UNIVERSIDADE DA BEIRA INTERIOR
Ciências da Saúde

Biological evidence of the protective role of regucalcin in breast cancer

Ricardo Jorge Fernandes Marques

Thesis for Doctoral Degree in

Biomedicine

(3rd cycle of studies)

Supervisor: Prof. Doutora Sílvia Cristina da Cruz Marques Socorro, PhD

Co-supervisor: Prof. Doutora Cecília Reis Alves dos Santos, PhD

Covilhã, February 2016

“Somewhere, something incredible is waiting to be known.”

Carl Sagan

“The scientist is not a person who gives the right answers,
he's one who asks the right questions.”

Claude Lévi-Strauss

Agradecimentos

Nesta altura é extremamente difícil expressar a minha gratidão, a todos aqueles que durante estes últimos anos, directa ou indirectamente me ajudaram ao longo deste percurso.

Antes de mais, quero expressar o meu especial agradecimento aos meus orientadores.
À Professora Doutora Sílvia Socorro a quem agradeço a oportunidade e a confiança em mim depositada, ao aceitar-me como aluno de doutoramento e pelo seu incansável apoio, conselhos, e dedicação. Muito obrigado, pela prontidão com que sempre se disponibilizou para me ajudar.
À Professora Doutora Cecília Santos, quero agradecer todo o apoio e a sua disponibilidade ao longo destes anos.

Ao Professor Doutor Cláudio Maia, por todos os ensinamentos transmitidos e esclarecimentos científicos.
Ao Professor Doutor Ignacio Verde e à Professora Doutora Elisa Cairrão, por toda a ajuda prestada com a medição da atividade das correntes de cálcio.

A todos os colegas e amigos de laboratório, Cátia Vaz, Sara Correia, Luís Pedro Rato, Margarida Gonçalves, Inês Gomes, Carlos, Carina Peres, Marília Figueira, Henrique Cardoso. Obrigado por todos os momentos que partilharam comigo.

A todas as pessoas do CICS, incluindo as técnicas Sofia Duarte e a Margarida Carrilho pelo apoio e boa disposição.

A ti Rute, um obrigado muito muito especial, por todo o apoio incondicional, tornando cada momento vivido bem mais leve. Contigo partilhei todas as alegrias, dúvidas e muitas frustrações, de quem sonha ser cientista e ainda assim ser feliz. Obrigado!

Finalmente a toda a minha família, aos meus pais, irmãos, cunhada, sobrinhas e sogros, agradeço-vos toda a paciência, compressão, confiança e apoio ao longo destes anos.

Por último, gostaria de agradecer à FCT por financiar o meu doutoramento (SFRH/BD/66875/2009).

Publications in International Peer-Reviewed Journals

Included in the thesis:

Ricardo Marques, Cláudio J. Maia, Cátia Vaz, Sara Correia, Sílvia Socorro (2014) The diverse roles of calcium-binding protein regucalcin in cell biology: from tissue expression and signaling to disease. Cell Mol Life Sci. **71**(1): 93-111.

Ricardo Marques, Cátia V. Vaz, Cláudio J. Maia, Madalena Gomes, Adelina Gama, Gilberto Alves, Cecília R. Santos, Fernando Schmitt, Sílvia Socorro (2015) Histopathological and *in vivo* evidence of regucalcin as a protective molecule in mammary gland carcinogenesis. Exp Cell Res. 330: 325-335.

Ricardo Marques, Cátia V. Vaz, Sílvia Socorro Glycolytic metabolism in the mammary gland of transgenic rats overexpressing calcium-binding protein regucalcin: new clues for the protective role against tumor development. Cellular Oncology (submitted)

Ricardo Marques, Carina G. Peres, Cátia V. Vaz, Inês M. Gomes, Marília I. Figueira, Elisa Cairrão, Ignacio Verde, Cláudio J. Maia, Sílvia Socorro (2015) 5α-dihydrotestosterone regulates the expression of L-type calcium channels and calcium-binding protein regucalcin in human breast cancer cells with suppression of cell growth. Med Oncol 32:228

Other publications during the PhD:

Marília I. Figueira, Sara Correia, Cátia V Vaz, Henrique J. Cardoso, Inês M Gomes, Ricardo Marques, Cláudio J Maia, Sílvia Socorro Estrogens down-regulate the stem cell factor (SCF)/c-KIT system in prostate cells: evidence of antiproliferative and apoptotic effects. (submitted)

Cátia V. Vaz, Ricardo Marques, Henrique J. Cardoso, Cláudio J Maia and Sílvia Socorro Suppressed glycolytic metabolism in the prostate of transgenic rats overexpressing calcium-binding protein regucalcin underpins reduced cell proliferation (submitted)

Cátia V. Vaz, Ricardo Marques, Claudio J Maia and Sílvia Socorro (2015) Aging-Associated Changes in Oxidative stress, Cell proliferation and Apoptosis are Prevented in the Prostate of Transgenic Rats Overexpressing Regucalcin. Transl Res doi:10.1016/j.trsl.2015.08.009

Cátia V. Vaz, Ricardo Marques, Marco G. Alves, Pedro F. Oliveira, José E. Cavaco, Cláudio J. Maia, Sílvia Socorro (2015) Androgens enhance the glycolytic metabolism and lactate export in

prostate cancer cells by modulating the expression of GLUT1, GLUT3, PFK, LDH and MCT4 genes. J Cancer Res Clin Oncol doi: 10.1007/s00432-015-1992-4

Henrique J. Cardoso, Cátia V. Vaz, Sara Correia, Marília I. Figueira, Ricardo Marques, Cláudio J. Maia, Sílvia Socorro (2015) Paradoxical and contradictory effects of Imatinib in two cell line models of prostate cancer. Prostate 75(9):923-935

Cátia V. Vaz, Claudio J Maia, Ricardo Marques, Gomes IM, Correia S, Alves MG, Cavaco JE, Oliveira PF, Socorro S (2014) Regucalcin is an androgen-target gene in the rat prostate modulating cell-cycle and apoptotic pathways. Prostate 74 (12):1189-98

Cátia V. Vaz, Marco G. Alves, Ricardo Marques, Pedro F. Oliveira, Claudio J. Maia, Sílvia Socorro (2013) 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 ISBN: 978-1-62618-670-5.

Cátia V. Vaz, Marco G. Alves, **Ricardo Marques**, Paula I. Moreira, Pedro F. Oliveira, Cáudio J. Maia, Sílvia Socorro (2012) Androgen-responsive and nonresponsive prostate cancer cells present a distinct glycolytic metabolism profile. Int J Biochem Cell Biol 44, 2077-2084

Resumo

O cancro da mama é uma doença heterogénea que compreende uma grande variedade de alterações moleculares e diferentes tipos de resposta em termos clínicos. Esta diversidade reside nos múltiplos fatores que podem levar à transformação maligna das células, em consequência da desregulação de diferentes processos fisiológicos.

A regucalcina (RGN) é uma proteína de ligação ao cálcio (Ca^{2+}) e cuja principal função conhecida é regular a homeostase do Ca^{2+} intracelular, mas podendo também estar envolvida na regulação da proliferação celular, apoptose e metabolismo das células. A RGN também foi identificada como um gene regulado por hormonas, incluindo os esteroides sexuais como os androgénios. Para além disso, foi anteriormente associada a determinadas patologias e tendo mesmo sido identificada como uma proteína subexpressa em casos humanos de cancro da mama, próstata ou fígado. No fígado, a subexpressão da RGN foi detetada em lesões pré-neoplásicas, ou seja, antes da aquisição do fenótipo neoplásico, o que sugere que a sua diminuição pode estar ligada ao início do processo de transformação tumorigénico. Apesar destas evidências, os mecanismos moleculares subjacentes às funções da RGN na mama permanecem por identificar. Nesta tese, colocámos a hipótese de que a sobreexpressão da RGN poderá exercer uma ação protetora em relação à carcinogénese mamária. De modo a avaliar esta questão, o composto 7,12 dimetilbenz[α]antraceneno, o qual é conhecido por induzir carcinogénese mamária em rato, foi administrado a ratos transgénicos que sobreepressam a RGN (Tg-RGN) e aos respetivos controlos (Wt, do inglês wild-type). Os ratos Tg-RGN apresentaram, notavelmente, uma menor incidência de tumores (25.8 %) comparativamente aos animais controlo (100 %). A classificação histológica também demonstrou uma clara resistência dos ratos Tg-RGN à tumorigénese, ao serem bastante mais resistentes à progressão dos tumores para estadios mais agressivos. Verificou-se uma muito menor percentagem de tumores do tipo invasivo nos animais transgénicos (3.8 % *vs* 45.8 % nos Wt). Para além disso, foi observado um aumento da atividade proliferativa nos tumores não-invasivos nos Wt comparativamente aos animais TG-RGN, o que indica a menor capacidade invasiva. A avaliação metabólica dos tumores benignos da glândula demonstrou que os tumores de ratos Tg-RGN possuem uma menor expressão e atividade da lactato desidrogenase (LDH), característica que normalmente se encontra associada a uma restrição da progressão tumoral e a um decréscimo da agressividade. Contudo, em tecido mamário não-neoplásico de ratos Tg-RGN observou-se uma restrição do metabolismo glicolítico, o que é indicativo de uma redução dos níveis energéticos no tecido. Estes resultados podem ser de extrema importância para a diminuição da proliferação celular e constituir um mecanismo adicional, pelo qual a RGN previne o desenvolvimento tumoral. De facto, a sobreexpressão da RGN originou uma diminuição da expressão de genes envolvidos na regulação ciclo celular e de oncogenes na glândula mamária de ratos Tg-RGN. Mais ainda, a expressão do P53 e a atividade da caspase-3 também foi encontrada aumentadas concomitantemente com a sobreexpressão

da RGN, o que sugere uma ação protetora da RGN no aparecimento do tumor, a qual pode ser mediada também pela regulação das vias apoptóticas. Em contrapartida, a expressão da RGN em células MCF-7 de cancro da mama diminui pela ação do androgénio não-aromatizável 5α-dihidrotestosterona, ao passo que a expressão do canal de Ca^{2+} do tipo L aumentou. Estes resultados sustentam a diminuição da viabilidade celular evidenciada nestas células e sugerem o envolvimento destes modeladores do Ca^{2+} no controlo da proliferação celular mamária, o que no caso do canal de Ca^{2+} do tipo L nunca antes tinha sido sugerido.

Em conclusão, o trabalho apresentado nesta tese destaca a preponderância da RGN numa diversidade de mecanismos fisiológicos e fisiopatológicos na glândula mamária. As evidências biológicas aqui apresentadas confirmam o papel protetor da RGN na carcinogénese mamária, ao restringir processos biológicos reconhecidos como fundamentais para o desenvolvimento do cancro, nomeadamente, a proliferação celular e as alterações no metabolismo celular, ao mesmo tempo que aumenta a morte celular por apoptose.

Palavras-chave

Apoptose; Cancro da mama; Glândula mamária; Metabolismo glicolítico; Proliferação; Regucalcina; Tg-RGN;

Resumo Alargado

O cancro da mama é uma doença heterogénea que compreende uma grande variedade de alterações moleculares e diferentes tipos de resposta, em termos clínicos. Apesar da evolução clinica registada nos últimos anos, o cancro da mama continua a ser a segunda maior causa de morte a nível mundial, com cerca 1.7 milhões de casos confirmados. A dificuldade na compressão desta doença reside, em boa parte, na ampla diversidade de fatores que podem levar à transformação maligna das células, em consequência da desregulação de diferentes processos fisiológicos. Alguns dos fatores envolvidos nas alterações celulares e transformação neoplásica incluem hormonas, fatores de crescimento, oncogenes, genes supressores de tumores, ou mesmo o estilo de vida, como por exemplo, a dieta, a obesidade ou a prática de exercício físico regular. Porém a própria composição estrutural da mama que, ao incluir células epiteliais, células estaminais e constituintes do estroma como os fibroblastos, constitui uma complexidade adicional para o desenvolvimento do cancro da mama. Esta heterogeneidade tem levado à procura incessante de componentes que permitam uma melhoria na deteção precoce da doença, na classificação fenotípica tumoral, assim como no desenvolvimento de terapêuticas mais eficazes.

A regucalcina (RGN) é uma proteína de ligação ao cálcio (Ca^{2+}), cuja principal função conhecida é regular a homeostase do Ca^{2+} intracelular, ao atuar através da modulação da atividade de canais e transportadores de Ca^{2+} na membrana celular, retículo endoplasmático e mitocôndria. Contudo, a RGN também está envolvida na regulação da proliferação celular, apoptose, stress oxidativo, metabolismo e sinalização celular, de forma independente ou dependente da calmodulina. A RGN também foi identificada como um gene regulado por hormonas, incluindo os esteróides sexuais como os androgénios, mas também pelo Ca^{2+} ou mesmo pelo stress oxidativo. Para além disso, a RGN foi anteriormente associada a determinadas patologias, como a distrofia muscular ou a doença de Parkinson e, determinante para este estudo, foi identificada como uma proteína subexpressa em casos humanos de cancro da mama, próstata ou fígado. No fígado, a subexpressão da RGN foi detetada em lesões pré-neoplásicas, ou seja, antes da aquisição do fenótipo neoplásico, o que sugere que a sua diminuição pode estar ligada ao início do processo de transformação tumorigénico. Apesar destas evidências, os mecanismos moleculares subjacentes às funções da RGN na mama permanecem por identificar. Nesta tese, colocámos a hipótese de que a sobreexpressão da RGN poderá exercer uma ação protetora em relação à carcinogénese mamária. De modo a avaliar esta questão, o composto 7,12 dimetilbenz[α]antraceneno, o qual é conhecido por induzir carcinogénese mamária em rato, foi administrado a ratos transgénicos que sobreepressam a RGN (Tg-RGN) e aos respetivos controlos (Wt, do inglês wild-type). Os ratos Tg-RGN apresentaram, notavelmente, uma menor incidência de tumores (25.8 %) comparativamente aos animais controlo (100 %). A classificação histológica dos tumores também demonstrou a clara resistência dos ratos Tg-RGN à

tumorigénese, ao serem bastante mais resistentes à progressão dos tumores para estadios mais agressivos. Verificou-se uma muito menor percentagem de tumores do tipo invasivo nos animais transgénicos (3.8 % *vs* 45.8 % nos Wt), e estes foram os únicos a desenvolverem tumores com características de lesões pré-neoplásicas. Nenhum animal Wt apresentou lesões pré-neoplásicas da glândula mamária. Para além disso, foi observado um aumento da atividade proliferativa nos tumores não-invasivos nos Wt, comparativamente aos animais TG-RGN, o que indica a menor capacidade invasiva. A avaliação metabólica dos tumores benignos da glândula demonstrou que os tumores de ratos Tg-RGN possuem uma menor expressão e atividade da lactato desidrogenase (LDH), característica que normalmente se encontra associada a uma restrição da progressão tumoral e a um decréscimo da agressividade. Estes resultados estão de acordo com a diminuição do índice proliferativo, avaliado pela marcação do Ki67 nestes tumores não-invasivos. Contudo, em tecido mamário não-neoplásico de ratos Tg-RGN observou-se uma restrição do metabolismo glicolítico, ao se verificar uma menor concentração de glucose no tecido, que se supõe dever-se à diminuição da expressão do transportador de glucose 3. Para além disso, o decréscimo dos níveis de expressão e atividade da fosfofructocinase, uma enzima limitante da glicólise, na glândula mamária dos ratos Tg-RGN, sugere uma restrição do metabolismo glicolítico e é indicativo de uma redução dos níveis energéticos no tecido. Adicionalmente, em tecido mamário não-neoplásico foram encontrados níveis elevados de lactato, o que se pensa poder estar associado ao decréscimo de expressão de um dos transportadores, o MCT4, e ao aumento da atividade da LDH. Níveis intracelulares elevados de lactato estão descritos como podendo promover a inibição da glicólise. Assim, estas alterações metabólicas podem ser de extrema importância para a diminuição da proliferação celular e constituir assim um mecanismo adicional, pelo qual a RGN previne o desenvolvimento tumoral. De facto, a sobreexpressão da RGN originou uma diminuição da expressão de genes envolvidos na regulação ciclo celular, como a Cdk1 e o oncogene Myc, na glândula mamária de ratos Tg-RGN. Mais ainda, a expressão do P53 e a atividade da caspase-3 também se encontraram aumentadas concomitantemente com a sobreexpressão da RGN, o que sugere uma ação protetora da RGN no aparecimento do tumor, a qual pode ser mediada também pela regulação das vias apoptóticas. Em contrapartida, a expressão da RGN em células MCF-7 de cancro da mama diminui pela ação do androgénio não-aromatizável 5α-dihidrotestosterona, ao passo que a expressão do canal de Ca^{2+} do tipo L aumentou. Estes resultados sustentam a diminuição da viabilidade celular evidenciada nestas células e sugerem o envolvimento destes modeladores do Ca^{2+} no controlo da proliferação celular mamária, o que no caso do canal de Ca^{2+} do tipo L nunca antes tinha sido sugerido.

