

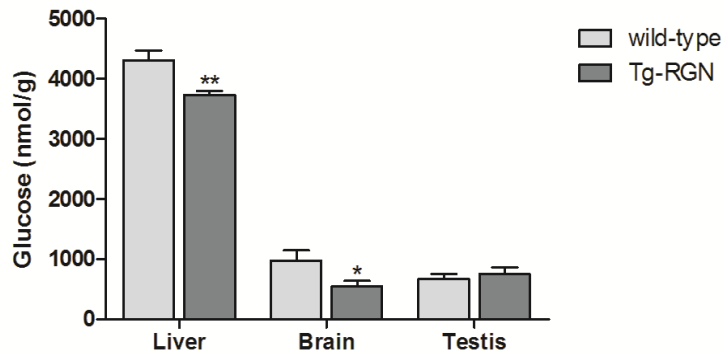


Figure 9.2. Concentration of glucose in the liver, brain and testis of transgenic rats overexpressing RGN (Tg-RGN) comparatively with their wild-type counterparts. Animals were 3 months old ($n \geq 6$). Error bars indicate mean $\pm$ SEM * $P < 0.05$; ** $P < 0.01$ relatively to the wild-type group.

Prostates of Tg-RGN rats presented decreased expression of GLUT3 and PFK

The consumption of glucose through glycolysis first depends on the uptake of glucose from the extracellular space, which is mediated by several GLUTs family members. GLUT1 and GLUT3 proteins specifically transport glucose and not fructose, and were previously identified in prostate cells [13, 18]. The influence of RGN on the regulation of glucose transport in rat prostate was determined by assessing the mRNA and protein expression of both GLUT1 and GLUT3 using qPCR and WB, respectively. The results presented herein showed that the prostate of Tg-RGN animals displayed lower mRNA and protein expression of GLUT3 (respectively, 0.44 ± 0.16 and 0.48 ± 0.06 fold-variation to wild-type, $P < 0.05$, Figure 9.3a, b), whereas no significant changes were observed in the expression of GLUT1 in comparison with the wild-type control group.

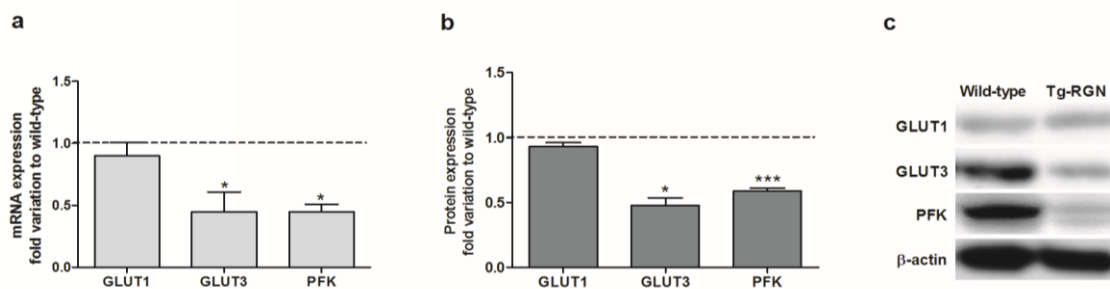


Figure 9.3. Expression of GLUT1, GLUT3 and PFK in the prostate of transgenic rats overexpressing RGN (Tg-RGN) comparatively with their wild-type counterparts. Animals were 3 months old ($n \geq 6$). (a) mRNA expression was determined by qPCR after normalization with CypA and β -actin housekeeping genes. (b) Protein levels determined by WB analysis after normalization with β -actin. Results are expressed as fold-variation relatively to the wild-type group, represented by the dashed line. Error bars indicate mean $\pm$ SEM. * $P < 0.05$; *** $P < 0.001$. (c) Representative immunoblots of three independent experiments showing protein levels in Tg-RGN and wild-type animals.

Phosphofructokinase involved in a crucial step of the glycolytic process, catalysing the first irreversible conversion of fructose-6-phosphate (F-6-P) to fructose-1-6-bis-phosphate (F-1,6-BP) [19]. The effect of RGN overexpression on the PFK levels and activity in rat prostate

was analyzed. It was demonstrated that both the mRNA and protein expression of PFK were significantly diminished in the prostate of Tg-RGN animals (respectively, 0.44 ± 0.06 and 0.58 ± 0.02 fold-variation to wild-type, Figure 9.3a and 9.3 b). Nevertheless, the activity of PFK was maintained unchanged between Tg-RGN and wild-type experimental groups (Figure 9.4a).

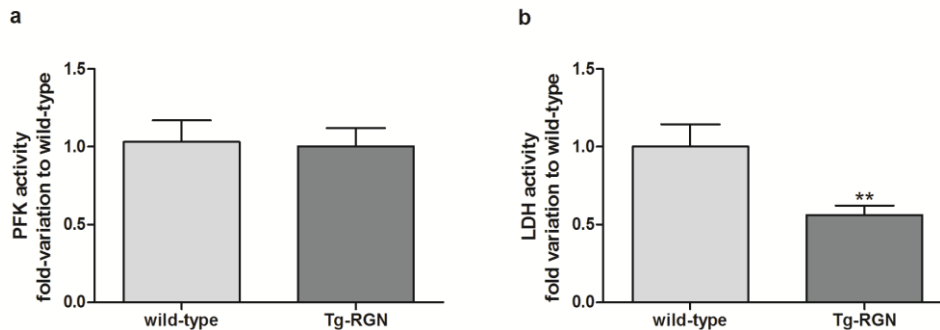


Figure 9.4. PFK (a) and LDH (b) activity in the prostate of transgenic rats overexpressing RGN (Tg-RGN) comparatively with wild-type animals. Animals were 3 months old ($n \geq 6$). Results are expressed as fold-variation relatively to wild-type group. Error bars indicate mean $\pm$ SEM ($n \geq 6$). ** $P < 0.01$.

Tg-RGN rats displayed lower expression and activity of LDH and decreased expression of MCT4

The reversible reaction that converts the final product of glycolysis pyruvate into lactate is catalyzed by the LDH enzyme family [20]. We evaluated the expression and activity of LDH in the prostate of animals overexpressing RGN and wild-type controls. It was observed that Tg-RGN animals displayed significantly lower enzymatic activity of LDH, as well as diminished protein expression relatively to their wild-type counterparts (respectively, 0.56 ± 0.06 and 0.63 ± 0.07 fold-variation, Figure 9.4b and 9.5b). MCT4 is the monocarboxylate transporter that controls lactate transport, and its activity has been mainly associated with the cellular efflux of lactate. The mRNA and protein expression of MCT4 were both significantly decreased in the Tg-RGN group relatively to wild-type animals (respectively, 0.26 ± 0.09 and 0.76 ± 0.07 fold-variation, Figure 9.5).

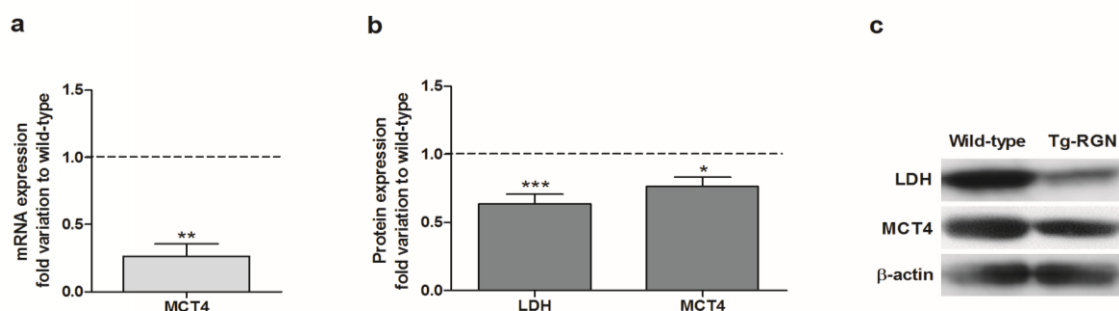


Figure 9.5. Expression of LDH and MCT4 in the prostate of transgenic rats overexpressing RGN (Tg-RGN) comparatively with their wild-type counterparts. Animals were 3 months old ($n \geq 6$). (a) mRNA expression was determined by qPCR after normalization with CypA and β -actin housekeeping genes. (b) Protein levels determined by WB analysis after normalization with β -actin. Results are expressed as fold-variation relatively to the wild-type group, represented by the dashed line. Error bars indicate mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (c) Representative immunoblots of three independent experiments showing protein levels in Tg-RGN and wild-type animals.

Suppressed glycolytic metabolism underpins reduced proliferation rates in the prostate of Tg-RGN

The cell proliferation index in the prostates of both Tg-RGN and wild-type animals was determined by Ki67 fluorescent immunohistochemistry. Considering the number of Ki67-stained cells relatively to total cells, the prostate of Tg-RGN animals displayed a lower index of proliferation in comparison to their wild-type counterparts (0.47 ± 0.05 fold-variation, Figure 9.6a).

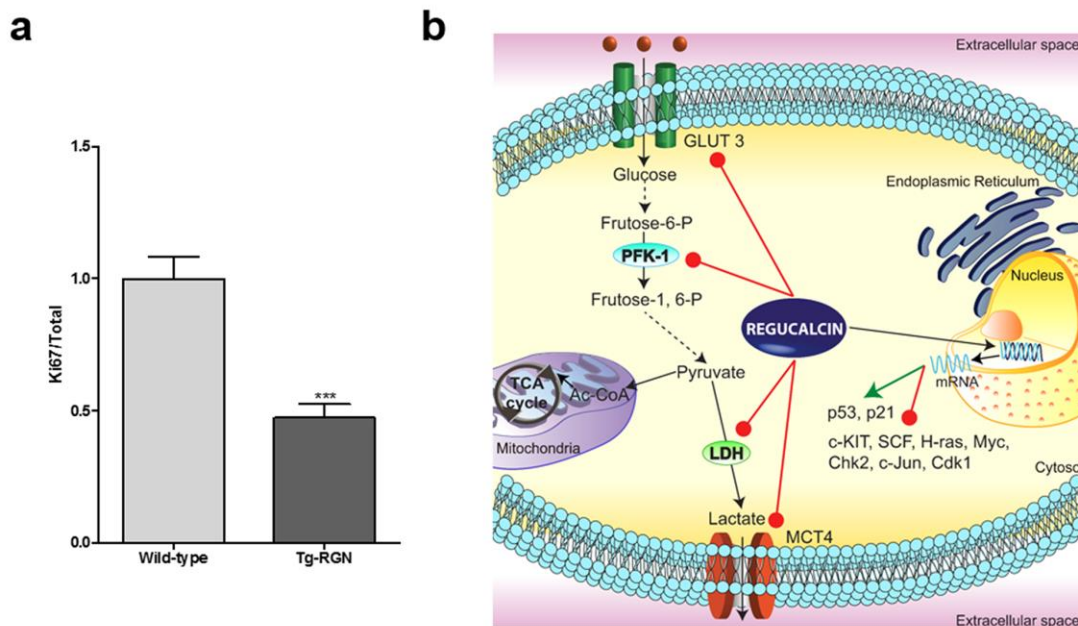


Figure 9.6. (a) Proliferation index in the prostate of transgenic rats overexpressing RGN (Tg-RGN) comparatively with wild-type animals determined by Ki67 fluorescent immunohistochemistry. Animals were 3 months old ($n \geq 6$). Results are expressed as fold-variation relatively to the wild-type group. Error bars indicate mean $\pm$ SEM *** $P < 0.001$. (b) Schematic diagram of RGN actions controlling the glycolytic metabolism in rat prostate cells. Altered expression of genes involved in glucose consumption (GLUT3 and PFK-1) and in lactate production (LDH) and export (MCT4) was found in Tg-RGN animals concomitantly with a diminished proliferation index. RGN protein can be translocated to cell nuclei and also has been implicated in the regulation of cell proliferation by modulating the expression of tyrosine kinase receptor c-kit and its ligand the stem cell factor (SCF), H-ras, Myc, Chk2, c-Jun, Cdk1, and p53 and p21 genes [4, 5, 35].