Em conclusão, o trabalho apresentado nesta tese destaca a preponderância da RGN numa diversidade de mecanismos fisiológicos e fisiopatológicos na glândula mamária. As evidências biológicas aqui apresentadas confirmam o papel protetor da RGN na carcinogénese mamária, ao restringir processos biológicos reconhecidos como fundamentais para o desenvolvimento do cancro, nomeadamente, a proliferação celular e as alterações no metabolismo celular, ao mesmo tempo que aumenta a morte celular por apoptose.

Abstract

Breast cancer is a heterogeneous disease that comprises a wide variety of molecular alterations and divergent clinical behaviors. This diversity resides in a plethora of factors that can drive cell malignant transformation, by the deregulation of basic physiological pathways that are recognized as the cancer hallmarks. Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein known to play an important role in intracellular Ca^{2+} homeostasis, but it is also involved in the regulation of multiple intracellular signaling pathways such as cell proliferation, apoptosis and metabolism. RGN also has been identified as a hormonally regulated gene, which includes the sex steroids androgens. Furthermore, RGN was previously associated with pathological conditions and described as a protein underexpressed in human breast, prostate and liver cancer cases. In the liver, it was demonstrated that RGN underexpression occurs in pre-neoplastic lesions before the acquisition of neoplastic phenotype, which thus implicates RGN loss in the tumorigenic transformation. In spite of this evidence, the molecular mechanisms underlying RGN actions in the breast remain to be identified. In the present thesis, we hypothesized that RGN overexpression may exert a protective action against mammary tumorigenesis. To address this issue, transgenic rats overexpressing RGN (Tg-RGN) and wild-type (Wt) controls were treated with 7,12-dimethylbenz[α]anthracene, a compound recognized to induce carcinogenesis of rat mammary gland. Tg-RGN rats displayed a remarkable lower incidence of tumors (25.8 %), comparatively with their Wt counterparts (100 %). Tumor histological classification also clearly showed that Tg-RGN rats are resistant to cancer progression into more aggressive stages, as indicated by the lower percentage of invasive tumors types (3.8 % vs 45.8 % in Wt). Moreover, higher proliferative activity was observed in non-invasive tumors of Wt comparatively with those of Tg-RGN animals. The metabolic evaluation of these tumors demonstrated a lower expression and activity of lactate dehydrogenase (LDH) in Tg-RGN rats, which is a feature associated with restricted tumor progression and lower aggressiveness. Notwithstanding, in the non-neoplastic mammary gland of Tg-RGN rats a restriction of the glycolytic metabolism was observed, which indicates a reduction of the energy levels in the tissue. These results may be quite relevant to slowdown cell proliferation, and may constitute an additional mechanism by which RGN prevent tumor development. Indeed, RGN overexpression suppressed the expression of cell cycle regulators and oncogenes in the mammary gland of Tg-RGN rats. Besides that, P53 expression and caspase-3 activity were also augmented in response to RGN overexpression, which suggests that the protective role of RGN against tumor onset may also be mediated by the modulation of apoptotic pathways. On the other hand, RGN and L-type Ca^{2+} channel were found to be down-regulated and up-regulated, respectively, by the non-aromatizable androgen 5α-didydrotestosterone in MCF-7 breast cancer cells. These results underpinned the decreased

viability of MCF-7 cells and suggest the involvement of Ca^{2+} regulators, RGN and L-type Ca^{2+} channels, in the control of breast cell proliferation.

In conclusion, the work presented in this thesis highlighted the influence of RGN in a plethora of physiologic and pathophysiologic mechanisms of the mammary gland. The biologic evidence presented herein confirmed the protective role of RGN in mammary carcinogenesis, by restraining biological processes recognized as the hallmarks of cancer, namely, cell proliferation and metabolism, or by enhancing apoptotic cell death.

Keywords

Apoptosis; Breast cancer; Glycolytic metabolism; Mammary gland; Proliferation; Regucalcin; Tg-RGN;

Table of Contents

List of Figures

List of Tables

List of Abbreviations

A23187	Ca^{2+} ionophore
AA	L-ascorbic acid
Ac2F	Rat liver cells
Acetyl CoA	Acetyl coenzyme A
ACs	Adenylyl cyclases
ADH	Atypical ductal hyperplasia
AP1	Activator protein 1
AR	Androgen receptor
BRCA1	Breast cancer suppressor gene 1
BRCA2	Breast cancer suppressor gene 2
BRCT	BRCA1 C-terminal
Ca^{2+}	Calcium
$CaCl_2$	Ca^{2+} chloride
CaIb	Calbindin
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
CaN	Calcineurin
$Ca_v1.2$	α_{1C} subunit of L-type Ca^{2+} channels
CK	Cytokeratin
CK14	Cytokeratin 14
CK5	Cytokeratin 5
CR	Caloric restriction
CTX TNA2	Rat astrocytes
DCIS	Ductal carcinoma in situ
DHT	5α-dihydrotestosterone
DMBA	7,12-dimethylbenz[α]anthracene
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
E_2	17β-estradiol
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
EREs	Estrogen response elements
ERs	Estrogen receptors
FBS	Fetal bovine serum
FEA	Flat epithelial atypia
FSH	Follicle stimulating hormone
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT1	Glucose transporter 1
GLUT3	Glucose transporter 3
GLUTs	Glucose transporters
GNL	Gluconolactonase
GnRH	Gonadotropin releasing hormone
GPER	G-protein coupled ER

HCC	Human hepatocellular carcinoma
HER-2	Human epidermal growth factor
HK	Hexokinase
I_{Ca}	Voltage dependent Ca^{2+} channels current
IDC	Infiltrating ductal carcinoma
KA	Kainate
LCIS	Lobular carcinoma in situ
LDH	Lactate dehydrogenase
LH	Luteinizing hormone
LLC-PK1	Pig kidney cells
LPS	Lipopolysaccharide
LTCCs	L-type Ca^{2+} channels
MAPK	Mitogenic-activated protein kinase
MCF-7	Human breast cancer cell line
MCT	Monocarboxylate transporter
MCT4	Monocarboxylate transporter 4
MCU	Mitochondrial Ca^{2+} uniporter
MSCs	Mammary stem cells
NADH	Nicotinamide adenine dinucleotide reduced
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NEM	N-ethylmaleimide
NLS	Nuclear localization signal
NO	Nitric oxide
NOS	Nitric oxide synthase
NRK52E	Rat kidney proximal tubular ephithelial cells
OXPHOS	Oxidative phosphorylation
PBST	FBS in phosphate buffer saline with 0.1% tween®-20
P-Cad	P-cadherin
PDH	Pyruvate dehydrogenase
PFK	Phosohofructokinase-1
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMCA	Plasma membrane Ca^{2+} ATPase
PR	Progesterone receptor
PTH	Parathyroid hormone
qPCR	Real-time PCR
RGN	Regucalcin
RGN-KO	Regucalcin knockout
ROS	Reactive oxidative species
RT	Room temperature
RT-PCR	Reverse transcription PCR
SERCA	Sarco/endoplasmic Ca^{2+} ATPase
SH	Sulfhydryl groups
SMP30	Senescence marker protein 30
SnoN	Ski-novel protein
SOCs	Store-operated channels
SOD	Superoxide dismutase
STIM	Stromal interaction molecules

T	Testosterone
TCA	Tricarboxylic acid
TEBs	Terminal end buds
TFP	Trifluoperazine
TGF-ß	Transforming growth factor-ß
Tg-RGN	Transgenic rats overexpressing regucalcin
TMAs	Tissue microarrays
TNF-α	Tumor necrosis factor-α
TPTX	Thyroparathyroidectomised
TRP	Transient receptor potential
TTCCs	T-type Ca^{2+} channels
VC	Vitamin C
VGCCs	Voltage-gated Ca^{2+} channels
WB	Western blot
Wt	Wild-type
B2M	B_2-microglobulin

Chapter I

General Introduction

Brief overview of mammary gland physiology

Breast cancer

Brief overview of mammary gland physiology

Breast cancer

- Development and histopathological classification
- The role of sex steroid hormones and genetic deviations
- Metabolic reprogramming and progression of disease
- Calcium players in breast carcinogenesis

Brief overview of mammary gland physiology

The size, shape and function of the mammary gland vary accordingly to the phase of human life. Breast development initiates in the fetus and undergoes similar processes both in male and female until puberty, when different hormonal actions start to regulate their size [1, 2]. During mammalian embryonic development the mammary buds, or anlagen, are formed after thickening of the epithelial layer, placode, and invagination into the mesenchyme (Figure I.1). In humans, only a pair of placodes is formed during the first trimester of pregnancy. Cell extension from the mammary bud through the fat pad establishes a rudimentary gland [2-4]. The terminal end bunds (TEBs) are sites constituted by high proliferative cells at the tip of growing ducts. Body and cap cells at TEBs differentiate, respectively, into the luminal and basal myoepithelial cells of mammary gland. The basal myoepithelial cells underlie luminal epithelial cells and duct formation [1, 2]. Myoepithelial cells also contribute to the synthesis of the basement membrane separating the epithelium from the connective tissue [5-7]. At puberty, female hormones trigger the expansion of ducts throughout the fat pad, and once ducts growth ceases TEBs structures disappear (Figure I.1) [1, 2].

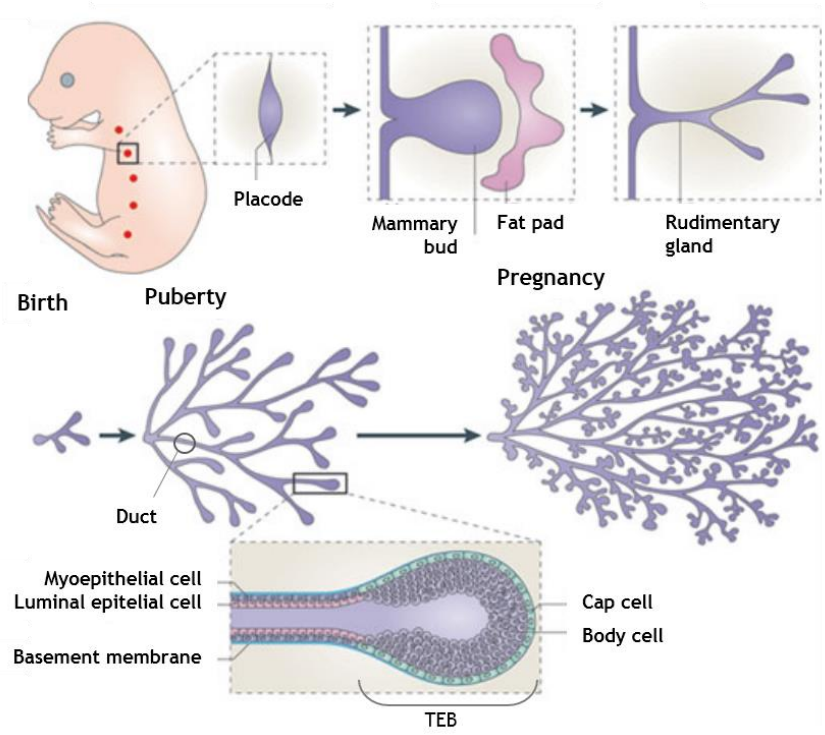

Figure I.1. Development of mouse mammary gland. Epithelium thickening in the embryo, placode, initiates mammary gland growth. Cells invagination give raise to mammary bud and latter invasion of fat pad establish a rudimentary gland maintained until puberty. Hormonal signaling stimulate formation of terminal end buds (TEB) whose extension leading to duct and epithelial tree formation (Adapted from Gjorevski and Nelson, 2011[2]).

Epithelial trees are formed by successive ducts elongation, bifurcation and lateral branching into numerous alveolus units that constitute the lobular structure of the mammary gland (Figure I.1). The adult human mammary gland is composed of 15 to 20 lobes embedded in stroma or connective tissue, also called fat pad, which include components as adipocytes, fibroblasts, neurons, blood vessels and immune cells. Each lobe is constituted by several alveoli and each alveolus is the functional unit of the mammary gland. Several epithelial collecting ducts draining alveolar secretions join in a single individual duct opening at the tip of nipple (Figure I.2) [3, 8].

After puberty, the mammary gland undergoes cycles of growth and involution, closely regulated by menstrual cycle, or cycles of pregnancy and lactation. A balance between cell proliferation and cell death occur to keep the mammary structure at the starting point of the menstrual cycle. However, this is not fully achieved since at each menstrual cycle, the development of mammary originates a slight promotion of new budding until the age of 35 years [9].

During pregnancy, the mammary gland undergoes maturation changes in branching development together with the alveologenesis. The luminal epithelial alveoli cells are the responsible for milk synthesis and secretion into the alveolar lumen, while the contractile myoepithelial cells participate in milk ejection and movement from the ducts to the nipple. As soon as lactation stimulation ends, the involution process initiates and the removal of alveoli by cell death mechanisms restore the normal ductal structure [1, 3, 4, 8, 10]. In each cycle of pregnancy and lactation, growth and involution is repeated whereas at post-menopausal period the cycle is finished and additional involution occurs [10].

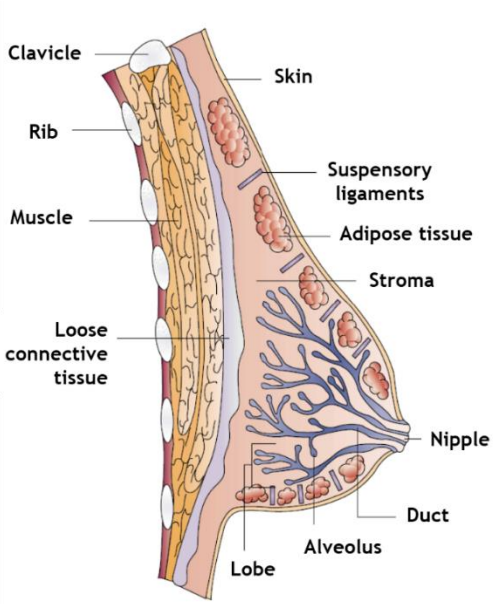

Figure I.2. Anatomy of the human mammary gland (Ali and Coombes, 2002 [8]).

The capacity of breast regeneration following each cycle of expansion and involution, led researchers to propose a model where the distinct cell lineages of mammary epithelium could be originated from the mammary stem cells (MSCs). It was postulated that MCSs generate themselves, maintaining the pool of stem cells, and also the epithelial precursor cells (EPCs). Subsequently, EPCs give rise to the progenitors of myoepithelial and luminal cells, which ultimately differentiate into the myoepithelial and luminal cells confined to the ductal or alveolar structures [1, 11-13]. Also, the activity of stem cells has been commonly accepted to be present on TEBs structures, therefore, the MSCs should be capable of regenerate the entire architecture of mammary gland epithelium [12]. This question has been a matter of debate but recent techniques allowed further elucidation on the subject, and several evidence support the stem cells hypothesis, though conflicting observations persist [4, 14].

Adipocytes, though viewed as a passive tissue, are quite active in the mammary gland and display the ability to modulated mammary epithelial growth and function. Adipocytes secrete the vascular endothelial growth factor that probably controls angiogenesis, and modulate the glandular epithelial function and breast development. Also, adipocytes are an important source of lipids during pregnancy and lactation, being observed a reduction of lipid content mostly during milk production [4, 15].

Fibroblasts are other fundamental cellular component of the stroma and their main functions involve the reciprocal signaling with epithelial cells, which includes the secretion of growth factors that support cell survival and branching morphogenesis in the fat pad [16, 4]. Additionally, fibroblasts influence cellular functions through the synthesis of collagen, proteoglycans or fibronectin, and metalloproteinases enzymes of the matrix. These enzymes besides promoting the degradation of extracellular matrix secrete growth factors and cytokines, and also affect morphogenesis [2, 4, 17].

The pronounced development of mammary gland occurs particularly during the hormone-dependent stages, namely, puberty and pregnancy. The female hormonal milieu is controlled by the hypothalamic-pituitary-ovarian axis, which is maintained under a feedback regulation. The gonadotropin releasing hormone (GnRH) released from the hypothalamus activates the pituitary gland to synthesize and secrete gonadotropins, the luteinizing hormone (LH) and the follicle stimulating hormone (FSH). In turn, LH and FSH act in the ovaries promoting the development and maturation of ovarian follicles that subsequently produces 17β-estradiol (E_2) and progesterone. E_2 is the main mitogenic player in the mammary gland during puberty responsible for inducing growth of ducts and glandular structures. In each menstrual cycle throughout woman reproductive life, though with less exacerbated effects, E_2 and progesterone cooperate to the development of mammary alveolar lobules. In pregnancy, prolactin released by the pituitary induces the proliferation of mammary epithelial cells and milk production at the alveoli during lactation. Finally, prolactin inhibits the release of GnRH suppressing the stimulation of the hypothalamus and preventing ovulation [18].

Breast cancer

Breast carcinoma is a multifaceted disease that affects millions of patients every year. Its complexity results of being a heterogeneous disease that embraces diverse biological features and divergent clinical behaviors [19]. Several factors are well recognized to affect the malignant transformation of breast cells, which comprises hormones, growth factors, oncogenes, tumor suppressor genes, or even lifestyle, as diet, obesity, and physical exercise [20-22]. Estimates of cancer incidence indicated that, in 2012, breast cancer was the second most common cancer worldwide, with 1.7 million cases reported [23]. In 2015, only in the United States, 232 000 new cases are expected to be diagnosed, being the most common type of cancer and the second leading cause of death among women. The probability of an individual to develop invasive breast cancer during its lifetime, determined between 2009 and 2011 in the United States, was one in eight (12.3 %), and represent the second highest only after prostate cancer (15 %) [24]. As will be detailed in the following topic, breast cancer can be histologically classified mainly as noninvasive (*in situ* carcinoma) or invasive carcinoma (ductal or lobular). The ductal subtype accounts about 75 % of all breast cancer cases [11]. Within the *in situ* carcinoma the ductal carcinoma in situ (DCIS) is foremost more common than the lobular carcinoma *in situ* (LCIS).The invasive carcinoma comprehend diverse subtypes, with the infiltrating ductal carcinoma (IDC) representing an astonishing proportion (80 %) of cases [25].

Development and histopathological classification

Breast carcinoma is a complex heterogeneous disease with different histopathological subtypes characterized by distinct molecular signatures, which may determine the therapeutic response and/or the clinical outcome. Some of the breast cancer diversity resides within the singular features of mammary gland, including the multiplicity of components present in their architecture, namely, the epithelial cells, MSCs or microenvironmental constituents that can play a role in tumor development [14, 26-29].

The traditional system of breast cancer classification is based on biologic findings and clinical behavior, accordingly to the histological grade and subtype. The histological grade encompasses the morphological evaluation of the tumor biological degree of differentiation (tubule formation and nuclear pleomorphism) and growth pattern [26]. Although had lost interest comparatively with other classification systems, the histological grade maintains clinical usefulness as it is correlated to molecular subtype and is viewed as a complement to new methodologies. The histological type refers to the proliferative pattern of tumors. Considering the existent histological diversity in breast cancer particular morphological and cytological patterns are applied to determine the clinical prognosis and outcome [26, 30].

Contrastingly with other human cancers, no definitive model of breast cancer development has been established. This is due to difficulties in applying markers and identifying features

specifically enough to characterize different stages of carcinoma progression [25]. However, based on epidemiologic and morphologic data, a classic model was proposed to illustrate the neoplastic evolutional steps. Normal tissue transformation generate flat epithelial atypia (FEA), that progresses into atypical ductal hyperplasia (ADH), advances to DCIS, considered as the precursor of IDC, and culminates in the metastatic ductal carcinoma [11]. Although considered for a long time as a non-obligate precursor of IDC, new evidence supports DCIS as a progressive stage to invasive breast cancer. For example, they share the same anatomical site and possess similar classification subtypes according with the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth receptor 2 (HER-2), among other molecular biomarkers. Despite this, and the fact that DCIS detection has increased in last years its usefulness to predict whose patients will actually develop invasive disease is still scarce. This is mostly explained by the absence of specific histopathological or molecular markers that may predict the transition of DCIS to invasive breast cancer types [29, 31]. However, the assessment of ER, PR, and HER-2 markers is clearly recommended in the evaluation of all invasive carcinomas, what is not totally established concerning DCIS [25].

The implementation of a classification system based on molecular biologic markers has implied a significant advance in understanding breast cancer and its clinical outcome, as well as, the application of the most appropriate therapies [25]. The classic molecular classification grouped breast cancer into the luminal A, luminal B, basal-like and HER-2 subtypes [32]. The luminal subtypes are ER positive and/or PR positive, and are characterized as luminal A or luminal B, accordingly to the HER negative or HER positive status, respectively. The basal-like subtype is triple negative for ER, PR and HER though it may be positive for epidermal growth factor receptor (EGFR). The HER-2 subtype is characterized by ER and PR negative status but overexpression of HER-2 [32, 33].

Breast tumors are further characterized by the analysis of other molecular markers. The luminal-like subtype expresses cytokeratins (CKs) 8 and 18 associated to luminal epithelial cells [26]. The luminal A is the most common subtype (60 %) presenting frequently, low histologic grade, mitotic rate and degree of nuclear pleomorphism, being the subtype with the best prognosis and lowest relapse rate [32]. Luminal B subtype is found in about 20 % of the tumors cases and displays a more aggressive phenotype of worse prognosis with higher histologic grade, proliferation and recurrence comparatively to luminal A [34]. As stated above, the HER-2 subtype is characterized by the overexpression of HER-2 protein that belongs to the tyrosine kinase receptor family, and represents 15 % to 20 % of all tumors subtypes [32] . HER-2 positivity confers a stronger aggressive state as clinic behavior with the tumors exhibiting higher histological and nuclear grade, and an elevated proportion of these tumors (~40 %) presenting p53 mutations [32, 34]. The basal-like tumors are identified in 8 % to 37 % of breast cancers that express high levels of myoepithelial markers, such as CK5, CK6, CK14, CK17, and laminin. The basal-like subtype also presents a very high frequency of p53 mutations (80 %), which is related to the genomic instability and inactivation of the retinoblastoma pathway, and presents the worst prognostic and clinical outcome. Despite being negative for ER, PR, and HER-2, the

basal-like is not synonymous of the so-called triple-negative breast cancer. The former is classified by microarray analysis of gene expression, and the triple-negative breast cancers are evaluated by the immunohistochemical determination of ER, PR and HER-2. In fact, studies indicate that there exists a variance of about 30 % among them [32, 34-36].

As mentioned above, no definitive model exists to explain the emergence of breast cancer. Indeed, the breast cancer origin has been a matter of debate, and two conceptual hypotheses have aroused, the clonal and the cancer stem cell or "tumor-initiating cells" model, which is supported by the concept of MSCs, that drive carcinoma initiation, progression and recurrence [11, 14]. The clonal model states that tumor initiation results from transforming insults that drive genetic and epigenetic alterations in a single cell, whose accumulation of events confer additional genetic advantages to their survival and abnormal progression [11, 29]. The cancer stem cell theory argues that a small subset of cells within the tumor can start and maintain tumor progression, while the remaining cells have low tumorigenic potential [11, 25]. Conceptually, MSCs should have a higher propensity to oncogenic transformation and to accumulate mutations over their long lifetime than differentiated cells [14]. Although the two models may compete, they may not be necessarily exclusive but are eventually complementary in the explanation of tumor initiation and progress, and it has been suggested that stem cells also may undergo clonal expansion [11, 25, 29]. Recent studies refer that tumors are heterogeneous identities with genetic variations [37], which may be consequence of different mutations within the same target cell or that distinct tumor subtypes result from distinct cells within the tissue that serve as the origin of cells [38].

The role of sex steroid hormones and genetic deviations

Breast cancer was established as a hormone-dependent disease long time ago. The association between cancer and hormones remounts back to the year 1880, upon the observation that the removal of the ovaries induced clinical benefits to breast cancer patients [39, 40]. In fact, prolonged exposure to estrogens as the consequence of an early menarche and late menopause is an important determinant factor associated with an increased risk of breast cancer development [39]. Another associated factor is the increased levels of circulating estrogens [41]. Augmented levels of estrogens may result from an overproduction by increased aromatase activity in the adipocytes or conversion of elevated circulating levels of androgens (androstenedione and testosterone (T)) [39, 41]. Another risk can include the exposure to hormones resulting from the use of oral contraceptives [13]. Additionally, findings on the appearance of estrogen-dependent breast cancer in postmenopausal women, displaying low circulating levels of estrogens, pointed to a local production of steroids hormones in tumor tissues [42]. This intratumoral localized production depends on the availability of precursors steroids such as dehydroepiandrosterone that is synthesized in the adrenal cortex but not in the ovary [42]. This evidence linked breast cancer and the hormonal actions, with estrogens, and predominantly E_2, playing a central role in breast carcinogenesis.

Estrogens actions are mediated by the classical estrogen receptors (ERs), by the metabolization of estrogens, or also by ER-independent pathways [39, 41, 43, 44]. Nonetheless, carcinogenesis may arise from E_2 oxidative metabolites, namely, the 4-hydroxycatechol and the 2-hydroxycathecol, which can also bind the ER, forming an active estrogen-ER complex able to exert biological effects [39, 41].

The ER subtypes ERα and ERβ have the same structural domain organization with six distinct functional domains (A-F). The DNA-binding domain (domain C) presents 96 % homology between ERα and ERβ proteins, whereas the ligand-binding domain (domain E) shows only 53 % of sequence identity [44, 45], which allow some ligand specificity and the development of specific agonists and antagonists. Nevertheless, the ERα and ERβ share the same mechanism of action characteristic of the nuclear receptor superfamily of ligand-activated transcription factors [44, 46]. In the absence of ligand the ERs are inactive and associated with heat-shock proteins [20, 47]. Upon ligand binding, the receptors undergo conformational changes, establish homodimers and/or heterodimers and are autophosphorylated becoming fully activated [20, 44, 45]. ER dimers then bind directly to the DNA through the highly conserved zinc-finger domains in the DNA-binding domain of the receptors, which recognize the estrogen response elements (EREs) consensus sequences, regulating gene transcription [44]. Alternatively, transcription of target genes could be indirectly activated or repressed by protein-protein interaction of ER with other transcription factors, as for example the activator protein 1 (AP1) or p53 [44, 45]. A substantial amount of data also has been demonstrating that estrogens may elicit rapid, non-genomic effects by interaction of ERs with components of the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), EGFR and HER-2 pathways, or through the activity of the G-protein coupled ER, the GPER. These actions may occur within few minutes after exposure to estrogens but also may lead to the regulation of gene expression through the activation of other transcription factors in the signaling cascade [45].

Despite sharing a common structure and the same mechanism of action, ERs exert distinct cellular functions and are widely expressed in several tissues, including the mammary gland. The distinct functions of ERs subtypes may depend on the relative abundance and cellular localization of both ERs. Nevertheless, ERα is commonly associated with the promotion of breast cancer cell proliferation by inducing the expression of Myc and cyclin D1 but positive expression of ERα is correlated with a better response to treatment and clinical prognosis [20, 45]. Indeed, in clinical diagnosis ER positivity refers only to the staining of ERα subtype and it is accepted as the intermediate in estrogen-mediated breast carcinogenesis [20, 39]. Contrarily, the ERβ seems to counteract ERα effects inhibiting cell proliferation, growth and angiogenesis [45].

Notwithstanding the central role of estrogens in breast development and carcinogenesis, also the sex steroid hormones androgens have an important function in breast physiology and pathology [48-51]. In fact, androgens were used as breast cancer therapy before the development of the anti-estrogenic therapeutic approaches, which led to loss of interest in the androgenic treatments [52]. Although the androgenic effects are far from generating a

consensus given the existence of conflicting results, a substantial amount of studies have shown the inhibitory effect of androgens on proliferation of breast cells [48, 52-55].

Androgens are produced by the ovaries, the adrenal glands but like estrogens also can be synthesized locally in the breast [50, 56, 57]. The main androgens present in the blood of premenopausal women are T and 5α-dihydrotestosterone (DHT), which are ligands of the androgen receptor (AR) [50, 58]. The potent androgen DHT [51] is a metabolite of T resulting from the enzymatic activity of 5α-reductase [49, 52] . T also can be converted to E_2 by the aromatase enzyme. The androgenic effects are mediated by the AR, a transcription factor of the nuclear receptor superfamily that regulates gene expression in several biological contexts [59], with a mechanism of action similar to that described for ERs. AR effects also involve the regulation of PI3K/AKT/MAPK signaling pathways and P53 or other cell cycle regulators [52]. For example, DHT inhibits cell growth by activation of P53 expression in MCF-7 cells [60]. Moreover, AR and ERα crosstalk is thought to antagonize the ERα signaling in breast cancer cells [51, 52].

In breast cancer, AR is detected in up to 90 % of primary breast cancers and 75 % of metastasis [51, 52], and its expression is correlated with several pathologic parameters, namely, lower histological grade and smaller tumor size, and is associated with a favorable prognosis and overall survival [51, 61, 62]. Moreover, AR is highly expressed in luminal types but more frequently in luminal A breast cancers, and AR positivity is the lowest in basal-like breast cancer though some studies suggested that AR may contribute to resistance to therapy.

Besides the hormonal factors, genetic susceptibility may also influence breast carcinogenesis, with some gene mutations increasing the risk to breast cancer. Hereditary breast carcinoma accounts for a small percentage, around 10 %, of all of breast cancer cases [63]. The first susceptible gene described was the breast cancer suppressor gene 1 (BRCA1) and afterwards the *BRCA2* [64-68]. A defective copy of one of BRCA1 or BRCA2 alleles in the germline is enough to cause predisposition to malignancy [65, 66]. The *BRCA1* gene is composed of 22 exons encoding a 220 kDa protein that structurally encompasses three main domains, the N-terminal RING domain, the nuclear localization signal domain (NLS) and the BRCA1 C-terminal domain (BRCT) [64]. The *BRCA2* gene consists in 27 exons and encodes a protein of 3418 amino acids [68]. Functionally, BRCA1 protein assumes diverse functions that include DNA repair, transcriptional activation, cell cycle regulation, chromatin remodeling and protein ubiquitination [63, 64], essential processes in the maintenance of genomic stability. Also, the BRCA2 protein is involved in DNA repair [68]. Whereas 90 % of breast cancer tumors are sporadic, the basal-like subtype accounts for 15-20 % of cases, and a significant fraction of these patients are BRCA1 mutation carriers [63]. BRCA1 has been shown to interact with ERα and AR. Its actions together with ERα seem to inhibit the downstream signaling by the down-regulation of expression of genes involved in the control of replication and maintenance of genome integrity, as well as, the diminishing estrogen synthesis via the inhibition of aromatase encoding genes [63, 64]. In the case of AR, the interaction with BRCA1 allow the enhancement

of AR activity, and studies have shown that BRCA1 mutations are correlated with a lower prevalence of AR [69].

Actually, multiple genes have been applied to estimate the risk of breast cancer and several predictive models have been proposed [70, 71], an issue that has been recently reviewed and is out of the scope of this thesis.

Metabolic reprogramming and progression of disease

The metabolic changes that occur in cell malignant transformation are distinctive and, currently, recognized as a hallmark of carcinogenesis [72]. Otto Warburg first reported that cancer cells produce energy preferentially by glycolysis in detriment of oxidative phosphorylation (OXPHOS), even in the presence of oxygen [73-75]. This metabolic switch in cancer cells towards the "aerobic glycolysis" increases glucose uptake, the glycolic flux and diverts pyruvate to the production of lactate, as a consequence of OXPHOS impairment [76, 77]. The hyperglycolytic phenotype is thought to be common to almost, if not, all human cancers and is known as the "Warburg effect" [77, 78]. In breast cancer, several mutations in the mitochondrial DNA were described, namely in complex I and complex II related genes, which supports the Warburg hypothesis [77]. A growing body of evidence is, in fact, contributing to improve the understanding of this heterogeneous disease on the basis of metabolic alterations [79].

Glycolysis is the pathway that converts glucose into pyruvate through multi-sequential enzymatic steps (Figure I.3). Normally, pyruvate is imported into the mitochondria where it is enzymatically oxidize to acetyl coenzyme A by the pyruvate dehydrogenase. Afterwards, acetyl coenzyme A enters the tricarboxylic acid (TCA) cycle with some of the resulting products flowing into the OXPHOS for an improved efficient energy yield [80].

The described process starts with the uptake of glucose, which is mediated via glucose transporters (GLUTs) family members [81]. GLUTs were shown to be highly expressed in diverse neoplastic conditions, and some of these transporters have been associated with breast cancer, namely GLUT1, GLUT3, GLUT5 or GLUT12 [82-85]. The expression of GLUT1 and GLUT3 have been strongly associated with poorly differentiated (grade 2 and grade 3) breast tumors [82], while negative GLUT1 expression was correlated with increase disease-free survival [86].

The enzymes that control the glycolytic pathway, such as the phosphofructokinase-1 (PFK), also have been linked to the "Warburg effect". PFK catalyzes the conversion of fructose 6-phosphate into fructose 1,6-bisphosphate, a rate-limiting step and irreversible reaction of glycolysis, and an overactivation of PFK, as well as, a resistance to its inactivation has been described in cancer cells [80]. Breast cancer tissues showed an increased glycolytic efficiency and a differential expression pattern of PFK isoforms, which followed the stage of tumors development [87, 88].

Besides the higher rates of glucose uptake and glucose metabolization, to be able to proliferate cancer cells also need to increase the biosynthesis of nucleotides, macromolecules and lipids. This is achieved by the consumption of NAD^+, NADPH, and ATP [78, 80, 89].

For nucleotide synthesis, glucose 6-phosphate obtained by conversion of glucose via hexokinase, enters the pentose phosphate pathway yielding ribose 5-phosphate and NADPH, both indispensable to the biosynthesis of DNA, fatty acid and redox system (Figure I.3) [76, 90, 91].