Discussion

In this study no differences were perceived on serum glucose levels between Tg-RGN and wild-type animals. This result is in agreement with other previous work also showing that serum glucose levels are unaltered both in male and female Tg-RGN rats [21]. The maintenance of plasma glucose levels requires a tight control of the glucose absorption in the intestine, the production by the liver, and the consumption and metabolism in peripheral tissues. These processes involve the action of several proteins, transporters and the integration of the complex signaling pathways that modulate insulin mechanism of action [22]. Our results indicate that the overall mechanisms contributing to maintaining constant plasma glucose levels are unchanged in Tg-RGN animals. However, the overexpression of RGN diminished both glucose and lactate content in rat prostate tissues. Also, in the liver, one of

the body's reservoirs of glucose (glycogen), and in the brain, an organ with a great demand for glucose, the overexpression of RGN decreased the levels of glucose. Recently, it was shown that blood glucose concentration was also similar in RGN knockout male mice and wild-type animals [23], which suggests that RGN acts locally at tissue level to modulate glucose content independently of serum levels. Therefore, the observed decrease in glucose concentration in the prostate of Tg-RGN rats should be the consequence of increased utilization of glucose or decreased uptake of glucose by prostate cells. The process of glycolysis starts precisely with glucose uptake from the extracellular space, which occurs through GLUTs present at the cell membrane. The results obtained demonstrated that, although GLUT1 expression did not change, the mRNA and protein expression of GLUT3 were significantly diminished in Tg-RGN animals relatively to their wild-type counterparts. This suggests that RGN negatively regulates the uptake of glucose in prostate cells by diminishing GLUT3 levels. The regulatory effect of RGN over other GLUT family members was previously reported in an hepatoma cell line [24]. H4-II-E cells overexpressing RGN displayed increased mRNA expression of GLUT2 [24], which indicates that RGN may also modulate the expression of other GLUTs. However, it is established that GLUT3 has a higher affinity for glucose than other GLUTs, namely GLUT1, GLUT2, or GLUT4. Also, the transport capacity of GLUT3 is much greater than that of GLUT1 and GLUT4 [25], which strongly supports the diminished glucose content found in the prostate of Tg-RGN animals concomitantly with diminished expression of GLUT3.

After enter the cell, glucose may be stored as glycogen or metabolized through the sequential enzymatic reactions of glycolysis that end with the production of pyruvate. Glycogen storage mainly occurs in the liver and skeletal muscle, which depends on the dynamic interplay between the activity of glycogen synthase (GS) and glycogen phosphorylase (GP) that converts the existing glycogen to glucose-1-phosphate when glucose is needed [26]. However, also prostate cells accumulate glycogen, which seems to depend on the androgenic response since androgens were able to regulate the activity of GS and GP [27]. Importantly, it was reported that overexpression of RGN decreased the concentration of glycogen in rat liver [28], which indicates that RGN overexpression inhibits glycogen storage. Thus, the reduced content of glucose found in the prostate of Tg-RGN should depend on the diminished uptake driven by the decreased levels of GLUT3 and would not be a consequence of its storage as glycogen.

The first rate-limiting step in glycolysis is the conversion of F-6-P to F-1,6-BP, an irreversible reaction catalysed by PFK1 [29]. Overexpression of RGN decreased the mRNA and protein levels of PFK in rat prostate though no changes were perceived on the overall activity of this enzyme. Non-concordant results between enzymatic activity and protein expression levels are commonly observed for different metabolic enzymes, including PFK [13], and may be a consequence of the complex regulatory mechanisms that control enzymatic activity. Ultimately, this includes the regulation of enzyme activity by the resultant metabolic products. However, previous work in rat liver have demonstrated that RGN suppresses the

activity of several enzymes involved in glycolysis, namely pyruvate kinase [30], fructose 1,6-diphosphatase [31] and glucose-6-phosphatase [32], an effect dependent on Ca^{2+} and/or calmodulin.

The present findings demonstrated that RGN overexpression negatively regulated the glycolytic flux in rat prostate cells by decreasing the glucose uptake and its metabolism to pyruvate, which was the consequence of the downregulated expression of GLUT3 and PFK observed in Tg-RGN animals.

The end-product of glycolysis pyruvate fuels the tricarboxylic acid cycle (TCA) in order to supply the cell with the required ATP. Alternatively, pyruvate can escape the TCA and be converted into lactate through an enzymatic reaction catalyzed by LDH, which is a characteristic adaptive condition developed by neoplastic cells. In turn, the lactate produced is exported to the extracellular space mainly per the activity of MCT4, and this is associated with fostering tumor progression and invasion. Considering that RGN was found to counteract cell proliferation and indicated as a protective molecule against cancer development [4, 5, 7, 10], we decided to investigate the expression of LDH and MCT4 in the prostate of Tg-RGN and wild type controls. The total amount of lactate in the prostate of Tg-RGN animals was inferior to that found in wild-type animals, which is consistent with the observed diminished uptake of glucose substrate. Furthermore, the decreased lactate content was underpinned by lowered LDH expression and activity, as well as diminished expression of MCT4, which suggests that RGN decreases the production and export of lactate in prostate cells. On the other hand, it was reported that the stimulatory effect of RGN over bone resorption in bone tissue culture was coincident with increased glucose consumption and lactate production, which further demonstrates the role of RGN in the regulation of glucose metabolism and lactate production, likely in a tissue-specific manner [33]. Nevertheless, we first report here the link between RGN and lactate production through the modulation of lactate handling proteins, such as LDH and MCT4.

In general, the results described above demonstrated that RGN negatively regulated the glycolytic metabolism in rat prostate lowering glucose uptake, glucose metabolism and lactate production. Moreover, the suppressed glycolytic metabolism in the prostate of Tg-RGN animals was underpinned by a decreased proliferation index, which is in line with previous findings of our research group showing that RGN counteracts cell proliferation through the regulation of the expression of important cell-cycle regulators, tumor suppressor genes, and oncogenes, such as p21, p53, Cdk, Chk2, c-Jun, SCF, c-kit, H-ras and Myc (Figure 9.6b) [4, 5]. The reprogramming of cell metabolism is a known hallmark of cancer and the hyper-glycolytic phenotype resulting from the increased utilization of glucose and exacerbate production of lactate is a common feature of neoplastic cells, which has been associated with increased proliferative ability, and development and progression of prostate cancer [13]. Very recently, we and others have shown that androgens stimulate the glycolytic flux in prostate cancer cells contributing to enhanced cell proliferation and cancer progression [13, 14, 34]. On the other hand, it is known that androgens negatively regulate the expression of RGN in prostate

cells [4, 10]. Also, it was established that the onset and development of prostate cancer are accompanied by the loss of RGN expression [10]. As demonstrated in this study, RGN overexpression inhibited the glycolytic metabolism in rat prostate, which was accompanied by a reduced proliferation index. Thus, it is liable to suggest that the reported antiproliferative effects of RGN may be partly achieved by its action decreasing glucose consumption to lactate production (Figure 9.6b). Accordingly, the diminished expression of RGN that has been found in human prostate cancer cases may predispose to the emergence of a hyper-glycolytic phenotype and aberrant cell proliferation favouring neoplastic transformation.

In conclusion, the present work is the first report showing that RGN regulates the production of lactate in rat prostate, through its action decreasing the expression and activity of important regulators of the glycolytic flux, such as GLUT3, PFK, LDH and MCT4. The obtained results helped to clarify the role of RGN in prostate physiology and indicated that the deregulated expression of RGN might enhance the glycolytic metabolism and cell proliferation, hence fostering development of prostate cancer.

Funding

This work was partially supported by Portuguese Foundation for Science and Technology (FCT) under Program COMPETE (PEst-OE/SAU/UI0709/2014). Cátia V. Vaz and Ricardo Marques were funded by FCT fellowships (SFRH/BD/70316/2010 and SFRH/BD/66875/2009, respectively).

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Chapter 10

Aging-associated changes in oxidative stress, cell proliferation and apoptosis are prevented in the prostate of transgenic rats overexpressing regucalcin

This chapter was published in:

Vaz, C.V., Marques, R., Maia, C.J., Socorro, S. Aging-Associated Changes in Oxidative stress, Cell proliferation and Apoptosis are Prevented in the Prostate of Transgenic Rats Overexpressing Regucalcin. *Translational Research*. 2015; 10.1016/j.trsl.2015.08.009.

Aging-associated changes in oxidative stress, cell proliferation and apoptosis are prevented in the prostate of transgenic rats overexpressing regucalcin

Abstract

Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein that displays a characteristic downregulated expression with aging in several tissues. Besides its role in regulating intracellular Ca^{2+} homeostasis, RGN has been associated with the control of oxidative stress, cell proliferation, and apoptosis. Thus, the diminished expression of RGN with aging may contribute to the age-associated deterioration of cell function. In the present study, we hypothesized that the maintenance of high expression levels of RGN may prevent age-related alterations in the processes mentioned above. First, we confirmed that RGN expression is significantly diminished in the prostate of 8 -, 9 -, 12 - and 24 months wild-type rats. Then, the effect of aging on lipid peroxidation, antioxidant defenses, cell proliferation and apoptosis in the prostate of wild-type controls and transgenic rats overexpressing RGN (Tg-RGN) was investigated. The activity of glutathione and the antioxidant capacity were increased in Tg-RGN rats in response to the age-associated increase in TBARS levels, an effect not seen in wild-type. Overexpression of RGN also counteracted the effect of aging increasing prostate cell proliferation. Contrarily to wild-type animals, the prostate weight of Tg-RGN did not change with aging and was underpinned by the diminished expression of SCF and c-kit and increased expression of p53. Also, aged Tg-RGN animals displayed increased expression (activity) of apoptosis regulators, therefore not showing the age-induced resistance to apoptosis observed in wild-type. Altogether, these findings indicate the protective role of RGN against the development of age-related pathologies, such as, for example, prostate cancer.

Keywords: aging; apoptosis; cell proliferation; oxidative stress; prostate; regucalcin.

Introduction

Regucalcin (RGN) was first described in 1978 as a calcium (Ca^{2+})-binding protein, that does not contain the typical EF-hand Ca^{2+} -binding motif [1]. Approximately two decades later, it was identified as senescence-marker protein-30 (SMP30) due to the down-regulated expression with aging in several rat tissues, namely, liver, kidney and testis [2, 3]. Despite the ability of RGN in regulating intracellular Ca^{2+} homeostasis [4, 5], different roles in cell physiology have been proposed for this protein in the last years. RGN seems to act as an antioxidant protein in rat liver, kidney and brain by exerting a dual function: inhibiting the activity of enzymes involved in the generation of reactive oxygen species (ROS) or enhancing the antioxidant defenses [4]. In addition, a role for RGN in the control of tissue cell

homeostasis has been established. Our previous work demonstrated that RGN decreases cell proliferation and apoptosis in rat prostate, concomitantly with altered expression of cell cycle (p21 and p53), and apoptosis (Bcl-2, Bax, caspase-8 and caspase-3) regulators [6]. On the other hand, diminished expression of RGN has been observed in a set of human cancer cell lines and also in liver, breast and prostate cancer tissues [7-9]. Altogether, the present data suggest that the diminished expression of RGN with aging may contribute to deregulated tissue physiology favoring the development of age-related diseases. Inversely, it is liable to assume that maintaining the expression levels of RGN may be a protective mechanism against age-related alterations in oxidative stress, cell proliferation and apoptosis.