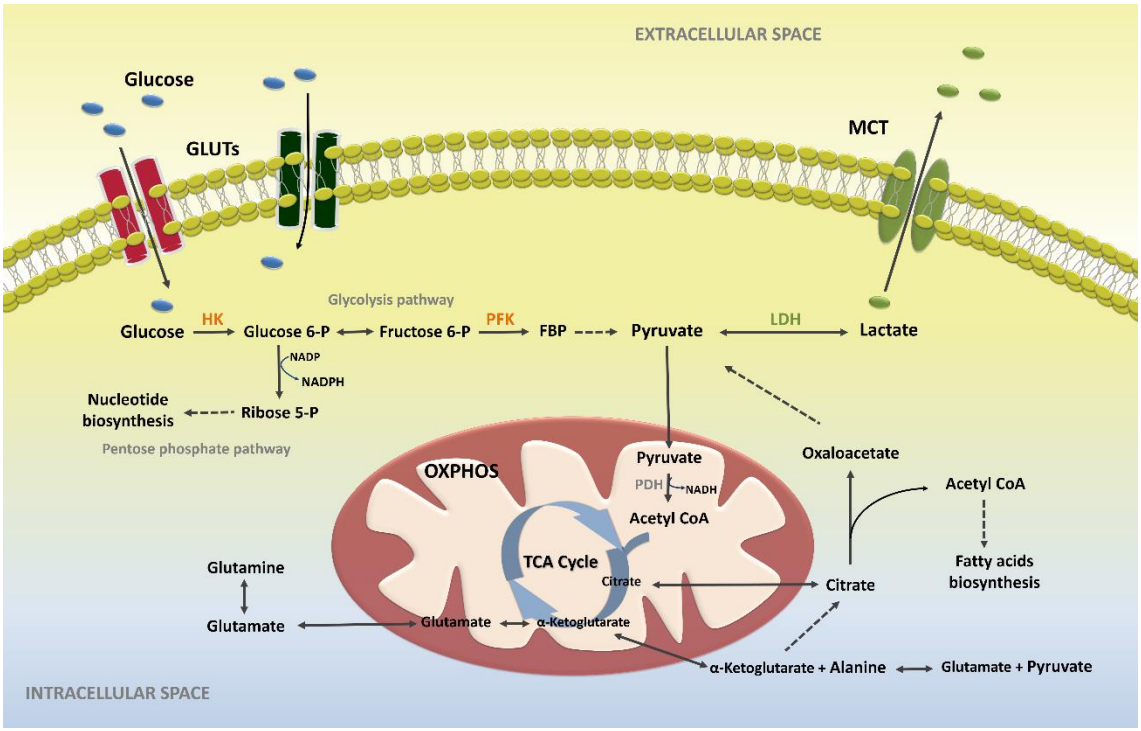

Figure I.3. Schematic representation of metabolic pathways. In glycolysis, glucose is transported to the cytosol by glucose transporters (GLUTs), where it is initially converted to glucose 6-phospahte (Glucose 6-P) by hexokinase (HK). Glucose 6-P is then isomerized to fructose 6-phospahte (Fructose 6-P), which is followed by an additional phosphorylation step to produce fructose 1,6-bisphosphate (FBP), a reaction catalyzed by phosphofructokinase (PFK), an irreversible and rate limiting step of glycolysis. After additional reaction steps (not shown) pyruvate is produced as the end product of the glycolytic pathway. In the mitochondria, pyruvate dehydrogenase (PDH) oxidizes pyruvate to acetyl coenzyme A (Acetyl CoA), which enters to the tricarboxylic acid (TCA) cycle. In the cytosol, pyruvate is also converted to lactate by lactate dehydrogenase (LDH). Lactate is the exported to the extracellular space by the monocarboxylate transporters (MCT). Nucleotide synthesis is achieved through entrance of glucose 6-P to the pentose phosphate. Metabolization of the amino acid glutamine supports energy production and fatty acids synthesis. Alanine conversion also can yield pyruvate. Legend: NADH, Nicotinamide adenine dinucleotide reduced; NADPH, Nicotinamide adenine dinucleotide phosphate reduced; *Solid arrows* indicate a single reaction step; *Dashed arrows*, symbolize multiple reactions steps

As lipid synthesis is dependent on the energy obtained from glycolysis, the TCA cycle, and the pentose phosphate shunt [78], the amount of pyruvate available in tumor cells could not sustain the overproduction of lactate. So, cancer cells make use of an additional strategy to obtain energy by metabolization of amino acids in the TCA cycle [78, 92]. Glutamine that is the most abundant amino acid, but also, alanine are alternative energetic fuel sources for the proliferation of cancer cells. In fact, it is well described that cancer cells utilize great amounts of amino acids to provide intermediates to TCA cycle used in the synthesis of lipid or to maintain the overproduction of lactate [93-96].

Pyruvate is the end product of glycolysis but the metabolic rate is dependent on the cytosolic availability of NAD^+, a cofactor of lactate dehydrogenase (LDH) enzyme. Thus, the maintenance

of higher rates of NAD^+ regeneration is achieved by the transformation of pyruvate into lactate with the simultaneous conversion of NADH and NAD^+ via LDH activity, which allows the continuity of a high glycolytic rate (Figure I.3) [80, 91, 97].

LDH silencing in breast cancer cells has been demonstrated to suppress tumor initiation and proliferation, both *in vivo* and *in vitro*, whereas it increases oxidative stress and apoptosis [98, 99]. Suppression of LDH inhibited metastasis, and reduced glucose consumption and glycolysis [99]. Others findings also referred that augmented glucose uptake and lactate production, and increased expression of LDH accompanied the transformation of breast cancer cell lines into more aggressive stages [100]. In the same way, triple negative breast tumors express high levels of LDH, which were correlated with poor clinical outcomes [101].

The lactate overproduced by the cancer cells is extruded into the extracellular space via monocarboxylate transporters (MCTs), which avoids its intracellular accumulation (Figure I.3). The lactate is transported together with a proton through a facilitated diffusion process [102, 103]. Thus, lactate removal from the cytoplasm of highly glycolytic cells promotes an acidification of the surrounding microenvironment, but since cancer cells are resistant to this conditions, this confers them a survival advantage against attacks from the immune system, which favors tumors invasion [89, 104, 105].

The family of MCTs is composed of 14 members of which the best characterized are the MCT1 to MCT4 [106]. Depending on the specific MCTs, the monocarboxylate can be imported or exported [105], and MCT4 is the MCT involved in the export of lactate [107]. The majority of tumors present an over-expression of MCTs proteins, and MCT4 was shown to be highly expressed in HER-2 positive breast cancers, as well as in triple negative tumors with association to overall survival decrease [108, 109]. Additionally, it was observed that MCT4 regulates cell survival since its depletion led to a reduced growth of breast cancer cells [109]. Also, the expression of MCT1 was associated to breast cancer subtypes, histological grade or proliferative status [110] whereas its inhibition decreased cell proliferation, migration and invasion [111]. Therefore, significant research efforts are using metabolomic approaches to understanding better the disease, to identify new therapeutic targets and to predict the therapeutic response in breast cancer cases.

Calcium players in breast carcinogenesis

Calcium (Ca^{2+}) ion is an intracellular secondary messenger commonly recognized in the control of diverse cellular processes depending on its location, concentration, and frequency of release. It is known that deviations in the intracellular Ca^{2+} levels following deregulated expression of Ca^{2+} handling proteins have implication in the physiology and pathophysiology of mammary gland [112]. Accordingly, Ca^{2+} signaling has been viewed as an important therapeutic target also given the involvement of different Ca^{2+} regulators in the known hallmarks of cancer, namely cell proliferation, apoptosis, angiogenesis, and invasion and metastasis [113].

The maintenance of intracellular Ca^{2+} levels is maintained by the orchestrated activity of several proteins (Figure I.4), which includes Ca^{2+} channels, Ca^{2+} pumps, and diverse Ca^{2+}-binding proteins.

Ca^{2+} channels are localized in the plasma membrane and intracellular Ca^{2+} storage organelles [112]. The plasma membrane Ca^{2+} channels control Ca^{2+} influx in response to different stimuli, mediating complex and diverse cellular signaling pathways [113-115], and are classified into four major classes: the transient receptor potential (TRP), the store-operated channels (SOCs), ligand-gated ion channels and the voltage-gated Ca^{2+} channels (VGCCs) [113].

Some of the most studied Ca^{2+} channels belong to the TRP family constituted by TRPC, TRPV, TRPM, TRPA, TRPML and TRPP subtypes that are activated by several compounds[116]. TRP channels have been shown to be involved in the tumorigenic process, and its altered expression in breast, ovarian, prostate and colon cancer was described [117-120]. Furthermore, silencing of a TRPC in MCF-7 breast cancer cells inhibited phosphorylation of ERK1/2 and cell proliferation [121].

The constituents of SOCs family are the ORAI1, ORAI2 and ORAI3 channel proteins that are activated by interaction with endoplasmic reticulum Ca^{2+} sensors, namely the stromal interaction molecules (STIM1 and STIM2) [122]. They represent the main Ca^{2+} entry pathway in non-excitable cells [114], and their expression was demonstrated to be extremely important for migration and metastasis of breast cancer cells [123].

Ligand-gated ion channels are mainly expressed in the nervous system, but they also can be located in other tissues, specifically the P2X channels [114]. The loss of P2X channels seems to impede Ca^{2+} influx necessary to promote apoptosis in cervical cancer cells [124]. In PC3 and DU145 prostate cancer cells lines, and in HT-1376 bladder carcinoma cells, P2X channels mediated the ATP growth inhibitory effects [125, 126].

Another class of Ca^{2+} permeable channels is the VGCCs or Ca_v family which is divided into three subfamilies (Cav1-Cav3) [127]. The VGCCs consist of a complex structure of five subunits (α1, α2, δ, ß and γ) where the α1 subunit is the pore forming protein. The activation of these channels is mediated upon membrane depolarization allowing Ca^{2+} influx into the cell [127, 128]. The VGCCs subfamilies due to their different localizations have distinct cellular functions and distinct kinetics of Ca^{2+} currents [127-129]. The L-type Ca^{2+} channels (LTCCs) subfamily presents several isoforms ($Ca_v1.1$–$Ca_v1.4$) and is characterized by high-voltage activation and long-lasting activity [127, 130, 131]. The expression of LTCCs is mainly associated with cardiac, skeletal muscle and neuronal cells [128]. However, despite the main localization in excitable cells, the $Ca_v1.2$ channel subunits of LTCCs, for example, have been identified in several non-excitable cell types, such as osteoblasts, osteoclasts, monocytes, macrophages and stem cell hair follicles [132-134]. $Ca_v1.1$ and $Ca_v1.2$ isoforms were demonstrated to be up-regulated in the colon and colorectal cancer cells [135, 136]. More recently, $Ca_v1.2$ and $Ca_v1.3$ channel subunits were shown in prostate cancer tissues [137]. Antagonists of LTCCs have inhibited breast cancer cell growth *in vivo* [138], and blockade of LTCCs in MDA-MB-231 human breast cancer cells and 4T1 mouse mammary tumor cells slows down migration [139].

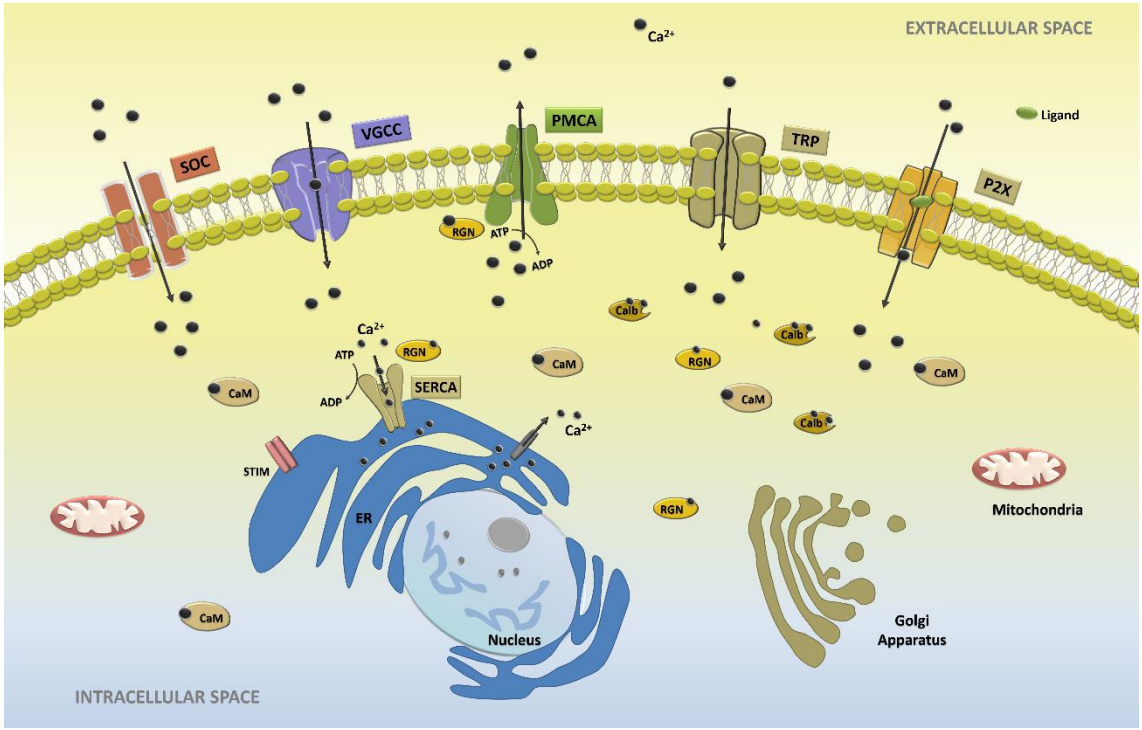

Figure I.4. Cellular regulators of Ca^{2+} homeostasis. Ca^{2+} entry occur through Ca^{2+} channels as the store-operated channels (SOC), voltage-gated Ca^{2+} channels (VGCCs), transient receptor potential (TRP) and ligand-gated ion channels, represented by P2X. The stromal interaction molecules (STIM) lead to the activation of SOC family. Intracellular Ca^{2+} levels are also regulated by the Ca^{2+} ATPases, namely plasma membrane (PMCA) and sarco/endoplasmic reticulum (SERCA), which are involved, respectively, in Ca^{2+} efflux to the extracellular space and influx to the endoplasmic reticulum (ER). Other proteins, like Calbindin (Calb), regucalcin (RGN) or calmodulin (CaM) bind to Ca^{2+} and function as Ca^{2+} buffers and/or Ca^{2+} modulators proteins.

Another well-characterized VGCCs belong to the subfamily of T-type Ca^{2+} channels (TTCCs) whose activation is mediated by low-voltage Ca^{2+} currents and are rapidly inactivated [127, 128]. TTCCs are mainly located in neurons but also are detected in cardiac tissue, which depends on the specific isoform ($Ca_v3.1$-$Ca_v3.3$) [127, 128]. Different isoforms of the TTCCs ($Ca_v3.1$ and $Ca_v3.2$ subunits) have been detected and associated with enhanced proliferation of breast cancers cells [140]. The $Ca_v3.1$ and $Ca_v3.3$ were also differentially expressed in colorectal cancer, gastric cancer and several cancer cells lines such as colon, breast, prostate or lung [120, 141]. The use of TTCCs inhibitors in HEK293 cells transfected with Cav3.2 channel has produced a reduction of cell proliferation [142].

Other players involved in the regulation of intracellular Ca^{2+} levels are the Ca^{2+} ATPases which can be found at plasma membrane (PMCAs) and sarco/endoplasmic reticulum (SERCAs) [112]. Beyond the physiologic role in Ca^{2+} handling, PMCAs are also associated to carcinogenesis [112, 143]. For example, suppression of PMCA in MCF-7 breast cancer cells promoted cell proliferation arrest [144] whereas its up-regulation is considered a protective mechanism to apoptosis, by decreasing intracellular Ca^{2+} levels [143].

Nevertheless, Ca^{2+} ion is an intracellular messenger that requires cross-talk players to transduce the signals, which includes several Ca^{2+} buffers and Ca^{2+}-modulated proteins. Calbindin is a cytosolic Ca^{2+}-buffer protein that acts by buffering Ca^{2+} and regulates intracellular Ca^{2+} raises

in order to maintain normal cell functions [112]. Ca^{2+}/calmodulin (CaM), the universal Ca^{2+}-sensor protein is one example of Ca^{2+}-modulated proteins that mediates Ca^{2+} signaling through kinase transduction pathways [112]. It is described that CaM-dependent kinases is required for cell cycle progression and cell growth in MCF-7 breast cancer cells [145, 146].

Regucalcin (RGN) is a Ca^{2+}-binding protein that does not contain the typical EF-hand Ca^{2+}-binding domain, and has an important function in the regulation of Ca^{2+}-homeostasis [147]. RGN was demonstrated to exert a regulatory effect in the control of intracellular Ca^{2+} levels by modulating the activity of Ca^{2+}-pumps, including PMCA and SERCA [148, 149]. Additionally, the enhanced expression of RGN was shown to diminish the mRNA levels of LTCCs [150].