Given that aging is a major risk factor for prostatic diseases, the present study aimed to establish the expression pattern of RGN along development and aging of the rat prostate. Furthermore, using a transgenic rats overexpressing RGN (Tg-RGN) we determined the impact of maintaining high expression levels of RGN in preventing the age-associated changes in lipid peroxidation and antioxidant defenses, as well as in cell proliferation and apoptosis in rat prostate.

Materials and methods

Animals and tissue collection

Sprague Dawley wild-type and Tg-RGN (n=7 in each group) were obtained from Charles River (Barcelona, Spain) and Japan SLC (Hamamatsu, Japan), respectively. Tg-RGN were originally generated by Yamaguchi M by oocyte transgene pronuclear injection [10] and have been used as a useful model to study the role of RGN in cell proliferation and apoptosis [6, 7, 11]. Also, the overexpression of RGN in the prostate of Tg-RGN was previously demonstrated [6].

Juvenile (20 days, 1 month [M], 2 M), young (3 M and 4 M), middle-aged (6 M, 8 M, 9 M and 12 M), and aged (24 M) rats were housed under a 12 h light, 12 h dark cycle, with food and water available ad libitum and handled in compliance with the NIH guidelines and the European Union rules for the care and handling of laboratory animals (Directive 2010\63\EU). Animals were euthanized under anesthesia (Clorketam 1000, Vetoquinol, Lure, France) and whole prostates were removed, weighted, and frozen in liquid nitrogen for RNA and protein extraction. The number of animals in each age group was n=7.

Quantitative RT PCR (qPCR)

Total RNA was extracted from rat prostates using TRI reagent (Sigma-Aldrich, St.Louis, Missouri) according to the manufacturer's instructions. After evaluating the quantity and integrity of total RNA [6], 1µg RNA was reverse transcribed in a final volume of 20 µl using the First Strand cDNA synthesis Kit (NZYtech, Lisboa, Portugal), following the manufacturer's instructions. 1 µl of synthesized cDNA was used in each qPCR reaction carried out in the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, California). The optimization strategy for each primer set, the specificity and efficiency of the amplification, as well as

primer details and cycling conditions were previously described [6, 12]. Cyclophilin A (CypA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping genes were used as internal controls for normalization of expression of RGN and apoptosis-related genes. Normalized expression values were calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta C_t}$ [13].

Total protein extraction

Total proteins were extracted from rat prostates using RIPA buffer (1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS in PBS) supplemented with protease inhibitors cocktail (Sigma-Aldrich). Protein concentration was determined by the Bradford assay (Bio-Rad) and protein extracts were used in Western blot (WB) and biochemical assays.

Western Blot (WB)

75 µg protein extracts were resolved by SDS-PAGE on 12% gels and electrotransferred to a PVDF membrane (Bio-Rad). Membranes were incubated overnight at 4 °C with mouse anti-RGN (1:1000, ab81721, Abcam, Cambridge, United Kingdom), rabbit anti-p53 (1:1000, sc-6243, Santa Cruz Biotechnology), rabbit anti-Bax (1:4000, no. 2772, Cell Signaling Technology, Beverly, MA, USA), rabbit anti-Bcl-2 (1:1000 no. 2876, Cell Signaling Technology), rabbit anti-Fas ligand (FasL, 1:500, sc-6237, Santa Cruz Biotechnology), rabbit anti-Fas receptor (FasR, 1:500, sc-6237, Santa Cruz Biotechnology), rabbit anti-caspase-9 (1:1000, sc-8355, Santa Cruz Biotechnology) and mouse anti-caspase-8 (1:1000, sc-5263, Santa Cruz Biotechnology). A mouse anti-β-actin (1:1000, A5441, Sigma-Aldrich) was used for normalization of protein expression. The membranes were then incubated for 1 h at room temperature with 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), used as secondary antibodies. At the end, membranes were incubated with ECL substrate (Bio-Rad) for 5 min, and visualized on the ChemiDoc™ MP Imaging System (Bio-Rad).

Thiobarbituric Acid Reactive Substances (TBARS) assay

Lipid peroxidation was assessed by the colorimetric measurement of malondialdehyde (MDA)-thiobarbituric acid (TBA) adduct formation using the TBARS Assay Kit (Cayman Chemical, Ann Arbor, MI). Briefly, the MDA-TBA adduct was formed by the reaction of MDA and TBA under high temperature and acidic conditions. The sample or standard was added to a vial with the appropriate volume of SDS and color reagent, and heated at 100 °C for 1 h. At the end, vials were placed on ice to stop reaction, and then centrifuged. 150 µl of each reaction were added to a clear plate and the absorbance at 530 nm was measured using a xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). TBARS content was expressed as µM/mg tissue relatively to wild-type 3- M animals.

Total antioxidant capacity (TAC) assay

The TAC was determined using a commercially kit according to the manufacturer's instructions (Cayman Chemical). Briefly, the antioxidant capacity depends on the ability of antioxidants present in the sample to inhibit the oxidation of 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS[®]) to ABTS[®].+ by metmyoglobin, which was compared with antioxidant capacity of Trolox, a water-soluble tocopherol analogue quantified as millimolar Trolox equivalents. The amount of oxidized ABTS[®] was determined spectrophotometrically at 405 nm using a xMark[™] Microplate Absorbance Spectrophotometer (Bio-Rad). Results were expressed as mM/mg tissue relatively to wild-type 3 M animals.

Glutathione S-Transferase (GST) assay

To determine the activity of GST, a commercial kit (Sigma-Aldrich) was used and the assay was performed following the manufacturer's instructions. Briefly, the reaction product resultant from the conjugation of the thiol group of glutathione to the 1-Chloro-2,4-dinitrobenzene substrate at 25 °C was detected spectrophotometrically at 340 nm (Pharmacia Biotech, Ultrospec 3000, Denmark). The increase in absorbance is proportional to the GST activity in the sample. Results were expressed as $\mu\text{mol}/\text{ml}/\text{min}/\text{mg}$ tissue relatively to wild-type 3 M animals.

Superoxide dismutase (SOD) assay

Superoxide dismutase activity was measured using the SOD Assay Kit-WST (Sigma-Aldrich) following manufacturer's instructions. Briefly, this kit uses the WST-1 substrate (tetrazolium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of formazan production is linearly related with the activity of xanthine oxidase, and inhibited by SOD. The reduction reaction occurred at 37 °C for 20 min, and the generation of formazan, which is proportional to the amount of superoxide anion, was monitored by measurement of absorbance at 450 nm in a xMark[™] Microplate Absorbance Spectrophotometer (Bio-Rad). The percentage of reaction inhibition rate indicates SOD activity. Results were expressed as a proportion of activity (percentage of inhibition) relatively to wild-type 3 M animals.

Caspase-3-like activity assay

The activity of caspase-3 was assessed determining the cleavage of a colorimetric substrate. Briefly, proteins (25 μg) were incubated with a reaction buffer (25 mM HEPES, pH 7.5, 0.1% CHAPS, 10% sucrose and 10 mM DTT) and 100 μM of caspase-3 substrate (Ac-DEVD-pNA) for 2 h at 37° C. The caspase-3-like activity was determined after cleavage of the labeled substrate by detection of the chromophore p-nitroaniline (pNA), measured spectrophotometrically at 405 nm. The amount of generated pNA was calculated by extrapolation with a standard curve of free pNA.

Statistical analysis

The statistical significance of differences among experimental groups was assessed by ANOVA, followed by the Tukey test. Significant differences were considered when $p < 0.05$. All experimental data are shown as mean $\pm$ SEM, and expressed as fold-variation relatively to 20 days or 3 M wild-type groups.

Results

Regucalcin expression is diminished in the prostate of aged animals

The expression pattern of RGN in the prostate of juvenile (20 days, 1 M and 2 M), young (3 M and 4 M), middle-age (6 M, 8 M, 9 M and 12 M) and aged (24 M) wild-type rats was analysed by means of qPCR and WB. Figure 10.1A shows that RGN mRNA expression reached a peak in young 3 M animals (3.18 ± 0.93 fold, $p < 0.05$), maintaining a plateau until the age of 6 M. In middle-age (8 M and 12 M) and aged (24 M) animals the RGN mRNA expression decreased to levels found in juveniles. The expression of RGN protein (Figure 10.1B) during developmental stages and aging of rat prostate followed the pattern found for mRNA. RGN protein levels were significantly increased in the prostate of 3 M and 6 M animals (1.33 ± 0.16 and 1.25 ± 0.08 fold, respectively), decreasing in 9 M and 24 M groups.

Our experimental hypothesis is that RGN overexpression may protect against age-associated deterioration of cell function. Therefore, Tg-RGN and wild-type animals, both of 3 M and 9 M, were selected for the subsequent analyses. We decided to use 9 M, instead of older animals, because wild-type animals of this age already display a significant reduction of RGN expression (Figure 10.1B and 10.1C), and it has been shown that the changes in metabolism, oxidative stress and apoptosis accompany aging process are already present at this age [14-16]. In addition, it was shown that the pattern in advanced ages seems to be an expansion of the alterations already set in motion in middle-age [14, 17]. Importantly, no changes were observed concerning the expression of RGN in 3 M and 9 M Tg-RGN rats (Figure 10.1C).

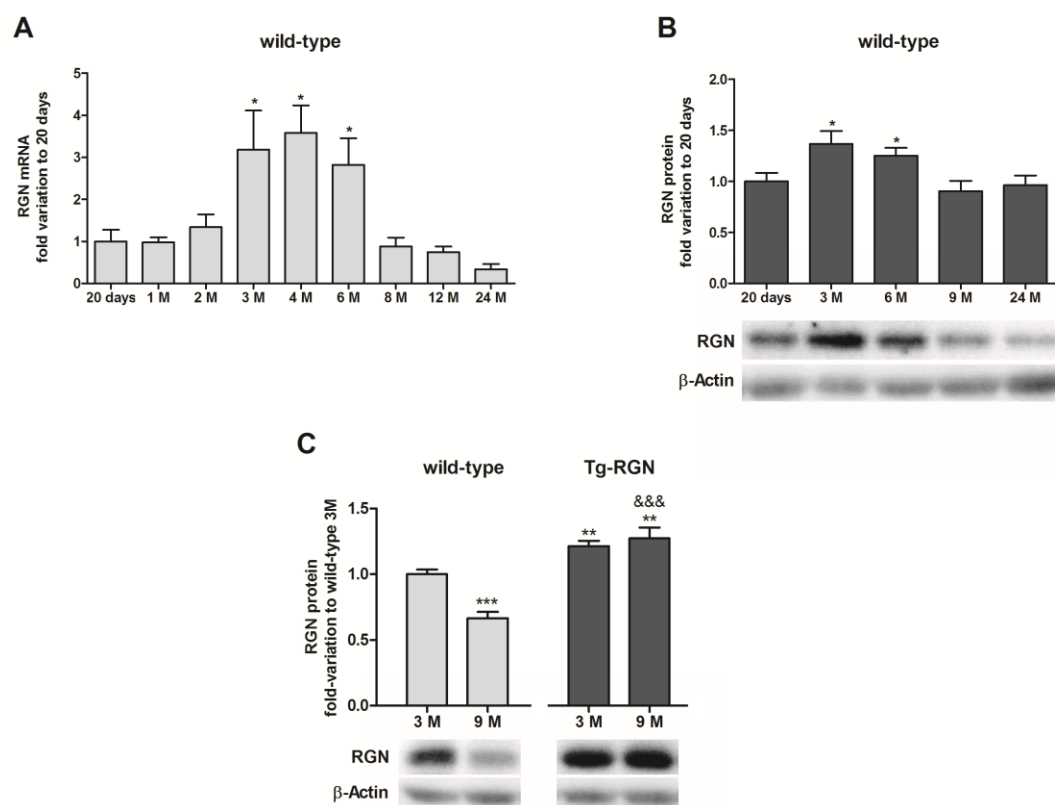


Figure 10.1. RGN mRNA (A) and protein (B) expression in the prostate of developing and aging wild-type animals, from 20 days to 24 month-old (24 M). Results are expressed as fold-variation relatively to the 20 days group. (C) Protein levels of RGN in the prostate of 3 M and 9 M wild-type and transgenic rats overexpressing RGN (Tg-RGN). Results are expressed as fold-variation relatively to the 3 M wild-type group. mRNA expression was determined by qPCR after normalization with CypA and GAPDH housekeeping genes. Protein expression was determined by WB analysis after normalization with β -actin. Error bars indicate mean $\pm$ SEM ($n \geq 5$). * $p < 0.05$, statistically significant relatively to the 20 days group. ** $p < 0.01$, *** $p < 0.001$, statistically significant difference relatively to 3 M wild-type group. &&& $p < 0.001$, statistically significant difference relatively to 9 M wild-type group. Representative immunoblots are shown as bottom panels.