The progresses made in the last years regarding Ca^{2+} signaling and homeostasis in the context of cancer and their relevance as possible therapeutic targets demonstrated that this is a promising area of research, which deserves to be further explored in a near future.

References

1. Hennighausen L, Robinson GW (2005) Information networks in the mammary gland. Nat Rev Mol Cell Biol 6(9):715-725

2. Gjorevski N, Nelson CM (2011) Integrated morphodynamic signalling of the mammary gland. Nat Rev Mol Cell Biol 12(9):581-593

3. Macias H, Hinck L (2012) Mammary gland development. Wiley Interdiscip Reviews Dev Biol 1(4):533-557

4. Inman JL, Robertson C, Mott JD, Bissell MJ (2015) Mammary gland development: cell fate specification, stem cells and the microenvironment. Development 142(6):1028-1042

5. Kedeshian P, Sternlicht MD, Nguyen M, Shao ZM, Barsky SH (1998) Humatrix, a novel myoepithelial matrical gel with unique biochemical and biological properties. Cancer Lett 123(2):215-226

6. Gudjonsson T, Ronnov-Jessen L, Villadsen R, Rank F, Bissell MJ, Petersen OW (2002) Normal and tumor-derived myoepithelial cells differ in their ability to interact with luminal breast epithelial cells for polarity and basement membrane deposition. J Cell Sci 115(Pt 1):39-50

7. Williams JM, Daniel CW (1983) Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis. Dev Biol 97(2):274-290

8. Ali S, Coombes RC (2002) Endocrine-responsive breast cancer and strategies for combating resistance. Nat Rev Cancer 2(2):101-112

9. Russo J and Russo IH Molecular basis of breast cancer: prevention and treatment. In: Springer-Verlag, editor. 1st edition ed2004.

10. Hassiotou F, Geddes D (2013) Anatomy of the human mammary gland: Current status of knowledge. Clin Anat 26(1):29-48

11. Bombonati A, Sgroi DC (2011) The molecular pathology of breast cancer progression. J Pathol 223(2):307-317

12. Smalley M, Ashworth A (2003) Stem cells and breast cancer: A field in transit. Nat Rev Cancer 3(11):832-844

13. Lanigan F, O'Connor D, Martin E, Gallagher WM (2007) Molecular links between mammary gland development and breast cancer. Cell Mol Life Sci 64(24):3161-3184

14. Skibinski A, Kuperwasser C (2015) The origin of breast tumor heterogeneity. Oncogene 1-8 doi:10.1038/onc.2014.475

15. Hovey RC, Aimo L (2010) Diverse and active roles for adipocytes during mammary gland growth and function. J Mammary Gland Biol Neoplasia 15(3):279-290

16. Makarem M, Kannan N, Nguyen LV, Knapp DJ, Balani S, Prater MD, Stingl J, Raouf A, Nemirovsky O, Eirew P et al. (2013) Developmental changes in the in vitro activated regenerative activity of primitive mammary epithelial cells. PLoS biology 11(8):e1001630

17. Wiseman BS, Werb Z (2002) Stromal effects on mammary gland development and breast cancer. Science 296(5570):1046-1049

18. Brisken C, O'Malley B (2010) Hormone action in the mammary gland. Cold Spring Harb Perspect Biol 2(12):a003178

19. Weigelt B, Reis-Filho JS (2009) Histological and molecular types of breast cancer: is there a unifying taxonomy? Nat Rev Clin Oncol 6(12):718-730

20. Keen JC, Davidson NE (2003) The biology of breast carcinoma. Cancer 97(3 Suppl):825-833

21. Umar A, Dunn BK, Greenwald P (2012) Future directions in cancer prevention. Nat Rev Cancer 12(12):835-848

22. Ferrini K, Ghelfi F, Mannucci R, Titta L (2015) Lifestyle, nutrition and breast cancer: facts and presumptions for consideration. Ecancermedicalscience 9:557

23. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136(5):E359-386

24. Siegel RL, Miller KD, Jemal A (2015) Cancer statistics, 2015. CA: a cancer journal for clinicians 65(1):5-29

25. Malhotra GK, Zhao X, Band H, Band V (2010) Histological, molecular and functional subtypes of breast cancers. Cancer Biol Ther 10(10):955-960

26. Weigelt B, Geyer FC, Reis-Filho JS (2010) Histological types of breast cancer: how special are they? Mol Oncol 4(3):192-208

27. Vargo-Gogola T, Rosen JM (2007) Modelling breast cancer: one size does not fit all. Nat Rev Cancer 7(9):659-672

28. Polyak K, Kalluri R (2010) The role of the microenvironment in mammary gland development and cancer. Cold Spring Harb Perspect Biol 2(11):a003244

29. Polyak K (2007) Breast cancer: origins and evolution. J Clin Invest 117(11):3155-3163

30. Rakha EA, Reis-Filho JS, Baehner F, Dabbs DJ, Decker T, Eusebi V, Fox SB, Ichihara S, Jacquemier J, Lakhani SR et al. (2010) Breast cancer prognostic classification in the molecular era: the role of histological grade. Breast Cancer Res 12(4):207

31. Cowell CF, Weigelt B, Sakr RA, Ng CK, Hicks J, King TA, Reis-Filho JS (2013) Progression from ductal carcinoma in situ to invasive breast cancer: Revisited. Mol Oncol 7(5):859-869

32. Yersal O, Barutca S (2014) Biological subtypes of breast cancer: Prognostic and therapeutic implications. World J Clin Oncol 5(3):412-424

33. Ahmad A (2013) Pathways to breast cancer recurrence. ISRN oncology 2013:290568

34. Eroles P, Bosch A, Perez-Fidalgo JA, Lluch A (2012) Molecular biology in breast cancer: intrinsic subtypes and signaling pathways. Cancer Treat Rev 38(6):698-707

35. Mullan PB, Millikan RC (2007) Molecular subtyping of breast cancer: opportunities for new therapeutic approaches. Cell Mol Life Sci 64(24):3219-3232

36. Koboldt DC, Fulton RS, McLellan MD, Schmidt H, Kalicki-Veizer J, McMichael JF, Fulton LL, Dooling DJ, Ding L, Mardis ER et al. (2012) Comprehensive molecular portraits of human breast tumours. Nature 490(7418):61-70

37. Zardavas D, Baselga J, Piccart M (2013) Emerging targeted agents in metastatic breast cancer. Nat Rev Clin Oncol 10(4):191-210

38. Visvader JE (2011) Cells of origin in cancer. Nature 469(7330):314-322

39. Russo J, Russo IH (2006) The role of estrogen in the initiation of breast cancer. J Steroid Biochem Mol Biol 102(1-5):89-96

40. Love RR, Philips J (2002) Oophorectomy for breast cancer: history revisited. J Natl Cancer Inst 94(19):1433-1434

41. Yager JD, Davidson NE (2006) Estrogen carcinogenesis in breast cancer. N Engl J Med 354(3):270-282

42. McNamara KM, Sasano H (2015) The intracrinology of breast cancer. J Steroid Biochem Mol Biol 145:172-178

43. Yue W, Yager JD, Wang JP, Jupe ER, Santen RJ (2013) Estrogen receptor-dependent and independent mechanisms of breast cancer carcinogenesis. Steroids 78(2):161-170

44. Liang J, Shang Y (2013) Estrogen and cancer. Annu Rev Physiol 75:225-240

45. Thomas C, Gustafsson JA (2011) The different roles of ER subtypes in cancer biology and therapy. Nat Rev Cancer 11(8):597-608

46. Bjornstrom L, Sjoberg M (2005) Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Mol Endocrinol 19(4):833-842

47. Richter K, Buchner J (2001) Hsp90: chaperoning signal transduction. J Cell Physiol 188(3):281-290

48. Dimitrakakis C, Bondy C (2009) Androgens and the breast. Breast Cancer Res 11(5):212

49. Suzuki T, Miki Y, Takagi K, Hirakawa H, Moriya T, Ohuchi N, Sasano H (2010) Androgens in human breast carcinoma. Med Mol Morphol 43(2):75-81

50. Fioretti FM, Sita-Lumsden A, Bevan CL, Brooke GN (2014) Revising the role of the androgen receptor in breast cancer. J Mol Endocrinol 52(3):R257-R265

51. Hickey TE, Robinson JL, Carroll JS, Tilley WD (2012) Minireview: The androgen receptor in breast tissues: growth inhibitor, tumor suppressor, oncogene? Mol Endocrinol 26(8):1252-1267

52. McNamara KM, Moore NL, Hickey TE, Sasano H, Tilley WD (2014) Complexities of androgen receptor signalling in breast cancer. Endocr Relat Cancer 21(4):T161-181

53. Chottanapund S, Van Duursen MB, Navasumrit P, Hunsonti P, Timtavorn S, Ruchirawat M, Van den Berg M (2013) Effect of androgens on different breast cancer cells co-cultured with or without breast adipose fibroblasts. J Steroid Biochem Mol Biol 138:54-62

54. Greeve MA, Allan RK, Harvey JM, Bentel JM (2004) Inhibition of MCF-7 breast cancer cell proliferation by 5alpha-dihydrotestosterone; a role for p21(Cip1/Waf1). J Mol Endocrinol 32(3):793-810

55. Ortmann J, Prifti S, Bohlmann MK, Rehberger-Schneider S, Strowitzki T, Rabe T (2002) Testosterone and 5 alpha-dihydrotestosterone inhibit in vitro growth of human breast cancer cell lines. Gynecol Endocrinol 16(2):113-120

56. Tiefenbacher K, Daxenbichler G (2008) The Role of Androgens in Normal and Malignant Breast Tissue. Breast Care 3(5):325-331

57. Labrie F, Luu-The V, Labrie C, Belanger A, Simard J, Lin SX, Pelletier G (2003) Endocrine and intracrine sources of androgens in women: inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone. Endocr Rev 24(2):152-182

58. Bennett NC, Gardiner RA, Hooper JD, Johnson DW, Gobe GC (2010) Molecular cell biology of androgen receptor signalling. Int J Biochem Cell Biol 42(6):813-827

59. Li J, Al-Azzawi F (2009) Mechanism of androgen receptor action. Maturitas 63(2):142-148

60. Wang Y, He X, Yu Q, Eng C (2013) Androgen receptor-induced tumor suppressor, KLLN, inhibits breast cancer growth and transcriptionally activates p53/p73-mediated apoptosis in breast carcinomas. Hum Mol Genet 22(11):2263-2272

61. Honma N, Horii R, Iwase T, Saji S, Younes M, Ito Y, Akiyama F (2013) Clinical importance of androgen receptor in breast cancer patients treated with adjuvant tamoxifen monotherapy. Breast Cancer 20(4):323-330

62. Ogawa Y, Hai E, Matsumoto K, Ikeda K, Tokunaga S, Nagahara H, Sakurai K, Inoue T, Nishiguchi Y (2008) Androgen receptor expression in breast cancer: relationship with clinicopathological factors and biomarkers. Int J Clin Oncol 13(5):431-435

63. Wang L, Di LJ (2014) BRCA1 and estrogen/estrogen receptor in breast cancer: where they interact? Int J Biol Sci 10(5):566-575

64. Ratanaphan A (2012) A DNA repair BRCA1 estrogen receptor and targeted therapy in breast cancer. Int J Mol Sci 13(11):14898-14916

65. Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W et al. (1994) A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 266(5182):66-71

66. Venkitaraman AR (2002) Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell 108(2):171-182

67. Njiaju UO, Olopade OI (2012) Genetic determinants of breast cancer risk: a review of current literature and issues pertaining to clinical application. Breast J 18(5):436-442

68. Ahmed M, Lalloo F, Evans DG (2009) Update on genetic predisposition to breast cancer. Expert review of anticancer therapy 9(8):1103-1113

69. Berns EM, Dirkzwager-Kiel MJ, Kuenen-Boumeester V, Timmermans M, Verhoog LC, van den Ouweland AM, Meijer-Heijboer H, Klijn JG, van der Kwast TH (2003) Androgen pathway dysregulation in BRCA1-mutated breast tumors. Breast Cancer Res Treat 79(1):121-127

70. Kurian AW, Kingham KE, Ford JM (2015) Next-generation sequencing for hereditary breast and gynecologic cancer risk assessment. Curr Opin Obstet Gynecol 27(1):23-33

71. Wuttke M, Phillips KA (2015) Clinical management of women at high risk of breast cancer. Curr Opin Obstet Gynecol 27(1):6-13

72. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5):646-674

73. Warburg O, Wind F, Negelein E (1927) The Metabolism of Tumors in the Body. J Gen Physiol 8(6):519-530

74. Warburg O (1956) On the origin of cancer cells. Science 123(3191):309-314

75. Warburg O (1956) On respiratory impairment in cancer cells. Science 124(3215):269-270

76. Icard P, Lincet H (2012) A global view of the biochemical pathways involved in the regulation of the metabolism of cancer cells. Biochim Biophys Acta 1826(2):423-433

77. Yadava N, Schneider SS, Jerry DJ, Kim C (2013) Impaired mitochondrial metabolism and mammary carcinogenesis. J Mammary Gland Biol Neoplasia 18(1):75-87

78. Hsu PP, Sabatini DM (2008) Cancer cell metabolism: Warburg and beyond. Cell 134(5):703-707

79. Oakman C, Tenori L, Biganzoli L, Santarpia L, Cappadona S, Luchinat C, Di Leo A (2011) Uncovering the metabolomic fingerprint of breast cancer. Int J Biochem Cell Biol 43(7):1010-1020

80. Lu J, Tan M, Cai Q (2015) The Warburg effect in tumor progression: mitochondrial oxidative metabolism as an anti-metastasis mechanism. Cancer Lett 356(2 Pt A):156-164

81. Mueckler M, Thorens B (2013) The SLC2 (GLUT) family of membrane transporters. Mol Aspects Med 34(2-3):121-138

82. Krzeslak A, Wojcik-Krowiranda K, Forma E, Jozwiak P, Romanowicz H, Bienkiewicz A, Brys M (2012) Expression of GLUT1 and GLUT3 glucose transporters in endometrial and breast cancers. Pathol Oncol Res 18(3):721-728

83. Macheda ML, Rogers S, Best JD (2005) Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. J Cell Physiol 202(3):654-662

84. Godoy A, Ulloa V, Rodriguez F, Reinicke K, Yanez AJ, Garcia Mde L, Medina RA, Carrasco M, Barberis S, Castro T et al. (2006) Differential subcellular distribution of glucose transporters GLUT1-6 and GLUT9 in human cancer: ultrastructural localization of GLUT1 and GLUT5 in breast tumor tissues. J Cell Physiol 207(3):614-627

85. Schmidt M, Voelker HU, Kapp M, Krockenberger M, Dietl J, Kammerer U (2010) Glycolytic phenotype in breast cancer: activation of Akt, up-regulation of GLUT1, TKTL1 and down-regulation of M2PK. J Cancer Res Clin Oncol 136(2):219-225

86. Kang SS, Chun YK, Hur MH, Lee HK, Kim YJ, Hong SR, Lee JH, Lee SG, Park YK (2002) Clinical significance of glucose transporter 1 (GLUT1) expression in human breast carcinoma. Jpn J Cancer Res 93(10):1123-1128

87. Wang G, Xu Z, Wang C, Yao F, Li J, Chen C, Sun S (2013) Differential phosphofructokinase-1 isoenzyme patterns associated with glycolytic efficiency in human breast cancer and paracancer tissues. Oncol Lett 6(6):1701-1706

88. El-Bacha T, de Freitas MS, Sola-Penna M (2003) Cellular distribution of phosphofructokinase activity and implications to metabolic regulation in human breast cancer. Mol Genet Metab 79(4):294-299

89. Lunt SY, Vander Heiden MG (2011) Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. Annu Rev Cell Dev Biol 27:441-464

90. Ramos-Montoya A, Lee WN, Bassilian S, Lim S, Trebukhina RV, Kazhyna MV, Ciudad CJ, Noe V, Centelles JJ, Cascante M (2006) Pentose phosphate cycle oxidative and nonoxidative balance: A new vulnerable target for overcoming drug resistance in cancer. Int J Cancer 119(12):2733-2741

91. Feron O (2009) Pyruvate into lactate and back: from the Warburg effect to symbiotic energy fuel exchange in cancer cells. Radiother Oncol 92(3):329-333

92. Ward PS, Thompson CB (2012) Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. Cancer Cell 21(3):297-308

93. DeBerardinis RJ, Cheng T (2010) Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. Oncogene 29(3):313-324

94. Icard P, Kafara P, Steyaert JM, Schwartz L, Lincet H (2014) The metabolic cooperation between cells in solid cancer tumors. Biochim Biophys Acta 1846(1):216-225

95. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB (2008) The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metab 7(1):11-20

96. DeBerardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, Thompson CB (2007) Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. Proc Natl Acad Sci U S A 104(49):19345-19350

97. Denko NC (2008) Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat Rev Cancer 8(9):705-713

98. Wang ZY, Loo TY, Shen JG, Wang N, Wang DM, Yang DP, Mo SL, Guan XY, Chen JP (2012) LDH-A silencing suppresses breast cancer tumorigenicity through induction of oxidative stress mediated mitochondrial pathway apoptosis. Breast Cancer Res Treat 131(3):791-800

99. Rizwan A, Serganova I, Khanin R, Karabeber H, Ni X, Thakur S, Zakian KL, Blasberg R, Koutcher JA (2013) Relationships between LDH-A, lactate, and metastases in 4T1 breast tumors. Clin Cancer Res 19(18):5158-5169

100. Kondaveeti Y, Guttilla Reed IK, White BA (2015) Epithelial-mesenchymal transition induces similar metabolic alterations in two independent breast cancer cell lines. Cancer Lett 364(1):44-58

101. McCleland ML, Adler AS, Shang Y, Hunsaker T, Truong T, Peterson D, Torres E, Li L, Haley B, Stephan JP et al. (2012) An integrated genomic screen identifies LDHB as an essential gene for triple-negative breast cancer. Cancer Res 72(22):5812-5823

102. Halestrap AP (2013) Monocarboxylic acid transport. Comprehensive Physiology 3(4):1611-1643

103. Halestrap AP, Wilson MC (2012) The monocarboxylate transporter family--role and regulation. IUBMB Life 64(2):109-119

104. Upadhyay M, Samal J, Kandpal M, Singh OV, Vivekanandan P (2013) The Warburg effect: insights from the past decade. Pharmacol Ther 137(3):318-330

105. Ganapathy-Kanniappan S, Geschwind JF (2013) Tumor glycolysis as a target for cancer therapy: progress and prospects. Mol Cancer 12:152

106. Halestrap AP (2012) The monocarboxylate transporter family--Structure and functional characterization. IUBMB Life 64(1):1-9

107. Dimmer KS, Friedrich B, Lang F, Deitmer JW, Broer S (2000) The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. Biochem J 350 Pt 1:219-227

108. Witkiewicz AK, Whitaker-Menezes D, Dasgupta A, Philp NJ, Lin Z, Gandara R, Sneddon S, Martinez-Outschoorn UE, Sotgia F, Lisanti MP (2012) Using the "reverse Warburg effect" to identify high-risk breast cancer patients: stromal MCT4 predicts poor clinical outcome in triple-negative breast cancers. Cell Cycle 11(6):1108-1117

109. Baenke F, Dubuis S, Brault C, Weigelt B, Dankworth B, Griffiths B, Jiang M, Mackay A, Saunders B, Spencer-Dene B et al. (2015) Functional screening identifies MCT4 as a key regulator of breast cancer cell metabolism and survival. J Pathol doi:10.1002/path.4562.

110. Pinheiro C, Albergaria A, Paredes J, Sousa B, Dufloth R, Vieira D, Schmitt F, Baltazar F (2010) Monocarboxylate transporter 1 is up-regulated in basal-like breast carcinoma. Histopathology 56(7):860-867

111. Morais-Santos F, Miranda-Goncalves V, Pinheiro S, Vieira AF, Paredes J, Schmitt FC, Baltazar F, Pinheiro C (2014) Differential sensitivities to lactate transport inhibitors of breast cancer cell lines. Endocr Relat Cancer 21(1):27-38

112. Lee WJ, Monteith GR, Roberts-Thomson SJ (2006) Calcium transport and signaling in the mammary gland: Targets for breast cancer. BBA-Rev Cancer 1765(2):235-255

113. Prevarskaya N, Skryma R, Shuba Y (2010) Ion channels and the hallmarks of cancer. Trends Mol Med 16(3):107-121

114. Prevarskaya N, Skryma R, Shuba Y (2011) Calcium in tumour metastasis: new roles for known actors. Nat Rev Cancer 11(8):609-618

115. Roderick HL, Cook SJ (2008) Ca2+ signalling checkpoints in cancer: remodelling Ca2+ for cancer cell proliferation and survival. Nat Rev Cancer 8(5):361-375

116. Zheng J (2013) Molecular mechanism of TRP channels. Compr Physiol 3(1):221-242

117. Prevarskaya N, Zhang L, Barritt G (2007) TRP channels in cancer. Biochim Biophys Acta 1772(8):937-946

118. Van Haute C, De Ridder D, Nilius B (2010) TRP channels in human prostate. ScientificWorldJournal 10:1597-1611

119. Lee JM, Davis FM, Roberts-Thomson SJ, Monteith GR (2011) Ion channels and transporters in cancer. 4. Remodeling of Ca(2+) signaling in tumorigenesis: role of Ca(2+) transport. Am J Physiol Cell Physiol 301(5):C969-976

120. Monteith GR, McAndrew D, Faddy HM, Roberts-Thomson SJ (2007) Calcium and cancer: targeting Ca2+ transport. Nat Rev Cancer 7(7):519-530

121. Cross BM, Breitwieser GE, Reinhardt TA, Rao R (2014) Cellular calcium dynamics in lactation and breast cancer: from physiology to pathology. Am J Physiol Cell Physiol 306(6):C515-526

122. Prakriya M (2013) Store-operated Orai channels: structure and function. Curr Top Membr 71:1-32

123. Yang S, Zhang JJ, Huang XY (2009) Orai1 and STIM1 are critical for breast tumor cell migration and metastasis. Cancer Cell 15(2):124-134

124. Feng YH, Li X, Wang L, Zhou L, Gorodeski GI (2006) A truncated P2X7 receptor variant (P2X7-j) endogenously expressed in cervical cancer cells antagonizes the full-length P2X7 receptor through hetero-oligomerization. J Biol Chem 281(25):17228-17237

125. Shabbir M, Ryten M, Thompson C, Mikhailidis D, Burnstock G (2008) Purinergic receptor-mediated effects of ATP in high-grade bladder cancer. BJU international 101(1):106-112

126. Shabbir M, Ryten M, Thompson C, Mikhailidis D, Burnstock G (2008) Characterization of calcium-independent purinergic receptor-mediated apoptosis in hormone-refractory prostate cancer. BJU international 101(3):352-359

127. Catterall WA (2011) Voltage-gated calcium channels. Cold Spring Harb Perspect Biol 3(8):a003947

128. Dolphin AC (2009) Calcium channel diversity: multiple roles of calcium channel subunits. Curr Opin Neurobiol 19(3):237-244

129. Zuccotti A, Clementi S, Reinbothe T, Torrente A, Vandael DH, Pirone A (2011) Structural and functional differences between L-type calcium channels: crucial issues for future selective targeting. Trends Pharmacol Sci 32(6):366-375

130. Fiske JL, Fomin VP, Brown ML, Duncan RL, Sikes RA (2006) Voltage-sensitive ion channels and cancer. Cancer Metastasis Rev 25(3):493-500

131. Hofmann F, Flockerzi V, Kahl S, Wegener JW (2014) L-type CaV1.2 calcium channels: from in vitro findings to in vivo function. Physiol Rev 94(1):303-326

132. Li F, Wang W, Gu M, Gyoneva S, Zhang J, Huang S, Traynelis SF, Cai H, Guggino SE, Zhang X (2011) L-type calcium channel activity in osteoblast cells is regulated by the actin cytoskeleton independent of protein trafficking. J Bone Miner Metab 29(5):515-525

133. Yucel G, Altindag B, Gomez-Ospina N, Rana A, Panagiotakos G, Lara MF, Dolmetsch R, Oro AE (2013) State-dependent signaling by Cav1.2 regulates hair follicle stem cell function. Genes Dev 27(11):1217-1222

134. Das R, Burke T, Van Wagoner DR, Plow EF (2009) L-type calcium channel blockers exert an antiinflammatory effect by suppressing expression of plasminogen receptors on macrophages. Circ Res 105(2):167-175

135. Wang XT, Nagaba Y, Cross HS, Wrba F, Zhang L, Guggino SE (2000) The mRNA of L-type calcium channel elevated in colon cancer: protein distribution in normal and cancerous colon. Am J Pathol 157(5):1549-1562

136. Zhang L, Zhou W, Velculescu VE, Kern SE, Hruban RH, Hamilton SR, Vogelstein B, Kinzler KW (1997) Gene expression profiles in normal and cancer cells. Science 276(5316):1268-1272

137. Chen R, Zeng X, Zhang R, Huang J, Kuang X, Yang J, Liu J, Tawfik O, Thrasher JB, Li B (2014) Cav1.3 channel alpha1D protein is overexpressed and modulates androgen receptor transactivation in prostate cancers. Urol Oncol 32(5):524-536

138. Taylor JM, Simpson RU (1992) Inhibition of Cancer Cell-Growth by Calcium-Channel Antagonists in the Athymic Mouse. Cancer Res 52(9):2413-2418

139. Yang S, Huang XY (2005) Ca2+ influx through L-type Ca2+ channels controls the trailing tail contraction in growth factor-induced fibroblast cell migration. J Biol Chem 280(29):27130-27137

140. Taylor JT, Huang L, Pottle JE, Liu K, Yang Y, Zeng X, Keyser BM, Agrawal KC, Hansen JB, Li M (2008) Selective blockade of T-type Ca2+ channels suppresses human breast cancer cell proliferation. Cancer Lett 267(1):116-124

141. Toyota M, Ho C, Ohe-Toyota M, Baylin SB, Issa JP (1999) Inactivation of CACNA1G, a T-type calcium channel gene, by aberrant methylation of its 5' CpG island in human tumors. Cancer Res 59(18):4535-4541

142. Gray LS, Perez-Reyes E, Gomora JC, Haverstick DM, Shattock M, McLatchie L, Harper J, Brooks G, Heady T, Macdonald TL (2004) The role of voltage gated T-type Ca2+ channel isoforms in mediating "capacitative" Ca2+ entry in cancer cells. Cell Calcium 36(6):489-497

143. Curry MC, Roberts-Thomson SJ, Monteith GR (2011) Plasma membrane calcium ATPases and cancer. Biofactors 37(3):132-138

144. Lee WJ, Robinson JA, Holman NA, McCall MN, Roberts-Thomson SJ, Monteith GR (2005) Antisense-mediated Inhibition of the plasma membrane calcium-ATPase suppresses proliferation of MCF-7 cells. J Biol Chem 280(29):27076-27084

145. Rodriguez-Mora OG, LaHair MM, McCubrey JA, Franklin RA (2005) Calcium/calmodulin-dependent kinase I and calcium/calmodulin-dependent kinase kinase participate in the control of cell cycle progression in MCF-7 human breast cancer cells. Cancer Res 65(12):5408-5416

146. Schmitt JM, Abell E, Wagner A, Davare MA (2010) ERK activation and cell growth require CaM kinases in MCF-7 breast cancer cells. Mol Cell Biochem 335(1-2):155-171

147. Yamaguchi M (2011) Regucalcin and cell regulation: role as a suppressor protein in signal transduction. Mol Cell Biochem 353(1-2):101-137

148. Yamaguchi M, Nakajima R (2002) Role of regucalcin as an activator of sarcoplasmic reticulum Ca2+-ATPase activity in rat heart muscle. J Cell Biochem 86(1):184-193

149. Lai P, Yip NC, Michelangeli F (2011) Regucalcin (RGN/SMP30) alters agonist- and thapsigargin-induced cytosolic [Ca2+] transients in cells by increasing SERCA Ca(2+)ATPase levels. FEBS Lett 585(14):2291-2294

150. Nakagawa T, Yamaguchi M (2006) Overexpression of regucalcin enhances its nuclear localization and suppresses L-type Ca2+ channel and calcium-sensing receptor mRNA expressions in cloned normal rat kidney proximal tubular epithelial NRK52E cells. J Cell Biochem 99(4):1064-1077

Chapter II

The diverse roles of calcium-binding protein regucalcin in cell biology: from tissue expression and signaling to disease

Chapter published in: Ricardo Marques, Cláudio J Maia, Cátia Vaz, Sara Correia, Sílvia Socorro (2014) The diverse roles of calcium-binding protein regucalcin in cell biology: from tissue expression and signaling to disease. Cell Mol Life Sci. **71**(1): 93-111.

The diverse roles of calcium-binding protein regucalcin in cell biology: from tissue expression and signaling to disease

Abstract

Regucalcin (RGN) is calcium (Ca^{2+})-binding protein widely expressed in vertebrate and invertebrate species, which is also known as senescence marker protein 30, due to its molecular weight (33 kDa) and a characteristically diminished expression with aging process. RGN regulates intracellular Ca^{2+} homeostasis and the activity of several proteins involved in intracellular signaling pathways, namely, kinases, phosphatases, phosphodiesterase, nitric oxide synthase and proteases, which highlights its importance in cell biology. In addition, RGN has cytoprotective effects reducing intracellular levels of oxidative stress, also playing a role in the control of cell survival and apoptosis. Multiple factors have been identified regulating the cell levels of RGN transcripts and protein, and an altered expression pattern of this interesting protein has been found in cases of reproductive disorders, neurodegenerative diseases and cancer. Moreover, RGN is a serum secreted protein and its levels have been correlated with the stage of disease, which strongly suggests the usefulness of this protein as a potential biomarker for monitoring disease onset and progression. The present review aims to discuss the available information concerning RGN expression and function in distinct cell types and tissues, integrating cellular and molecular mechanisms in the context of normal and pathological conditions. Insight into the cellular actions of RGN will be a key step towards deepening the knowledge of the biology of several human diseases.

Introduction

Regucalcin (RGN) was initially discovered in 1978 by Yamaguchi [1] and, although classified as a calcium (Ca^{2+})-binding protein, it does not contain the typical EF-hand Ca^{2+}-binding motif [2]. The overall structure of RGN protein contains 24 ß-strands forming six ß-sheets able to bind diverse divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}) [3-6]. The RGN ability to bind Ca^{2+} was recently confirmed by X-ray diffraction studies which have allowed the resolving of the crystal structure of human RGN protein bound to Ca^{2+} or Zn^{2+} cations. Although Ca^{2+} and Zn^{2+} ions bind to the same amino acid residues forming a unique metal binding site in a nearly identical coordination, an very much higher level of dissociation constant is documented for Ca^{2+} which could be relevant under non physiological conditions, whereas elevated Ca^{2+} levels can occur [4].

The RGN gene is localised in the p11.3-q11.2 and q11.1-12 segments of the human and rat X chromosome, respectively [7,8]. In both cases, the gene consists of seven exons [9-11] encoding a protein of 299 amino acid residues with an approximate molecular weight of 33 kDa [2,12]. For this reason, together with the diminished expression of RGN in tissues of aged animals, Fujita and co-authors [12-14] named it senescence marker protein 30 (SMP30).

RGN is highly expressed in the liver and kidney cortex [12,15,16], but it has been detected in several others tissues [16,17] in a broad range of vertebrate and invertebrate species [18-20]. The transcription of RGN gene is enhanced by several regulatory transcription factors upstream of the 5'flaking region, namely the AP1, NF1-A1, RGPR-p117 and ß-catenin [21]. Ca^{2+} levels modulate RGN expression in a process involving, for example, calmodulin (CaM) or protein kinase C (PKC) [22-24]. Also, Ca^{2+}-independent mechanisms [25], hormonal factors and others have been described as regulating the levels of RGN in cells [11,26-30]. Moreover, altered expression patterns of RGN have been associated with several disease conditions in both human and animal models [11,31-39], which highlights for the importance of this protein in cell biology.

RGN has been localized to the cell nucleus and cytoplasm [26,40,41], as well as in mitochondrial fraction [42], and multiple physiological functions have been assigned to this curious protein. Among them is the ability of RGN to influence Ca^{2+} homeostasis through the regulation of Ca^{2+}-pumping activity in cell membrane, nucleus, microsomes, endoplasmic reticulum and mitochondria of various cell types [43]. It has been also associated to intracellular signaling pathways, since it regulates several Ca^{2+}-dependent enzymes such as protein kinases, tyrosine kinases, phosphatases, phosphodiesterase, nitric oxide synthase and proteases [43-48].

In addition, the antioxidant properties of RGN in reducing intracellular levels of oxidative stress have also been described. This effect is achieved through modulation of the activity of enzymes involved in generation of oxidative stress as well as in the antioxidant defence [49-52].

Several reports using gene-silencing and overexpression approaches have pointed out a role of RGN regulating cell death and proliferation. Although the mechanisms implicated in this control are not completely understood, it has been demonstrated that RGN can regulate DNA synthesis and fragmentation [53-56], modulate the expression of oncogenes, tumor suppressor genes and cell cycle regulators [53,54,57], influencing survival and apoptotic pathways [58-60].

This review discusses the current knowledge about the expression and function of RGN in several cell types and tissues, exploring concepts from molecular biology point of view in signaling pathways and systems biology. The potential roles of RGN in pathological situations will also be discussed.