Prostates of Tg-RGN animals displayed increased GST activity and TAC in response to the age-associated augment of TBARS levels

The increase in oxidative stress and the weakening of oxidative defense system have been considered a major cause of cell physiological deterioration with aging, and consequently associated with the onset of age-related pathologies [18]. The TBARS levels, the TAC, and the activity of GST and SOD enzymes were used to compare oxidative stress levels and the antioxidant defenses in the prostate of wild-type and Tg-RGN animals along aging.

Lipid peroxidation is a common signal of cell injury and for this reason TBARS levels are usually used as indicative of oxidative stress [19]. Our results demonstrated that the prostate of 3 M Tg-RGN animals presented significant lower levels of TBARS relatively to wild-type counterparts at the same age (0.85 ± 0.09 fold, Figure 10.2A). With increasing age, the levels of these substances increased both in wild-type and Tg-RGN animals (1.22 ± 0.18 and 1.12 ± 0.10 fold, respectively, $p < 0.05$, Figure 10.2A).

Figure 10.2B shows the variation in TAC with aging in wild-type and Tg-RGN animals. The prostate of 9 M wild-type animals showed decreased overall antioxidant capacity comparatively with young (3 M) wild-type animals (0.89 ± 0.10 fold, $p < 0.05$). Although both 3 M and 9 M Tg-RGN rats displayed significantly lower antioxidant capacity comparatively with the 3 M wild-type group (0.70 ± 0.08 and 0.81 ± 0.16 fold, respectively), interestingly, the antioxidant capacity in the prostate of Tg-RGN animals increased with aging ($p < 0.05$, Figure 10.2B).

Glutathione-S-transferase is an antioxidant enzyme that participates in the detoxification of a range of substances resultant from ROS-mediated reactions [20]. The activity of GST significantly decreased in the prostate of 9 M wild-type animals comparatively with the 3 M group (0.81 ± 0.16 fold, Figure 10.2C). 3 M Tg-RGN animals showed lower GST activity relatively to their age-matched wild-type counterparts (0.48 ± 0.15 fold, $p < 0.001$). Nevertheless, the GST activity is significantly higher in aged Tg-RGN animals comparatively with wild-type (3 M and 9 M), and 3 M Tg-RGN groups (1.24 ± 0.25 fold in 9 M Tg-RGN vs. 0.48 ± 0.15 fold in 3 M Tg-RGN, Figure 10.2C).

Superoxide dismutase is a metallo-enzyme with superoxide scavenger functions, providing a first line of defense against oxidative stress [21] but no significant differences were found in the activity of SOD between all experimental groups (Figure 10.2D).

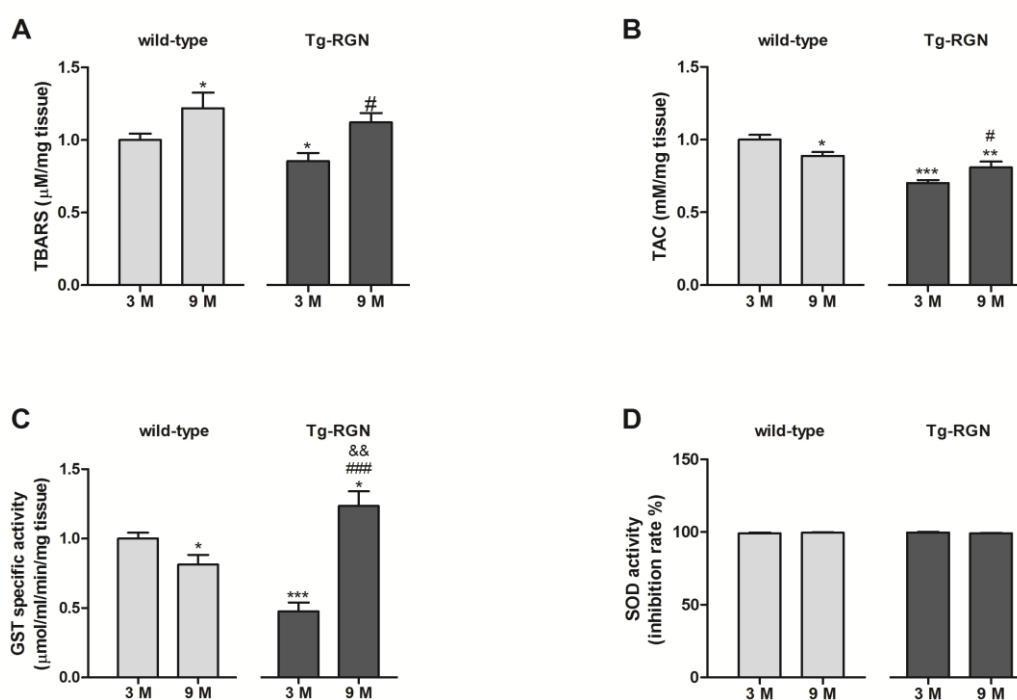


Figure 10.2. Thiobarbituric acid reactive substances (TBARS) (A), total antioxidant capacity (TAC) (B), and glutathione S-transferase (GST) (C) and superoxide dismutase (SOD) activity (D) in the prostate of 3 M and 9 M wild-type and transgenic rats overexpressing RGN (Tg-RGN). Results are expressed as fold-variation relatively to the 3 M wild-type group. Error bars indicate mean $\pm$ SEM ($n \geq 5$). * statistically significant difference relatively to 3 M wild-type group (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). & statistically significant difference relatively to 9 M wild-type group (& $p < 0.01$). #statistically significant difference relatively to 3 M Tg-RGN group (# $p < 0.05$, ### $p < 0.001$).

Overexpression of RGN modulates the expression of p53 and other regulators of cell proliferation in the prostate of aged animals

The stem cell factor (SCF) and its tyrosine kinase receptor c-kit are powerful regulators of cell proliferation, which also have been associated with the control of prostate cell growth [22]. The mRNA expression of SCF is significantly lower in aged wild-type animals (0.65 ± 0.10 fold, Figure 10.3A). Also, 3 M and 9 M Tg-RGN groups displayed reduced expression of SCF mRNA relatively to their age-matched counterparts (0.62 ± 0.07 and 0.36 ± 0.05 fold, respectively, Figure 10.3A), which means an approximately 40% reduction of SCF with increasing age ($p < 0.05$). Regarding c-kit, its mRNA expression is highly diminished in the prostate of 9 M Tg-RGN (0.20 ± 0.05 fold) in comparison with 3 M ($p < 0.001$) and 9 M ($p < 0.01$) wild-type animals (Figure 10.3B). Also, c-kit expression is markedly reduced with aging in Tg-RGN animals (0.20 ± 0.05 fold in 9 M vs. 0.8 ± 0.27 fold in 3 M, Figure 10.3B). The mRNA expression of c-kit did not suffered age-associated alterations in wild-type animals (Figure 10.3B).

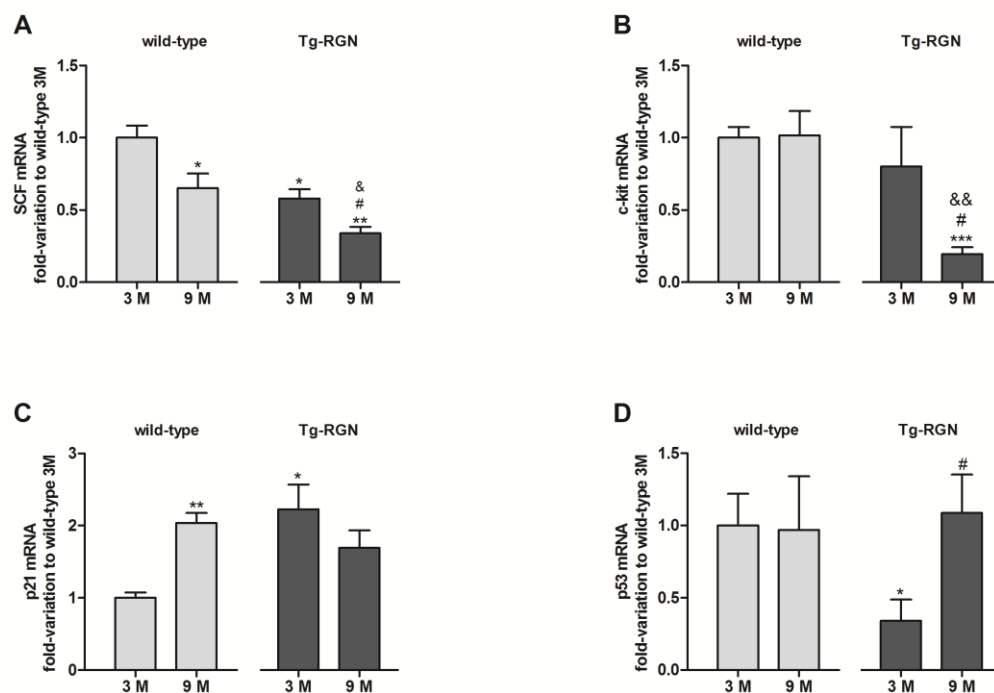


Figure 10.3. mRNA expression of SCF (A), c-Kit (B), p21 (C) and p53(C) in the prostate of 3 M and 9 M wild-type and transgenic rats overexpressing RGN (Tg-RGN). Expression was determined by qPCR after normalization with CypA and GAPDH housekeeping genes. Results are expressed as fold-variation relatively to the 3 M wild-type group. Error bars indicate mean $\pm$ SEM ($n \geq 5$). * statistically significant difference relatively to 3 M wild-type group (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). & statistically significant difference relatively to 9 M wild-type group (& $p < 0.05$, && $p < 0.01$). # statistically significant difference relatively to 3 M Tg-RGN group (# $p < 0.05$).