RGN in non-pathological and pathological tissues and cell lines

RGN has been identified in a wide range of species from invertebrates to mammalian and non-mammalian vertebrates, also including fungi and bacteria [10,12,18-20,61-65]. Protein sequence alignment and determination of amino acid identities show that RGN is highly conserved throughout evolution (Table II.1). Human RGN (NP_690608) is highly homologous with other primate proteins showing 97 % identity with that of orang-utan (*Pongo abelii*, NP_001127502). Percentages of amino acid identity with other mammals range from 88 to 91

Table II.1. Overall percentage of amino-acid identities of RGN protein among vertebrate, invertebrate, bacteria and fungi species, determined by Genedoc software[a] after performing Clustalw alignment[b]

	Pongo abelii	*Homo sapiens*	*Bos taurus*	*Sus scrofa*	*Oryctolagus cuniculus*	*Rattus norvegicus*	*Mus musculus*	*Gallus gallus*	*Xenopus laevis*	*Danio rerio*	*Ictalurus punctatus*	*Haliotis discus*	*Drosophila Melanogaster*	*Acyrthosiphon pisum*	*Bacillus cereus*	*Aspergillus fumigatus*	*Agrobacterium tumefaciens*
Pongo abelii[c]	299																
Homo sapiens	97%	299															
Bos taurus	90%	91%	299														
Sus scrofa	88%	88%	93%	299													
Oryctolagus cuniculus	88%	89%	89%	89%	299												
Rattus norvegicus	87%	88%	87%	85%	85%	299											
Mus musculus	87%	88%	87%	85%	85%	94%	299										
Gallus gallus	77%	77%	77%	76%	76%	76%	75%	299									
Xenopus laevis	69%	70%	69%	68%	71%	71%	70%	73%	299								
Danio rerio	61%	62%	62%	61%	62%	61%	61%	61%	61%	295							
Ictalurus punctatus	61%	62%	62%	59%	62%	62%	62%	64%	61%	74%	299						
Haliotis discus	40%	41%	41%	42%	40%	43%	42%	43%	41%	43%	45%	305					
Drosophila melanogaster	32%	32%	32%	32%	33%	32%	33%	32%	31%	32%	31%	29%	303				
Acyrthosiphon pisum	30%	30%	31%	31%	31%	30%	31%	30%	31%	32%	32%	33%	41%	326			
Bacillus cereus	32%	32%	32%	32%	32%	32%	32%	32%	33%	32%	33%	31%	28%	26%	300		
Aspergillus fumigatus	26%	26%	26%	26%	26%	26%	25%	25%	27%	24%	25%	24%	20%	21%	24%	281	
Agrobacterium tumefaciens	22%	22%	23%	23%	23%	23%	23%	25%	25%	21%	22%	22%	19%	21%	26%	19%	295

[a] Nicholas KB and HB Nicholas GeneDoc: a tool for editing and annotating multiple sequence alignments. *EMBNEW.NEWS* 1997 **4**:p. 14; [b] Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic acids research* 1997 **25**:4876-82; [c] Common names and protein accession numbers are provided in the text.

%: 88 % with pig (*Sus scrofa*, NP_001070688), rat (*Rattus norvegicus*, NP_113734) and mouse (*Mus musculus*, NP_033086); 89 % with rabbit (*Oryctolagus cuniculus*, NP_001075472), and 91 % with cow (*Bos taurus*, NP_776382.1). The overall identity decreases in comparison with non-mammalian vertebrates showing 77 % identity with chicken (Gallus gallus, NP_990060), 70 % with frog (*Xenopus laevis*, NP_001079124) and 62 % with fish species (catfish, *Ictalurus punctatus*, NP_001187297 and zebrafish, *Danio rerio*, NP_991309). Homology with disk abalone

(*Haliotis discus*, ABO26616), fruit fly (*Drosophila melanogaster*, NP_727586) and louse *(Acyrthosiphon pisum*, *NP_001155519)* RGN proteins range from 41 to 30 %, what still is noticeable high since these are invertebrate species. Also with fungi *(Aspergillus fumigates*, XP_751966*)* and bacteria (*Bacillus cereus,* NP_978918 and *Agrobacterium tumefaciens*, NP_353727) the percentage of amino acid identities are very high, being 26, 32 and 22 %, respectively. This demonstrates that RGN gene is highly conserved among various vertebrates and invertebrates species which corroborates the idea of its well-conserved basic biologic function throughout evolution.

RGN was first identified in the liver where it is highly expressed [1,2,15], but it has also been found in a variety of pathological and non-pathological tissues and cell lines [10,11,18,24,26,32,66-68]. Table II.2 summarises the distribution of RGN mRNA and/or protein in non-pathological tissues and body fluids of several species. It is present in a variety of reproductive [26,60,66] and non-reproductive tissues [12,16,17,38,60,69-75], as well as in plasma [16,35,37,76-78], seminiferous tubules fluid [66] and insect saliva [79].

Moreover, RGN was identified in several non-pathological cell lines such as pig kidney cells (LLC-PK1) [80], rat kidney proximal tubular epithelial cells (NRK52E) [57,67], rat astrocytes (CTX TNA2) [32] and rat liver cells (Ac2F) [68].

One distinctive characteristic of RGN expression pattern is the significant diminished expression in tissues of aged animals [13,14]. Studies on the expression of RGN from embryonic to senescent stages of life revealed that, in rat liver and kidney, a maximum of expression is reached within the first month after birth. Substantial amounts of mRNA and protein are maintained up to 3 or 6.5 months, respectively, in kidney and liver, and a marked decrease of RGN expression is found in older animals [14]. In addition, it is interesting to note the existence of gender differences in RGN expression levels. Hepatic RGN mRNA expression is higher in male rats [81] and mice [82]. RGN protein levels are lower in female liver, kidney and serum, but no significant alteration was found in spleen or cerebral cortex [16,71]. As an exception, stomach of females presents higher RGN levels [71]. In any case, in aged animals, where a down-regulation of RGN expression is expected to occur, female rat livers still present minor levels in comparison with males [13,83].

Several reports have described an altered expression of RGN in distinct pathological conditions. Proteomic analysis studies identified RGN as a down-regulated gene in a muscular dystrophy mouse model [38] and in acute liver failure [35]. In contrast, RGN was up-regulated in human brain of Parkinson's disease patients [75]. Also, human testicular tissues with defective phenotypes of spermatogenesis displayed an increased expression of RGN in comparison with normal cases [34].

Table II.2. Regucalcin expression in non-pathological tissues and body fluids of distinct species

Tissue	Species	Biomolecule	Reference
Liver	h, r, m	mRNA/Protein	[1,2,12,13,15-18,60,71]
Kidney	h, r, m	mRNA/Protein	[12,13,15-18,60,71]
Adrenal gland	r	mRNA	[60]
Lung	r	mRNA/Protein	[16,60]
Heart	h, r	mRNA/Protein	[16,17,69,74]
Bone	r	mRNA/Protein	[70,72]
Skeletal muscle	r	Protein	[16,71]
Diaphragm muscle	m	Protein	[38]
Epidermis	r	mRNA	[60]
Brain	r	mRNA/Protein	[17,60]
Cerebral Cortex	r, m	Protein	[16,18]
Hippocampus	r	Protein	[16]
Locus ceruleus	h	Protein	[78]
Stomach	r, m	mRNA/Protein	[60,71]
Pancreas	h	?	[69]
Duodenum	r	Protein	[16]
Submandibular gland	m	Protein	[73]
Spleen	r	Protein	[16]
Mammary gland	h, r	mRNA/Protein	[11,26]
Uterus	r	mRNA	[60]
Ovary	r	mRNA	[60]
Prostate	h, r	mRNA/Protein	[11, 26,66]
Testis	h, r	mRNA/Protein	[16,60,66]
Epididymis	r	mRNA/Protein	[66]
Seminal vesicles	r	mRNA/Protein	[66]
Seminiferous tubules fluid	r	Protein	[66]
Plasma	h, r	Protein	[16,35,37,76,77,78]
Saliva	ap	Protein	[79]

r, rat; m, mouse; h, human; ap, pea aphid, *Acyrthosiphon pisum*

Concerning tumoral conditions, RGN expression was analyzed in hepatomas [84,85], breast and prostate cancer tissues [11], as well as in cancer cell lines of these and other tissues (see Table II.3) [86-92]. Under-expression of RGN mRNA was firstly reported in rat chemical-induced hepatomas [84]. More recently, RGN was found to be under-expressed in human hepatocellular carcinoma (HCC) [37] and breast and prostate cancers [11]. Moreover, the diminished expression of RGN was associated with histological grade of infiltrating ductal carcinoma of breast and cellular differentiation of prostate adenocarcinoma [11]. High RGN immunoreactivity was detected in 60 % of non-neoplastic prostate tissues, while only 40 % and 12 % of well-differentiated and poorly differentiated adenocarcinomas, respectively, displayed this expression pattern. Likewise, 90 % of non-neoplastic tissues of human breast showed high RGN immunoreactivity contrasting with 12 and 0 % of grade I and grade III human breast infiltrating ductal carcinomas, respectively [11]. A gene expression profile study of rat liver by

means of cDNA microarrays demonstrated that down-regulated expression of RGN starts occurring in pre-neoplastic lesions before acquisition of a tumoral phenotype [93]. Other report also established a correlation between detection of RGN in serum and cellular differentiation of HCC [37], with 52.6 % of positivity in well differentiated tumors (grade I-II) as opposed to 19 % in poorly differentiated tumors (grade III-IV).

Also, altered expression patterns of RGN were observed in non-tumoral liver diseases. Liver biopsies from patients with non-alcoholic fatty liver disease showed diminished RGN levels, which seems to be dependent of the stage of the disease [39]. On the other hand, human patients with acute liver injury [35] or chronic liver failure presented high serum levels of RGN [94]. Induced liver failure in mice by administration of galactosamine [77,78], carbon tetrachloride [76] or lipopolysaccharide (LPS) [78] is also accompanied by elevated plasma levels of RGN.

Collectively, available data raised much evidences supporting the idea that RGN may be a useful biomarker tracking onset and/or progression of tumor and non-tumor pathologies.

Table II.3. Regucalcin expression in human and murine cancer cell lines

Cell Line	Cell Type	Biomolecule	Expression	Reference
HepG2	Human Hepatocarcinoma	mRNA/Protein	↓	[51,85,88,89,90,91]
Transplantable Morris	Rat Hepatocarcinoma	mRNA	-	[84,85]
H4-II-E		mRNA/Protein	↓	[24 ,86,87]
MC3T3-E1	Mouse Osteoblast	mRNA/Protein	-	[92]
MCF-7	Human Breast cancer	mRNA/Protein	↓	[11]
LNCaP	Human Prostate cancer	mRNA/Protein	↓	[11]

↓, downregulated; -, data not available

Hormonal factors and others regulating RGN expression

Several cell-signaling factors have been shown to regulate RGN gene expression (Figure II.1) in a variety of tissues. The cell-response triggered by a specific signaling factor can be different from tissue to tissue, and several studies have shown that the regulation of RGN expression may be tissue-specific, thereby presenting different responses to the same signaling factor.

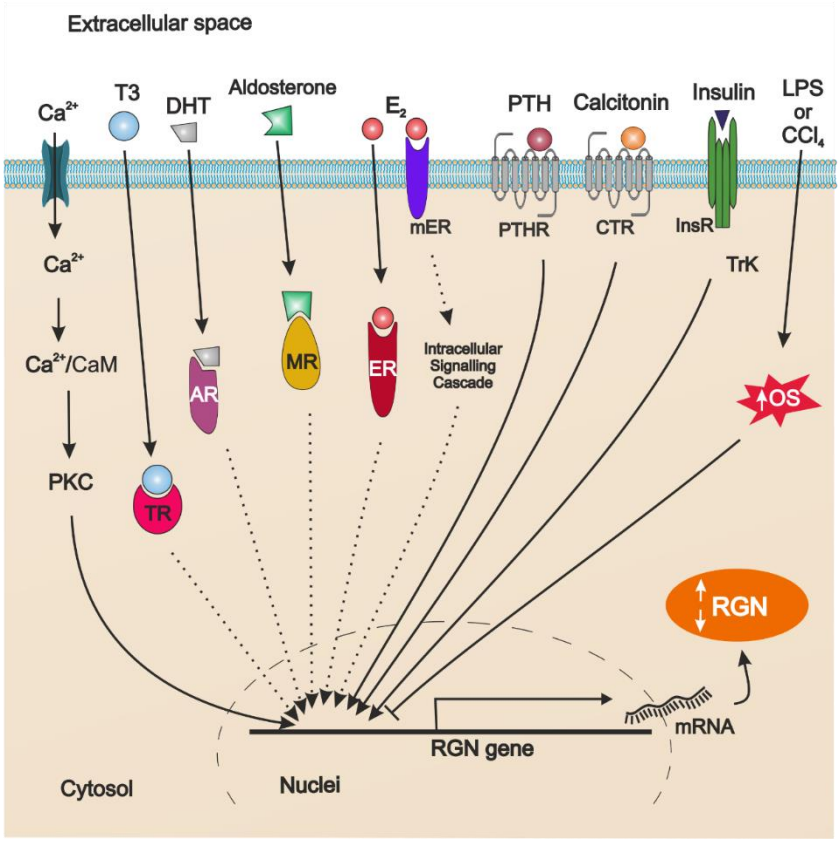

Figure II.1. The myriad of factors regulating regucalcin (RGN) gene expression. Some exert upregulation effects (*solid arrows*) while others upregulated or downregulated RGN expression (*dashed arrows*) depending on the cell type, doses and/or time of stimulation. *Bar-headed arrow* represents inhibition. Legend: DHT, 5α-dihydrotestosterone; E_2, 17β-estradiol; PTH, parathyroid hormone; LPS, lipopolysaccharide; CCl_4 carbon tetrachloride; CaM, calmodulin; PKC, protein kinase C; ER, estrogen receptor; PTHR, parathyroid hormone receptor; CTR, calcitonin receptor; InsR, insulin receptor; TrK, tyrosine kinase; TR, thyroid hormones receptor; AR, androgen receptor; MR, mineralocorticoid receptor; OS, oxidative stress

Calcium

Ca^{2+}, a second messenger triggering important cell signaling pathways, is one of the main factors involved in the regulation of RGN gene expression in liver and kidney. Several reports have showen that rats treated with Ca^{2+} chloride ($CaCl_2$) present higher levels of RGN mRNA at 30, 60 and 120 min after administration [15,22,29,82,95,96]. The role of Ca^{2+} regulating RGN expression is also observed in H4-II-E hepatoma cells [24,25].

Regarding the mechanisms underlying Ca^{2+} regulation of RGN expression, it was hypothesized that it could involve the Ca^{2+}-binding protein, CaM. When Ca^{2+} and trifluoperazine (TFP), an antagonist of CaM, were simultaneously administrated, the effect of Ca^{2+} increasing RGN mRNA expression was blocked, which suggests that expression of RGN mRNA is mediated by CaM [22,23]. A Ca^{2+}/CaM complex regulates the activation of several enzymes involved in signal transduction, such as cyclic adenosine monophosphate (cAMP) phosphodiesterase or PKC. The effect of phorbol 12-myristate 13-acetate (PMA), an activator of PKC, was evaluated on the expression of RGN. Different doses of PMA do not produced any effect on RGN mRNA expression, suggesting that the downstream effect of CaM is not triggered by PKC [23]. Although the effect

of Ca^{2+} in rat liver was not mediated by PKC, it was demonstrated in H4-II-E cells that it is mediated by CaM and involves PKC activation [24,25].

Thyroid and parathyroid hormones

It is well known that calcitonin and parathyroid hormone (PTH) play an important role in maintenance of Ca^{2+} homeostasis [97]. M. Yamaguchi's group have investigated the role of calcitonin regulating RGN expression. In rat liver, the effect of $CaCl_2$ in RGN mRNA expression is completely abolished in thyroparathyroidectomised (TPTX) rats, but calcitonin administration to TPTX rats treated with $CaCl_2$ induced an increase of RGN mRNA expression. These results suggested that the Ca^{2+} effect in RGN mRNA expression is dependent on calcitonin [29]. On the other hand, experiments using HepG2 cells did not find any effect on RGN mRNA expression triggered by calcitonin [90]. Regarding kidney, the administration of calcitonin or PTH to TPTX rats treated with $CaCl_2$ did not cause any alteration in RGN mRNA levels, suggesting that RGN expression is not stimulated by hormones involved in Ca^{2+} metabolism [22,96]. In normal rat kidney proximal tubular epithelial NRK52E cells, the RGN mRNA expression was stimulated by treatment with PTH, but no effect was detected using calcitonin [30,67].

RGN seems to play an important role maintaining bone homeostasis [98], since it has been described that bones of transgenic rats overexpressing RGN (RGN knock-in) are more fragile than that of wild-type animals [70]. This again raised the question of whether PTH may regulate RGN expression, and, in fact, treatment of osteoblastic MC3T3-E1 cells with PTH induced an increase in RGN mRNA transcripts [99]. On the other hand, both male and female RGN knock-in rats display significantly decreased Ca^{2+} levels in femoral-diaphyseal and –metaphyseal [70]. A recent report described that exogenous RGN stimulates osteoclastogenesis and suppresses osteoblastogenesis which occurs through the activation of the nuclear factor-kappa B (NF-kB) signaling transduction pathway [100]. Thus, the known effects of PTH in bone reabsorption may be mediated by increased expression of RGN.