Deregulated expression of cell cycle regulatory genes, namely p21 and p53, has been a common event associated with aging [23-25]. qPCR and WB analysis showed that young 3 M

Tg-RGN animals presented significantly increased mRNA expression of p21 (2.23 ± 0.19 fold, Figure 10.3C) and diminished levels of p53 mRNA and protein (respectively, 0.34 ± 0.07 and 0.44 ± 0.04 fold, Figure 10.3D and Figure 10.4) relatively to their age-matched wild-type counterparts. However, the mRNA and protein expression of p53 were highly increased in the prostates of aged Tg-RGN animals relatively to the 3 M Tg-RGN group (1.09 ± 0.12 vs. 0.34 ± 0.07 fold and 0.71 ± 0.11 vs. 0.44 ± 0.04 fold for mRNA and protein, respectively, $p < 0.05$, Figure 10.3D and Figure 10.4). Also, p53 protein is significantly increased in the prostate of 9 M Tg-RGN relatively to their age-matched wild-type counterparts (respectively, 0.71 ± 0.11 and 0.35 ± 0.06 fold, Figure 10.4). Contrary, the prostatic expression of p53 protein diminished with aging in wild-type rats (0.34 ± 0.07 fold, Figure 10.4). The mRNA expression of p21 is strongly enhanced in 9 M wild-type animals comparatively with the 3 M group (2.03 ± 0.08 fold, $p < 0.01$), an effect not seen in Tg-RGN (Figure 10.3C).

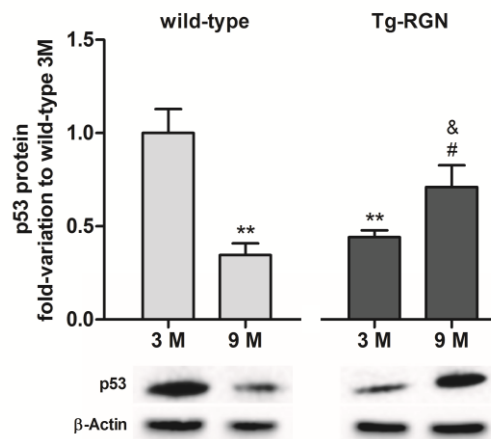


Figure 10.4. Protein levels of p53 in the prostate of 3 M and 9 M wild-type and transgenic rats overexpressing RGN (Tg-RGN), determined by WB analysis after normalization with β -actin. Results are expressed as fold-variation relatively to the 3 M wild-type group. Error bars indicate mean $\pm$ SEM ($n \geq 5$). Representative immunoblots are shown as bottom panels. * statistically significant difference relatively to 3 M wild-type group (** $p < 0.01$). & statistically significant difference relatively to 9 M wild-type group (& $p < 0.05$). # statistically significant difference relatively to 3 M Tg-RGN group (# $p < 0.05$).

Aging enhances apoptosis in the prostate of Tg-RGN animals

Programed cell death by apoptosis is a critical process in maintaining tissue homeostasis, and increased resistance to apoptosis is a common hallmark in aging, which is determined by altered expression of apoptosis regulators [26]. Apoptosis can be triggered through two pathways, the mitochondrial (or intrinsic) and the receptor-mediated (or extrinsic), both converging at the activation of effector caspase-3 [27]. The enzymatic activity of caspase-3, as well as, caspase-3 mRNA expression were significantly lower in 3 M Tg-RGN animals comparatively with the age-matched wild-type group (0.39 ± 0.06 and 0.57 ± 0.08 fold, respectively, Figure 10.5 and Figure 10.6A). Inversely, the mRNA expression of caspase-3 was strongly increased in 9 M Tg-RGN animals (3.25 ± 0.26 fold, Figure 10.6A) relatively to 3 M ($p < 0.001$) and 9 M ($p < 0.01$) wild-type groups. Although not significantly different from age-matched wild-type animals, also the activity of caspase-3 is augmented with aging in Tg-RGN

animals (0.38 ± 0.06 fold in 3 M vs. 0.73 ± 0.14 fold in 9 M, Figure 10.5). In wild-type animals, no differences were perceived on caspase-3 mRNA expression with aging (Figure 10.6A) but caspase-3 activity was diminished in the prostate of 9 M animals (0.56 ± 0.08 fold, $p < 0.05$, Figure 10.5).

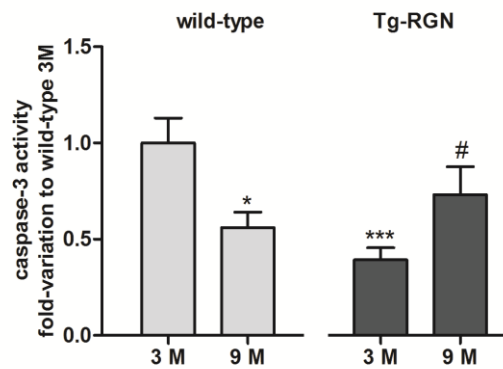


Figure 10.5. Caspase-3 activity in the prostate of 3 M and 9 M wild-type and transgenic rats overexpressing RGN (Tg-RGN). Results are expressed as fold-variation relatively to the 3 M wild-type group. Error bars indicate mean $\pm$ SEM ($n \geq 5$). * statistically significant difference relatively to 3 M wild-type group (* $p < 0.05$; *** $p < 0.001$). # statistically significant difference relatively to 3 M Tg-RGN group (# $p < 0.05$).

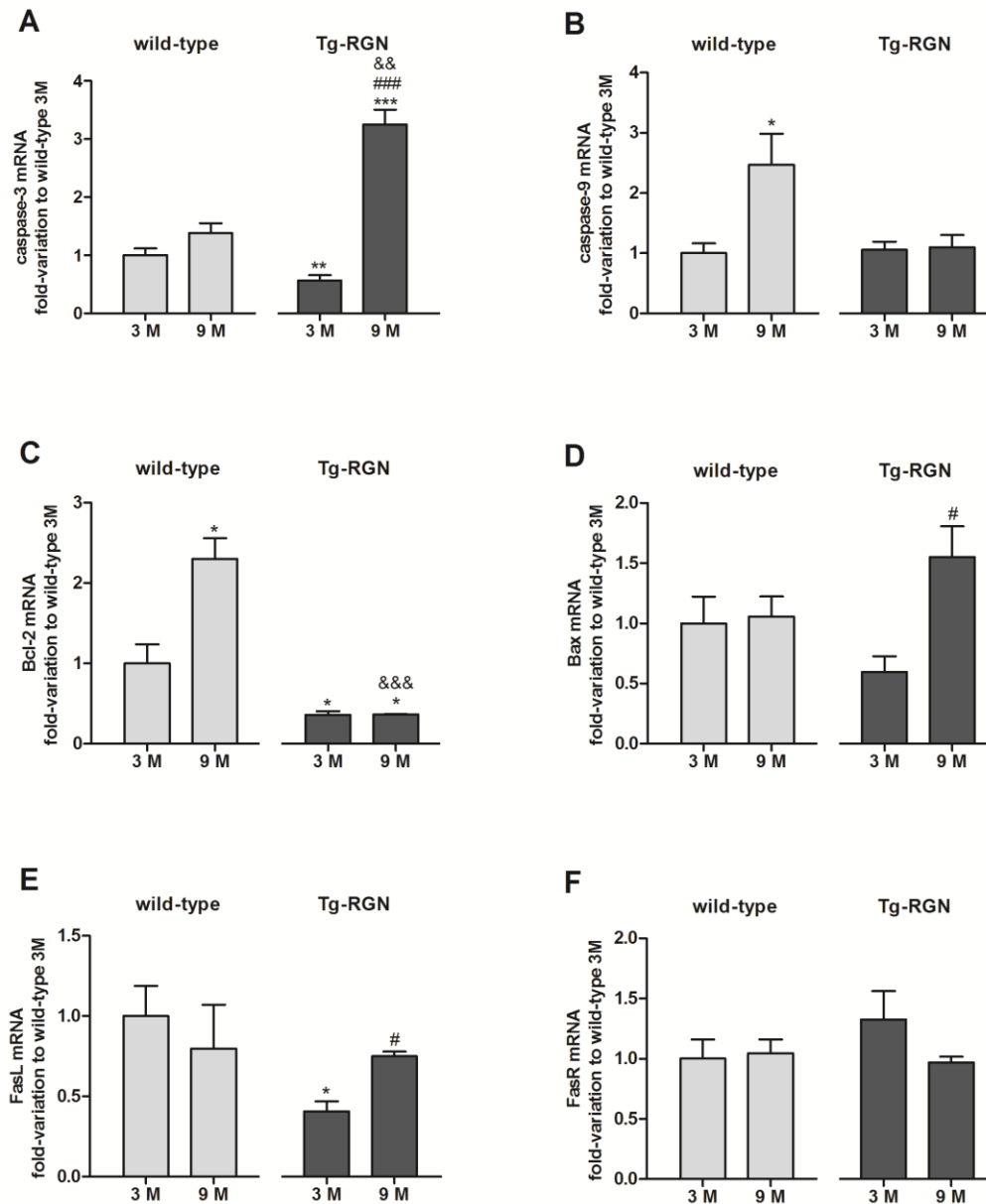


Figure 10.6. mRNA expression of caspase-3 (A), caspase-9 (B), Bcl-2 (C), Bax (D), FasL (E) and FasR (F) and in the prostate of 3 M and 9 M wild-type and transgenic rats overexpressing RGN (Tg-RGN). Expression was determined by qPCR after normalization with CypA and GAPDH housekeeping genes. Error bars indicate mean $\pm$ SEM ($n \geq 5$). Results are expressed as fold-variation relatively to the 3 M wild-type group. * statistically significant difference relatively to 3 M wild-type group (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). & statistically significant difference relatively to 9 M wild-type group (&& $p < 0.01$, &&& $p < 0.001$). # statistically significant difference relatively to 3 M Tg-RGN group (# $p < 0.05$; ### $p < 0.001$).

Caspase-9 and caspase-8 are initiators caspases involved, respectively, in the intrinsic and extrinsic pathways of apoptosis [27]. As shown in Figure 10.7A, protein levels of caspase-8 markedly increased with aging in wild-type animals (1 ± 0.08 fold in 3 M vs. 5.3 ± 1.2 fold in 9 M, $p < 0.001$). Both 3 M and 9 M Tg-RGN rats presented diminished expression of caspase-8 comparatively with their age matched wild-type counterparts (0.72 ± 0.08 fold, $p < 0.05$), but also in transgenics the expression of caspase-8 increased with aging (0.72 ± 0.08 fold in 3 M vs. 1.97 ± 0.42 fold in 9 M, $p < 0.01$). Caspase-9 mRNA expression significantly increased with

aging in wild-type animals (2.47 ± 0.51 fold, Figure 10.6B), an effect not observed at protein level. No significant differences were observed for caspase-9 mRNA and protein expression between others experimental groups (Figure 10.6B and Figure 10.7B).

Bcl-2 and Bax are, respectively, anti- and pro-apoptotic proteins involved in the regulation of the mitochondrial pathway of apoptosis [28]. Aging significantly increased Bcl-2 mRNA and protein expression in the prostate of wild-type animals (2.3 ± 0.26 and 3.62 ± 0.78 fold, respectively, Figure 10.6C and Figure 10.7C). Although the mRNA levels of Bcl-2 were significantly decreased in 3 M and 9 M Tg-RGN animals (0.36 ± 0.04 and 0.36 ± 0.003 fold respectively, Figure 10.6C), its protein expression was increased in 3 M Tg-RGN animals (2.52 ± 0.57 , $p < 0.05$, Figure 10.7C) relatively to the 3 M wild-type group.

Concerning Bax, no significant alterations were observed on the mRNA expression in all experimental groups when compared to the wild-type control group (3 M) (Figure 10.6D). However, the expression of Bax protein decreased with aging in wild-type animals (0.50 ± 0.08 fold, $p < 0.01$, Figure 10.7D). 3 M Tg-RGN animals displayed lower levels of Bax protein (0.63 ± 0.05 fold, $p < 0.01$, Figure 10.7D) relatively to their age matched wild-type counterparts, but advancing age significantly increased Bax protein expression in Tg-RGN (1.07 ± 0.09 fold, Figure 10.7D) both comparatively with wild-type 9 M animals ($p < 0.01$) and 3 M Tg-RGN ($p < 0.01$). This effect on Bax expression was also seen at mRNA level (0.60 ± 0.13 fold in 3 M Tg-RGN vs. 1.55 ± 0.25 fold in 9 M Tg-RGN, $p < 0.05$, Figure 10.6D).

The extrinsic pathway of apoptosis is triggered by death receptors at cell membrane, namely, the FasR, which is activated by its ligand the FasL [29]. No differences were detected on mRNA and protein levels of FasL and FasR between 3 M and 9 M wild-type animals (respectively, Figure 10.6E and Figure 10.7E; Figure 10.6F and Figure 10.7F). 3 M Tg-RGN animals displayed lower mRNA levels of FasL (0.41 ± 0.06 fold, Figure 10.6E) relatively to their wild-type counterparts. However, FasL mRNA and protein levels were significantly increased in 9 M Tg-RGN animals (0.75 ± 0.03 and 1.51 ± 0.26 fold, respectively) relatively to 3 M Tg-RGN animals (0.41 ± 0.06 and 0.94 ± 0.10 fold respectively, Figure 10.6E and Figure 10.7E). Also, FasL protein expression was highly augmented in 9 M Tg-RGN relatively to their age-matched wild-type controls (1.51 ± 0.26 fold vs. 0.64 ± 0.03 , $p < 0.01$). Concerning FasR, its protein expression was increased in Tg-RGN animals along aging (0.79 ± 0.02 fold in 3 M vs. 1.09 ± 0.08 fold in 9 M, $p < 0.05$, Figure 10.7F), and it was also significantly higher in 9 M Tg-RGN animals in relation to 9 M wild-type controls (1.09 ± 0.08 fold vs. 0.81 ± 0.05).

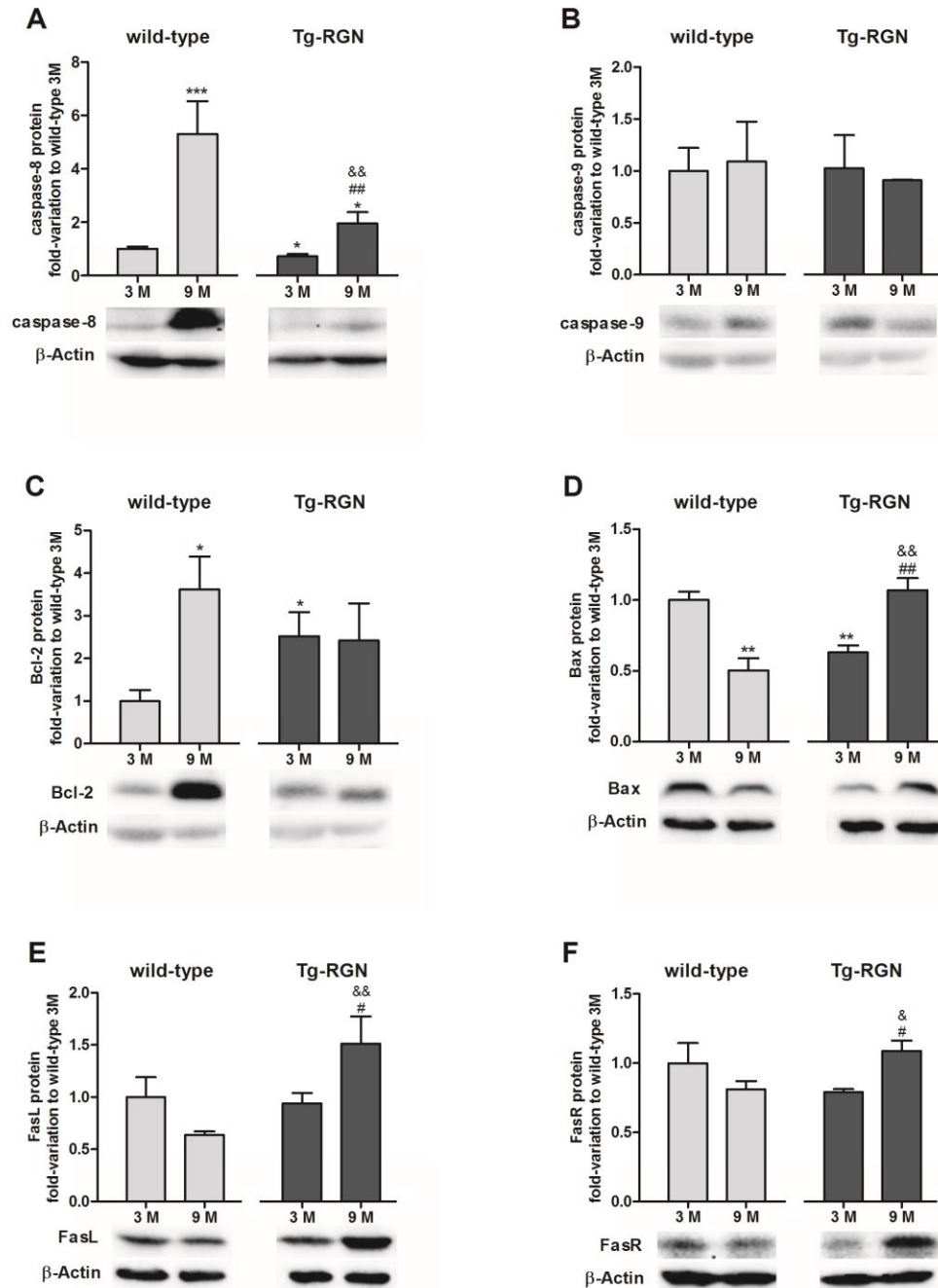


Figure 10.7. Protein levels of caspase-8 (A), caspase-9 (B), Bcl-2 (C), Bax (D), FasL (E) and FasR (F) in the prostate of 3 M and 9 M wild-type and transgenic rats overexpressing RGN (Tg-RGN), determined by WB analysis after normalization with β -actin. Results are expressed as fold-variation relatively to the 3 M wild-type group. Error bars indicate mean $\pm$ SEM ($n \geq 5$). Representative immunoblots are shown as bottom panels. * statistically significant difference relatively to 3 M wild-type group (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). & statistically significant difference relatively to 9 M wild-type group (& $p < 0.05$, && $p < 0.01$). # statistically significant difference relatively to 3 M Tg-RGN group (# $p < 0.05$; ## $p < 0.01$).

Age-related increase in prostate weight is suppressed in Tg-RGN animals

The variation of prostate weight with aging in wild-type and Tg-RGN animals was also evaluated. Figure 10.8 shows that both 3 M and 9 M Tg-RGN rats display lower prostate weight relatively to their wild-type counterparts with the same age (0.76 ± 0.07 and 0.79 ± 0.07 fold,

respectively, $p < 0.05$). Moreover, aging significantly increased prostate weight in wild-type animals (1.60 ± 0.08 fold), but such variation was not observed in Tg-RGN animals.

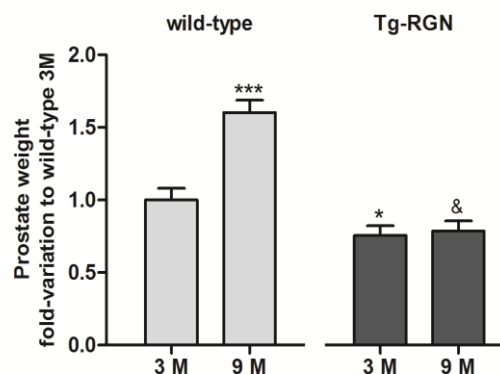


Figure 10.8. Prostate weight in young (3 M) and aged (9 M) wild-type rats and transgenics overexpressing RGN (Tg-RGN). Results are expressed as fold-variation relatively to the 3 M wild-type group. Error bars indicate mean $\pm$ SEM ($n \geq 5$). * statistically significant difference relatively to 3 M wild-type group (* $p < 0.05$; *** $p < 0.001$). & statistically significant difference relatively to 9 M wild-type group (& $p < 0.05$).

Discussion

In the present study, we demonstrated that the expression of RGN in rat prostate is significantly diminished with aging (Figure 10.1A, B). The decreased expression of RGN was first visible for 8 M animals and maintained after that in the prostate of older animals (9 M, 12 M and 24 M). This distinctive expression pattern of RGN accompanying aging was also reported in other rat tissues, namely, liver, kidney and testis [2, 3], and is a distinctive feature of RGN, which for this reason is also named SMP30. Besides its function in the maintenance of intracellular Ca^{2+} concentration, RGN seems to modulate oxidative stress levels, and control cell proliferation and apoptosis [4, 6], all known processes accumulating defects with aging. This led us to hypothesize that RGN may protect against age-associated deterioration of cell function. With this purpose, changes in oxidative stress, and proliferative and apoptotic status in the prostates of Tg-RGN and wild-type animals during aging were investigated. Since RGN protein levels are maintained unaltered in the prostate of Tg-RGN rats with aging (Figure 10.1C) any protective effect might be attributed to the overexpression of RGN.

The relationship between aging and increased oxidative stress is unquestionable, and, interestingly, as stated above, RGN has been considered to have antioxidant properties, contributing markedly to decrease cell oxidative stress levels [30-32]. In vitro and in vivo approaches have demonstrated that RGN exerts antioxidant effects by reducing the activity of enzymes related to the production of ROS, and increasing the activity of those involved in the antioxidant defense, namely, GST and SOD (reviewed in [4]). Accordingly, our results demonstrated that the prostate of young rats overexpressing RGN (3 M) presented lower levels of oxidative stress comparatively with their wild-type counterparts, as indicated by the

diminished lipid peroxidation measured by the TBARS assay (Figure 10.2A). Though, the TAC and the activity of GST were also decreased in 3 M Tg-RGN animals. In addition, no changes were perceived on SOD activity between 3 M Tg-RGN and wild-type groups. Altogether, these results indicate that the diminished oxidative stress in the prostate of 3 M Tg-RGN may be the consequence of decreased production of ROS instead of augmented antioxidant defense or scavenge of ROS.

Aging significantly increased TBARS levels both in wild-type and Tg-RGN rats (Figure 10.2A), which is concordant with previous findings showing that prostate of 9 M senescent rats display reduced capability in detoxification of oxygen-radicals due to decreased activity of catalase and glutathione peroxidase, and consequently increased lipid peroxidation [15]. Indeed, diminished activity of GST and decreased total antioxidant potential were observed in 9 M wild-type animals (Figure 10.2C). Curiously, however, the 9 M Tg-RGN group showed augmented TAC and a great increase in GST activity relatively to 3 M Tg-RGN animals, concomitantly with the characteristic increase in TBARS levels with advanced age. No response was observed concerning the activity of SOD in aged Tg-RGN rats, and also no significant differences were observed between Tg-RGN and wild-type groups (Figure 10.2D). Similar results were found in the testis and mammary gland of Tg-RGN comparatively with wild-type (results to be published elsewhere). Nonetheless, the results obtained strongly support the role of RGN as a regulator of oxidative stress levels in prostate and indicate that this protein may modulate the antioxidant defenses in response to the oxidative status. Senescent animals overexpressing RGN seem to be able to respond to age-related increase in oxidative stress, as suggested by the observed increase in antioxidant capacity and GST activity (Figure 10.2B and Figure 10.2C). Another evidences exist indicating the protective role of RGN in response to cell injury. Elevated levels of RGN were detected in rat brain in response to injury induced by administration of the neurodegenerative agent kainate [33]. Also, the upregulation of RGN was implicated in the attenuation of diet-induced liver injury after administration of 17 β -estradiol [34].

In a previous work, we found that prostates of 3 M Tg-RGN animals displayed reduced Ki67 cell proliferation index relatively to their wild-type counterparts [6]. This observation was further confirmed in this study by the increased levels of cell cycle inhibitor p21 (Figure 10.3C) and the lower prostate weight (Figure 10.8) detected in 3 M Tg-RGN, which support the lower proliferative activity described in the prostate of these animals. Moreover, the work presented here demonstrated that the overexpression of RGN in rat prostate counteracted age-related alterations on cell proliferation. As described by others, rat prostate weight significantly increase with age in consequence of cell overgrowth and independently of decreased testosterone levels [35], which is concordant with our results showing an approximately 60% increase in prostate weight in 9 M wild-type animals comparatively with 3 M (Figure 10.8). On the contrary, prostate weight in Tg-RGN did not changed with aging, and this was underpinned by a distinct expression pattern of SCF, c-kit system and p53. It is known that the tyrosine kinase receptor c-kit upon binding of SCF

activates several intracellular signaling cascades that promote cell proliferation [22]. Interestingly, the mRNA expression of SCF and c-kit is diminished in 9 M Tg-RGN relatively to the corresponding wild-type group. In addition, aging reduced the expression of SCF and c-kit in Tg-RGN, while c-kit levels remained unchanged in wild-type animals (Figure 10.3A and Figure 10.3B). Therefore, the overexpression of RGN seems to suppress age-associated overgrowth of rat prostate by diminishing the expression of SCF and c-kit.

The tumor suppressor p53 protein might drive apoptosis and cellular senescence [25, 36], and is also capable of detecting damage and act as an inducer of defense responses, decreasing the accumulation of ROS [36]. Therefore, the increased levels of p53 mRNA and protein in 9 M Tg-RGN support both the reduced cell proliferation and the augmented antioxidant activity observed in these animals.

We cannot avoid mentioning that in wild-type animals the mRNA expression of p53 (Figure 10.3D) do not followed the reduced protein levels of p53 observed in 9 M animals (Figure 10.4), and that this discrepancy between RNA and protein also occurs to other genes analyzed. This fact is not an uncommon finding [37, 38], which can be related to the great complexity of gene expression regulatory mechanisms in eukaryotes, namely, mRNA retention in the nucleus, processing, stability and half-life time of mRNA molecule, control of protein translation efficiency, post-translational modifications, and protein degradation. Nevertheless, whenever protein variations were detected these superimposed to mRNA results for discussion purposes since the physiological effects depend on protein activity.

Besides the augment on p53, aged Tg-RGN animals displayed increased expression of Bax, which is indicative of an apoptotic status in the prostate of Tg-RGN with aging. Indeed, aged animals overexpressing RGN showed increased expression of FasL, FasR and caspase-8 (Figure 10.7), as well as increased enzymatic activity of caspase-3 (Figure 10.5). These findings suggest a role for RGN in stimulating apoptosis with increasing age, which seems to occur mainly through the activation of the extrinsic pathway. In contrast, wild-type animals displayed the classical resistance to apoptosis with increasing age supported by the reduced activity of caspase-3, increased expression of Bcl-2 and diminished expression of Bax observed in 9 M relatively to 3 M (Figure 10.7). The lower rate of apoptosis also may be contributing to the huge increase in prostate weight observed in aged wild-type animals.

The molecular damage that leads to enhanced resistance to apoptosis, is a crucial point in accelerating aging process and tumor development [26]. In the light of the results obtained, the effect of RGN stimulating prostate cell apoptosis with aging may favor the elimination of cells with accumulated damage, which can be viewed as a protective mechanism against the development of prostate cancer. Indeed, we previously showed that in vivo overexpression of RGN had a protective role against the development of carcinogen-induced mammary gland tumors [7]. On the other hand, it has been shown that genetically engineered mice with augmented expression of p53 are highly resistant to cancer [39], which, considering the increased levels of p53 found in the prostate of 9 M Tg-RGN animals, further supports the role of RGN restricting tumor development. These findings also are concordant

with the past work of our group describing the diminished expression of RGN in human prostate cancer cases, and its negative association with the cellular differentiation of adenocarcinoma [9].

In conclusion, transgenic overexpression of RGN in the prostate of aged rats improved the antioxidant defenses, counteracted age-associated prostate overgrowth and prevented the age-induced resistance to apoptosis. These findings indicate a role for RGN protecting from age-related pathologies. Moreover, augmented oxidative stress, deregulated cell proliferation and resistance to apoptosis are known hallmarks of cancer. Therefore, though it is not known whether human prostate display age-associated decrease in RGN expression, the results presented herein also support that the diminished expression of RGN found in human prostate cancer cases may be an important mechanism favoring tumor development. This assumption raises the curiosity about the strategies to increase RGN levels in prostate tissues, which may be achieved by the encapsulation of RGN gene or protein plus an expedite model of delivery, such as nanoparticles. Nevertheless, much work has to be done to determine the best methods to encapsulate RGN and effectively promote its delivery to the prostate alone or in combination with anti-cancer drugs. Moreover, both in vitro and in vivo studies have to be done to ascertain and exclude non-expected side effects. Finally, the development of strategies to manipulate RGN levels in target tissues may have potential application in the future to alleviate age deterioration of cell function and prevent against progression of prostate cancer.

Funding

This work was partially supported by Portuguese Foundation for Science and Technology (FCT) under Program COMPETE (PEst-OE/SAU/UI0709/2014). Cátia V. Vaz and Ricardo Marques were funded by FCT fellowships (SFRH/BD/70316/2010 and SFRH/BD/66875/2009, respectively).

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Chapter 11

Summarizing discussion and future perspectives

Summarizing discussion and future perspectives

Prostate cancer (PCa) continues to be a worrying disease and, although several research efforts have contributed to improving diagnosis and treatment methods, it persists as a source of considerable morbidity and mortality for men worldwide. At the initial stages of PCa, androgens are the principal regulators of cell growth, but advanced stages are characterized by the emergence of an androgen-independent phenotype that usually is associated with a lethal stage of disease. Moreover, it is known that PCa is a complex disease that requires several genetic modifications and a set of metabolic alterations for the neoplastic transformation and to evolve for more aggressive stages.

Nowadays, the metabolic reprogramming is considered a hallmark of cancer, which was first established by the Warburg's studies. Early in the 1920s, Warburg proposed that tumor cells have the capacity to change their metabolism increasing the glycolytic activity with a high consumption of glucose and production of lactate to produce energy, which is now established as an important metabolic feature in tumor development. Also, it has been described that the lactate produced and exported to extracellular space contributes for the acidification of tumor environment, and may be used as a metabolic fuel for the neighboring cells, and so conferring a selective advantage that enhances survival and proliferation.

Several studies demonstrated that PCa cells have unique and distinctive metabolic features comparatively with normal cells. However, the metabolic alterations acquired in the transition to androgen-independent stages, namely those concerning the glycolytic activity, were poorly studied. In Chapter 5 of this thesis, the glycolytic metabolism between androgen-responsive (LNCaP) and androgen-nonresponsive PCa cell lines (PC3) was compared, and we demonstrated that LNCaP and PC3 cells present a distinct glycolytic profile. Although no changes were observed on glucose consumption between both cell lines, PC3 cells showed enhanced production and export of lactate as a result of increased activity of lactate dehydrogenase (LDH) activity and augmented expression of monocarboxylate transporter (MCT) 4. In line with these results, a higher lactate/alanine ratio was also detected in PC3 cells suggesting an increase in oxidative stress levels in this cell line model of more aggressive stages of PCa. These findings suggest that in the progression to androgen-independent stages PCa cells acquire new metabolic strategies, enhancing the glycolytic metabolism (and also oxidative stress), by altering the expression and activity of key regulators of this metabolic pathway, such as LDH and MCT4 (Figure 11.1).

Taking into account the described results, and the well-known role of androgens in controlling PCa cell growth, it became imperative to decipher the role of this sex steroids in the regulation of glycolytic metabolism in PCa cells, especially in lactate handling. This study was performed and included as Chapter 6. Androgens treatment increased the glucose uptake, glycolysis rate and the export of lactate in androgen-responsive LNCaP cells, by upregulating the expression of glucose transporters (GLUTs) 1 and 3 (GLUT1 and GLUT3), phosphofructokinase (PFK) and MCT4 (Figure 11.1). Moreover, we showed that the androgen receptor (AR) seems to be directly involved in this regulation. The reprogramming of cell

metabolism that occurs in response to androgens seems to favor survival and proliferation of PCa cells.

Although studies presented in Chapters 5 and 6 relied on *in vitro* approaches, they highlighted the importance of controlling glucose and lactate metabolism as possible therapeutic approaches in PCa. Together with studies demonstrating that AR stimulation and inhibition, respectively, increase and decrease glucose utilization in PCa cases [1, 2], our results suggest that combined therapies targeting simultaneously AR signaling and glycolysis may represent an effective strategy to improve the outcomes of PCa treatment.

We cannot exclude from the discussion the existing evidence indicating that other metabolic alterations, namely in the fatty acids and glutamine metabolism, occur in the transition of PCa cells from androgen-dependent to nondependent stage. It has been shown that metabolization of glutamine, and also fatty acids, contributes to the generation of complementary metabolic pathways that lead to the uncontrolled tumor cell proliferation, survival and invasion capacity. However, it remains to be clarified whether androgens also have the ability to modulate these alternative metabolic pathways. Additionally, further research is needed to understand the overall metabolic pathways altered in PCa cells in the diverse stages of tumor development, and to unveil how androgens trigger these metabolic adaptations. Disclosing this issues will certainly improve the therapeutic options available for PCa.

The importance of the glycolytic genes not only in the regulation of glycolytic flux, but also in carcinogenesis, in the regulation of cell cycle and cell death, and in cell response to oxidative stress has been reported [3]. As an example, LDH controls the production of lactate, but it also can be translocated to the nucleus where it facilitates the progression in cell cycle coupled with GAPDH and other transcription factors [3, 4]. GAPDH and LDH are both enzymes involved in glycolytic steps associated with the oxidation or reduction reaction of NADH to NAD⁺ and vice-versa. NAD⁺ and NADH, respectively, stimulate and inhibit LDH and GAPDH binding to the DNA. It was proposed that the inhibition of the catalytic activity of NADPH and LDH may result in a deregulation of NADH/NAD⁺ ratio, and the translocation of these proteins to the nucleus, may stimulates cell cycle progression and cell proliferation [3]. So, the inhibition of glycolytic genes as therapeutic option needs to be carefully studied in different cells and physiological contexts. Nevertheless, the use of combined therapies targeting glycolytic regulators and the proteins involved the regulation of the prostate cell growth also emerges as a promising option to treat PCa. Interestingly, a couple of proteins have been referred to exert multifunctional roles in the control of tissue cell homeostasis and assume a front standing in this field. This is the case of the tumor suppressor protein p53 and regucalcin (RGN).

Regucalcin is a calcium (Ca²⁺)-binding protein playing an important role in the maintenance of intracellular Ca²⁺ homeostasis, but also in the regulation of other important physiologic processes, such as cell proliferation and apoptosis. Our research group, previously have demonstrated that RGN expression is lost in cases of human PCa comparatively with the

normal tissues, and that the RGN immunoreactivity is negatively correlated with the cellular differentiation of prostate adenocarcinoma [5]. In Chapter 8, we have demonstrated that RGN expression also is lower in human neoplastic prostate cell lines (LNCaP and PC3) in comparison to the non-neoplastic PNT1A cell line. The available information indicates that loss of RGN expression is related to tumor onset, progression and aggressiveness of PCa. On the other hand, both androgens and Ca^{2+} are well-known regulators of the prostate physiology, and also of PCa cell growth. Previous studies of our research group showed that RGN is an androgen-target gene in PCa cells, being down-regulated in response to androgens stimulation [5]. A finding that we confirmed to be translated *in vivo*, as demonstrated by the obtained results in rat prostate (Chapter 7).

Other hormonal and non-hormonal factors, including Ca^{2+} , have been described to control RGN expression in different cell lines and tissues [6]. The effect of extracellular Ca^{2+} (Ca^{2+}_o) on RGN expression was studied in different prostate cell lines (Chapter 8). We verified that the Ca^{2+}_o differentially regulates the expression of RGN in LNCaP and PNT1A cells, and that this regulation is time- and dose-dependent. Furthermore, PNT1A cells seem to exhibit more pronounced effects in up-regulating the expression of RGN in response to Ca^{2+} administration. The fact that both androgens and Ca^{2+} regulate RGN levels in prostate cells (Figure 11.1) strongly points this protein as an important target in the regulation of prostatic physiology. Interestingly, in Chapter 8, we also observed that high Ca^{2+}_o concentrations trigger different effects on cell proliferation of PNT1A and LNCaP cells, which seems to be related to the RGN expression levels. The effects of Ca^{2+}_o in regulating cell proliferation, differentiation and apoptosis also has been linked to the activity of its cognate receptor, the Ca^{2+} -sensing receptor (CaSR), which is found to be overexpressed in PCa cases [7, 8]. Still in Chapter 8, we verified that the effect of Ca^{2+}_o on the expression of CaSR may depend on the expression of RGN, a conclusion that was drawn by the fact that the silencing of RGN increased the levels of CaSR, whereas its overexpression down-regulated CaSR expression. These results demonstrated that RGN is involved in the regulation of Ca^{2+} -dependent signaling pathways in prostate cells and suggested that the up-regulation of RGN expression may act protecting cells from accelerated proliferation (Figure 11.1).

Altogether the presented findings highlighted the importance of RGN in prostate physiology and pathophysiology, but the molecular mechanisms underlying its actions need full clarification. Therefore, the influence of RGN on cell proliferation, and cell cycle and apoptosis pathways in prostatic tissues was demonstrated under conditions of protein overexpression *in vivo* (Chapter 7). Overexpression of RGN suppressed cell proliferation and diminished the expression of the oncogene H-ras, whereas the expression of the cell-cycle inhibitor p21 was increased. In addition, RGN diminished apoptosis in rat prostate as a result of the decreased expression of caspase-8 and reduced activity of caspase-3, concomitantly with an increase in Bcl-2/Bax protein ratio. Thus, we demonstrated that RGN exerted a dual role in reducing both cell proliferation and apoptosis in rat prostate and thus, its influence in maintaining prostate growth balance was established (Figure 11.1).

Another interesting function that has been attributed to RGN is their ability to regulate several metabolic pathways, including the activity of some regulators of glycolytic metabolism, which we demonstrate to be crucial in the progression of PCa cells (Chapters 5 and 6). In Chapter 9 of this thesis, the effect of RGN on the glycolytic activity of the rat prostate was evaluated. A decreased consumption of glucose and lactate production and export were observed in the prostate of animals overexpressing RGN, which was a consequence of the diminished expression (and activity) of important regulators of the glycolytic flux, such as GLUT3, PFK, LDH and MCT4 (Figure 11.1). These results suggest that the RGN actions controlling proliferation of rat prostate cells may be partly achieved by the regulation of glycolytic flux. In a future work, it will be of interest to undisclosed the effect of RGN in the other metabolic pathways that also have been associated with the development of prostate pathophysiology.

Regucalcin also has been linked with the decrease of oxidative stress levels and the increase of antioxidant defenses. Since abnormal oxidative stress is a crucial issue in malignant cell transformation, Chapter 10, was dedicated to the analysis of oxidative status in the prostate of rats overexpressing RGN. These animals displayed decreased lipid peroxidation, which indicates that RGN can reduce the production of reactive oxygen species in rat prostate.

The deregulation of Ca^{2+} -signaling pathways, the uncontrolled cell proliferation and resistance to apoptosis, the reprogramming of energy metabolism, and the continuous induction of oxidative stress, are all known mechanisms involved in tumor onset and invasion. The present work demonstrated that RGN is a protein involved in the regulation of all these biological processes in prostate cells. Also, androgens and Ca^{2+} , which have a crucial role in maintaining cell tissue homeostasis, were demonstrated to be regulators of RGN expression in prostate cells. Altogether these findings support the idea that deregulated expression of RGN may lead to loss of antioxidant defenses and may predispose to a hyper glycolytic phenotype fostering the proliferation of prostate cells, which is halfway to the development of PCa (Figure 11.1).

Age is considered a major risk factor for the appearance of PCa. Interestingly, RGN is also known as senescence marker protein-30 due its down-regulated expression with aging in the liver, kidney and testis [6, 9]. In Chapter 10 we demonstrated that also in rat prostate the expression of RGN diminishes with the increasing age. As we have been discussing, RGN has a role in controlling oxidative stress levels and also in cell proliferation and apoptosis in rat prostate, all known processes deregulated with aging. This suggests that the diminished expression of RGN with aging may contribute to the age-associated deterioration of cell function. For this reason, the impact of maintaining high expression levels of RGN in preventing age-associated changes in oxidative stress, as well as, in cell proliferation and apoptosis in rat prostate was analyzed in Chapter 10. We demonstrated that the overexpression of RGN augmented the total antioxidant capacity and the activity of the antioxidant protein, glutathione S-Transferase, in response to the age-characteristic increase

in lipid peroxidation in rat prostate. In addition, prostates of aged animals overexpressing RGN presented higher levels of p53 and reduced expression of the pro-proliferation stem cell factor/c-kit system, together with the increased expression of apoptosis inducers, such as Bax, FasL, FasR and caspase-8, as well as, augmented activity of caspase-3. These results suggest a role for RGN in preventing the development of aged-associated diseases in rat prostate, improving the antioxidant defenses, preventing the age-induced resistance to apoptosis, and the excessive proliferation usually found in the prostate of aged animals and in PCa (Figure 11.1).

The obtained results suggest that would be interesting to increase RGN levels to counteract age deterioration of cell function, prostate overgrowth and progression of PCa, which raises the question about the measures to be undertaken to achieve this goal. The idea that promptly comes up is the use of RGN protein as a nutritional supplement. However, the use of proteins as therapeutics has some limitations due to their complex structures, degradation rate, *in vivo* stability, low permeability and immunogenicity. These constraints have been overcome by the development of expedite modes of delivery, which include, among others, several nanoparticle platforms, namely, liposomes and polymer-based nanoformulations [10]. Recently, the use of exosomes-based delivery systems have been gaining importance, but further investigation is needed before their application to clinical studies [11]. Thus, the encapsulation of RGN gene or protein in nanoparticles, alone or in combination with other anti-cancer drugs, as metabolic inhibitors, positions itself as good strategy for prevention/treatment of PCa. Nevertheless, much work has to be done to determine the best methods to encapsulate RGN and effectively promote its delivery to the prostate. Moreover, both *in vitro* and *in vivo* studies have to be done to ascertain and exclude other non-expected side effects.

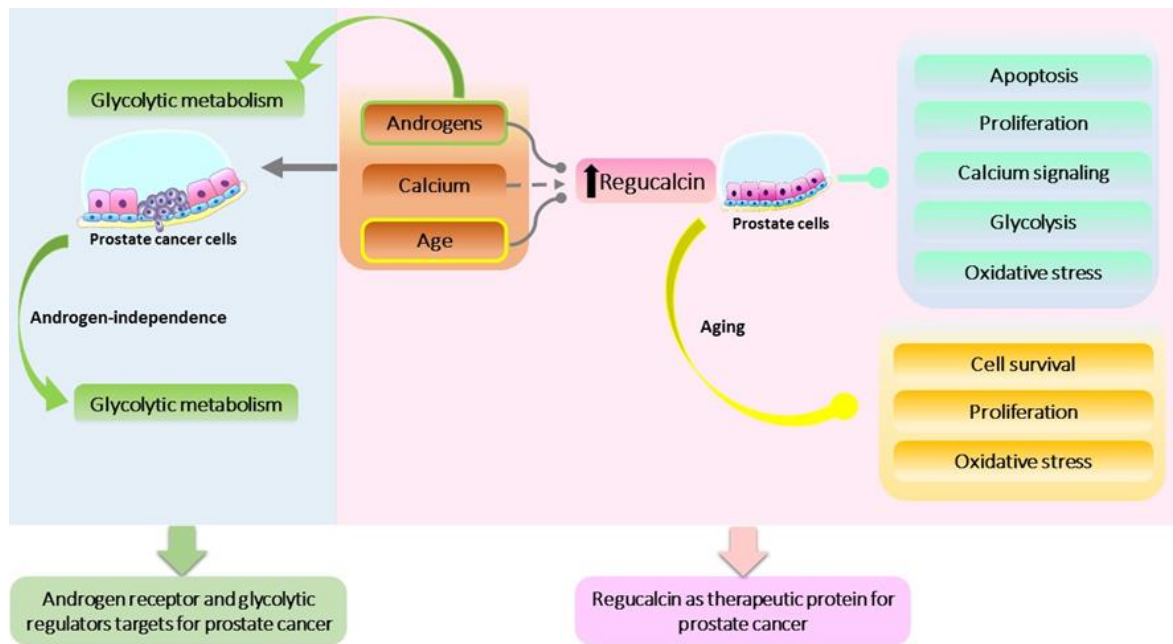


Figure 11.1. An integrative view of the potential actions of androgens and regucalcin (RGN) in the control of glycolytic metabolism and cell survival in prostate cells, and the implications in carcinogenesis. Androgens regulate the glycolytic metabolism in prostate cancer (PCa) cells through increasing the expression/activity of metabolic regulators, and androgen-nonresponsive PCa cells display a higher glycolytic flux comparatively with androgen-responsive cells. Thus, the androgen receptor signaling and glycolytic regulators represent attractive targets for PCa treatment. Androgens, calcium signaling, and age, well-known factors in the development of PCa, regulate the expression of RGN in prostate cells. Also, PCa tissues/cells display diminished expression of RGN comparatively with nonneoplastic cells. In human prostate cells *in vitro* or in rat prostate *in vivo*, RGN regulates several proteins involved in the control of important biological processes, inhibiting apoptosis, proliferation, calcium signaling, oxidative stress and glycolytic metabolism. Interestingly, the overexpression of RGN counteracts the effect of aging in the deregulation of the physiologic processes in rat prostate, improving antioxidant defenses, preventing the age-induced resistance to apoptosis, and the excessive proliferation usually found in the prostate of aged animals, and in PCa. These results suggest that in the future, RGN may be an interesting therapeutic protein to be use in PCa. Legend: Arrows and ball-ended arrows indicate activation and inhibition, respectively. Dashed arrows indicate that up- or down-regulation depends on the dose and time of stimulation.

The work presented in this thesis advanced the existing knowledge on the androgens actions in the development of PCa, and demonstrated that they stimulate the alterations in the glycolytic metabolism during PCa progression, further establishing the role of androgens as “the good friends” of prostate tumor cells. On the other hand, the loss of RGN with aging appeared as a “powerful foe” of prostate cells contributing to the aged-associated pathophysiologic alterations, and to the neoplastic transformation. Even more, the present findings launch new challenges for the development of future therapies for PCa, and to prevent their evolution to more aggressive stages, based on the use of combined therapies, targeting AR and glycolytic metabolic regulators, and on the potential of RGN as therapeutic protein.

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