Concerning T3 and T4 hormones, T3 treatment of female rats induced an increase in RGN mRNA and protein levels up to 12h of stimulation, which declined after 24h and disappearing after 5 days [28]. No effect has been observed in response to T4 treatment [101], likely explained by the low biologic activity of this hormone. Recently, it was demonstrated that RGN mRNA is down-regulated by T3 in MCF-7 cells needing activated thyroid hormone receptors (TRs), but does not requiring high affinity between TR and thyroid-responsive elements on RGN gene promoter [102]. Down-regulation of RGN expression seems to be mediated through modification of histone acetylation triggered by T3 treatment [102].

Steroid hormones

RGN mRNA expression in rat kidney is suppressed by saline administration [103], and Ca^{2+}-induced up-regulation of RGN mRNA expression is weakened by saline ingestion [96], suggesting the involvement of adrenal hormones on the regulation of RGN expression. The levels of RGN mRNA in the kidney were clearly diminished by administration of aldosterone. On the other

hand, dexamethasone induced an increase in RGN mRNA levels, and hydrocortisone administration had no effect. The effect of dexamethasone is inhibited by administration of cycloheximide, suggesting that the effect of dexamethasone is dependent of newly synthesized proteins [27]. However, these effects are not clearly understood because adrenalectomy in rats caused a decrease in RGN mRNA levels, an effect not restored by dexamethasone administration [103]. On the contrary, treatment of kidney NRK52E cells with aldosterone stimulated RGN mRNA expression [30,67]. These results suggested that others hormones synthesized by adrenal gland may be involved, or a synergetic effect between them are required to restore or regulate the levels of RGN mRNA in cells. More studies are needed to clarify the role of adrenal hormones regulating RGN expression. Vitamin D has no effect on RGN expression in NRK52E cells [30,67], while it seems to decrease its expression in MC3T3-E1 cells [99].

The effect of sex steroid hormones, androgens and estrogens, on RGN expression has been evaluated in liver, kidney, bone, prostate, breast, and testis tissues or cell lines. In rat liver, the expression of RGN was not altered by orchidectomy or treatment with testosterone, suggesting that RGN expression in the liver is androgen-independent [13]. Also, in female rats, the ovariectomy did not cause a significant modification of RGN mRNA levels in the liver. In addition, the administration of 17β-estradiol (E_2) to ovariecomised rats did not induced alterations in RGN mRNA expression [81]. However, other studies have showed that administration of E_2 induced a remarkable increase of RGN mRNA levels both in rat and mice liver [101,104]. This up-regulation in response to E_2 is also observed in MC3T3-E1 cells [99]. One report demonstrated that E_2 decreases RGN mRNA levels in rat kidney [27]. The levels of RGN mRNA increased in the prostate of orchidectomized rats, an effect abrogated by E_2 treatment for 7 days. The levels of RGN mRNA in the prostate of E_2-treated rats are similar to those found in intact animals, suggesting that normal levels of E_2 may down-regulate RGN mRNA expression [26]. However, it is possible that the levels of RGN mRNA in the prostate of intact animals cloud also be maintained by the paracrine effect of testosterone metabolite 5α-dihydrotestosterone (DHT). In fact, another study showed that DHT down-regulates RGN mRNA expression in human prostate cancer LNCaP cells by direct action of androgen receptor (AR), but requiring *de novo* protein synthesis [11].

RGN expression is higher in the mammary gland of ovariectomised rats in comparison with intact animals, but this effect is inhibited by treatment with E_2 for 7 days [26]. In human breast cancer MCF-7 cells, E_2 had a biphasic effect controlling RGN mRNA expression. Initially, E_2 induced an increase in RGN mRNA levels at 6h and 12h, but a down-regulation was observed after 24h and 48h of stimulation. Moreover, the effects of E_2 on RGN mRNA expression were not abrogated in presence of ICI 182,780 (Estrogen Receptor (ER) antagonist), and E_2-bound to BSA produced the same effect as E_2, suggesting the involvement of a membrane-bound ER [11]. These results demonstrated that long exposure to E_2 decrease the expression of RGN mRNA in both rat mammary gland and MCF-7 cells.

Also, in the testis, the effect of sex steroid hormones regulating RGN expression has been reported. DHT up-regulates RGN expression in rat seminiferous tubules cultured *ex vivo*, an

effect blocked in the presence of flutamide (AR antagonist) suggesting the involvement of classical genomic mechanism of gene expression through AR [66].

Oxidative stress

Oxidative stress reduction trough calorie restriction (CR) is known to have anti-aging and antioxidative properties [105]. It has been shown that CR inverts the characteristic down-regulation of RGN expression in the liver and kidney of aged rats [106]. Rats fed *ad libitum* for 6, 12, 18 and 24 months showed a decrease of RGN expression when compared to animals under CR. Moreover, rats treated with LPS, which stimulates the production of reactive oxygen species (ROS), presented lower levels of RGN [106]. It has also been reported that treatment with carbon tetrachloride, an acute oxidative stress inducer, suppresses the RGN expression in rat liver during the necrotic phase [107]. These results suggest that the down-regulation of RGN expression in older animals is eventually due to the increased oxidative stress characteristic of the aging process.

Effects of RGN on calcium homeostasis

A tight regulation of intracellular Ca^{2+} concentrations is essential for maintenance of fundamental biological functions and oscillations between 50 and 150 nM promote activation of specific and diverse signaling pathways that are involved in both physiological and pathophysiological conditions [108-111]. Several studies have been demonstrating that RGN plays a role regulating Ca^{2+} homeostasis through direct and/or indirect regulatory actions at plasma membrane Ca^{2+}-ATPase (PMCA), sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), nuclear outer membrane SERCA pumps, and increasing mitochondrial Ca^{2+}-uptake by the mitochondrial Ca^{2+} uniporter (MCU) [43]. Although no effects are described for RGN on Ca^{2+} channels activity, RGN overexpression in NRK52E normal rat kidney proximal tubular epithelial cells suppressed L-type Ca^{2+} channels and Ca^{2+}-sensing receptor mRNA expression [112].

RGN transfection in HepG2 cells [89] and addition of RGN to rat liver plasma membranes significantly increased PMCA activity [113,114]. This effect was inhibited by N-ethylmaleimide (NEM) [115], a modifying reagent of sulfhydryl groups (SH), which suggests that PMCA activity induced by RGN implies the regulation of ATPase SH groups. Accordingly, NEM blocked the activator effect of dithiothreitol (DTT), which is a SH group protective compound [116]. In addition, it is thought that RGN regulates PMCA activity by direct binding to the plasma membrane [113], since the stimulatory effect is blocked by digitonin, a solubilising reagent of membrane lipids [116]. Elevation of Ca^{2+} levels in the liver induced by oral administration to rats also increases PMCA activity. This effect is abolished in presence of anti-RGN antibody [117,118], reflecting the role of endogenous RGN on the control of PMCA activity.

CaM activates PMCA through direct interaction with a specific CaM-binding domain located in the cytosolic tail of the pump [119]. Interestingly, some reports have pointed out that the RGN effect regulating PMCA activity may be CaM-dependent. The CaM inhibitor TFP has been shown to inhibit RGN effect on PMCA activity in HepG2 cells overexpressing the protein [89].

Administration of carbon tetrachloride increased cytosolic Ca^{2+} levels in rat liver as a consequence of tissue injury and impairment of the RGN effect on PMCA activity [120].

The RGN role regulating PMCA activity seems to be Ca^{2+} dependent. RGN-induced Ca^{2+} uptake and increased PMCA activity in rat kidney cortex basolateral membranes is enhanced in the presence of Ca^{2+} in a dose-dependent manner [121].

Considering RGN influence on SERCA function, its effect has also been shown in enhancing pump activity [74,121-123]. Moreover, increased mRNA and protein levels of SERCA were observed in COS-7 cells overexpressing RGN [124]. Thapsigargin, a specific microsomal ATPase inhibitor, and digitonin clearly decrease RGN-induced SERCA activity in rat liver microsomes [123], suggesting a membrane association. In opposition, A23187, a Ca^{2+} ionophore, increased RGN-induced ATPase activity [123]. RGN presumably acts on SERCA SH groups, since NEM and DTT, respectively, promote a decrease and an increase in RGN-induced SERCA activity [123]. Similar results were described for rat kidney cortex [121] and heart microsomes [74]. In addition, vanadate, a phosphorylation inhibitor, significantly decreased RGN-induced SERCA activity in kidney microsomes, suggesting a phosphorylation effect of RGN at enzyme sites [121]. Contrastingly with the previous observations, in rat brain microsomes, RGN decreased SERCA activity, an effect that was weakened with increasing age [125].

It has been reported that RGN can be found in cell nucleus [26,40,41,126] and SERCA pumps are also located in nuclear outer membrane which is an extension of endoplasmic reticulum [127]. RGN did not change Ca^{2+}-uptake into rat liver nucleus [128] but reduced nuclear SERCA activity, while anti-RGN antibody caused the opposite effect [129]. Moreover, thapsigargin, NEM or vanadate prevented the effect of anti-RGN antibody increasing nuclear SERCA activity [129]. On the other hand, CaM enhanced the increased SERCA activity by anti-RGN antibody, an effect that is reduced in the presence of TFP. Thus, RGN seems to modulate CaM effects on nuclear SERCA and to promote nuclear Ca^{2+} release in a way not clarified so far [128,129].

RGN also regulates cytosolic Ca^{2+} concentration by stimulation of Ca^{2+} uptake into the mitochondria matrix of rat liver [130,131] and kidney cortex [132] cells, likely through MCU. In fact, it has been reported in liver [131], kidney cortex [132], heart [133] and brain [134], that MCU inhibitors, such as ruthenium red or lanthanum chloride, prevent Ca^{2+} uptake as well as RGN-induced mitochondrial ATPase activity. In the same way, increased mitochondrial ATPase activity was observed in heart and brain of RGN knock-in rats [133,134]. In liver and kidney cortex of wild-type animals, digitonin and vanadate reduced mitochondrial ATPase activity even in presence or absence of RGN, whereas CaM and DTT promoted an opposite effect [131,132]. This means that RGN may regulate cytosolic Ca^{2+} homeostasis by acting on SH groups of mitochondrial ATPase and/or MCU channel, depending on CaM, since ATPase activity is decreased by TFP in kidney cortex [132].

The described actions of RGN controlling Ca^{2+}-pumps activity and intracellular Ca^{2+} levels highlight the importance of this protein maintaining homeostasis and appropriate signaling for this ion, which may have profound implications in pathophysiologic conditions as a result of Ca^{2+} deregulation. Nevertheless, the regulatory role of RGN in Ca^{2+} homeostasis and signaling

needs to be further explored and extended to contemplate potential effects on Ca^{2+} channels or Ca^{2+}-sensing receptor activities.

RGN and calcium-dependent intracellular signaling

Beyond its capability to regulate cytosolic Ca^{2+} levels, RGN is also able to modify the activity of a wide range of Ca^{2+}-dependent enzymes involved in intracellular signaling and cell metabolism (Figure II.2).

A Ca^{2+}-dependent enzyme that is regulated by RGN is the 5'-nucleotidase. Ca^{2+} inhibits 5'-nucleotidase activity which is reverted by RGN [135]. At metabolic level, mitochondrial succinate dehydrogenase activity is increased by Ca^{2+}, whereas RGN induced an opposite effect [136]. So, mitochondrial Ca^{2+} regulation by RGN has an indirect effect on cell energy production. Also, enzymes such as glycogen phosphorylase a, an enzyme involved in glycogen hydrolysis in liver and muscle (glucogenolysis), pyruvate kinase (glycolysis) and microsomal glucose-6-phosphatase (gluconeogenesis), which are activated by Ca^{2+}, have their activities reverted to control levels by RGN [137-139]. Moreover, ATP hydrolysis by adenosine 5'-triphosphatase in rat brain is increased by Ca^{2+}, while RGN promoted an inhibitory effect as demonstrated by RGN-antibody administration [140]. This RGN action seems to be independent of CaM, since it is not inhibited by CaM or TFP [140]. In contrast, Ca^{2+}-induced rat liver pyruvate kinase activity is reverted by RGN, and also by CaM [138]. Fructose-1.6-diphosphatase enzyme activity in rat and rabbit liver is found to be increased by Ca^{2+} and CaM, and diminished by the addition of RGN or CaM inhibitor, suggesting that effects may be mediated by Ca^{2+}-CaM [141]. On the other hand, cytosolic deoxyuridine 5'-triphosphatase activity is decreased by Ca^{2+} and stimulated by RGN [142]. Altogether, available data indicate diverse regulatory roles of RGN on enzymes involved in different cellular energy production pathways, such as oxidative phosphorylation, glucogenolysis, gluconeogenesis and glycolysis, as well as in energy conversion enzymes. Moreover, it was also suggested that RGN exert its effects by direct actions on the regulation of CaM or CaM-dependent proteins.

cAMP, as well as Ca^{2+}, is an ubiquitous second messenger essential to the control of cellular homeostasis [143]. The adenylyl cyclases (ACs) that synthesize cAMP are regulated by Ca^{2+} signaling pathways [143,144] and activated by heterotrimeric G proteins [144]. In turn, cAMP phosphodiesterases are responsible for cAMP degradation. Thus, levels of cAMP are regulated by the activity balance of ACs and cAMP phosphodiesterases both activated by Ca^{2+}/CaM [143,145]. In rat liver and kidney, RGN inhibited Ca^{2+}/CaM dependent activation of cAMP phosphodiesterase [146,147], an effect abolished by high Ca^{2+} levels and in the presence of TFP [146,147]. Thus, RGN action on phosphodiesterase appears to be related to the capacity of Ca^{2+} binding, as it seems to be dependent of CaM.

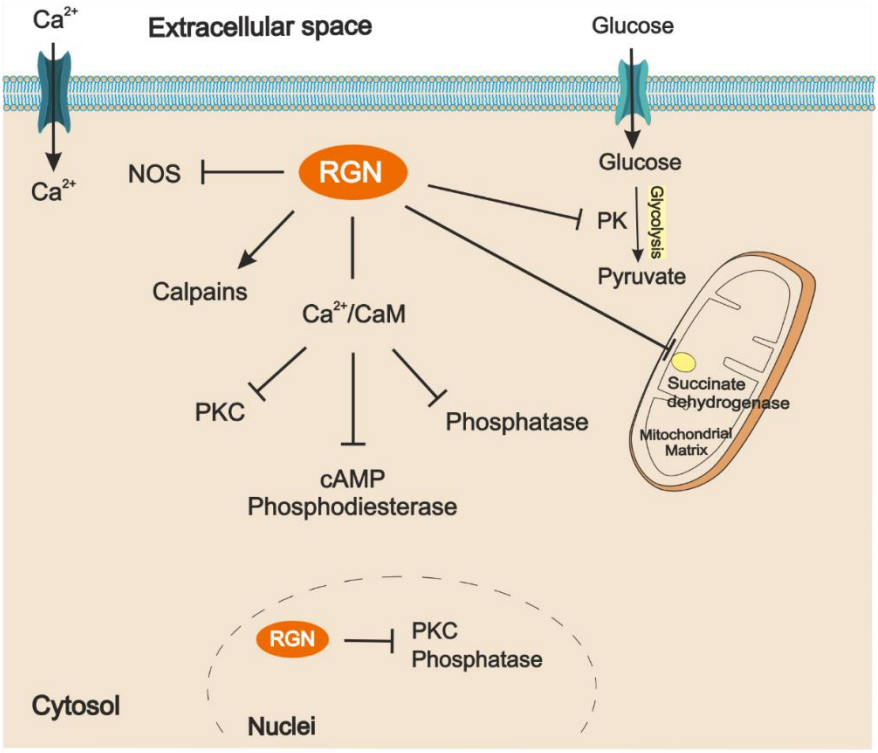

Figure II.2. Schematic representation of regucalcin (RGN) actions on enzymes involved in intracellular signaling and metabolism. *Arrows* indicate activation by RGN and *bar-headed arrows* represent inhibition. RGN decreases NOS, PK and succinate dehydrogenase enzymes activity. RGN also inhibits Ca^{2+}/CaM dependent activation of PKC, cAMP phosphodiesterase and phosphatases. Legend: NOS, nitric oxide synthase; PK, pyruvate kinase; CaM, calmodulin; PKC, protein kinase C.

Nitric oxide (NO) is a signaling agent produced by the nitric oxide synthase (NOS), which is regulated by free intracellular Ca^{2+} concentrations and CaM [148]. The addition of RGN to cytosol preparations from rat liver, kidney, heart and brain lead to a significant decrease of NOS activity [48,149-151]. Furthermore, both Ca^{2+} and anti-RGN antibody stimulated NOS activity in rat liver, heart and brain cytosol, while it is blocked in RGN knock-in rats [150-152]. RGN overexpression in kidney proximal tubular epithelial NRK52E cells [153] also demonstrated the decrease in NOS activity even in presence of Ca^{2+} and CaM, while, in MC3T3-E1 cells anti-RGN antibody reverted this effect [92]. The mechanism by which RGN regulates NOS activity may be related with CaM, since, in liver and kidney, it is impaired in the presence of CaM antagonist, TFP [48,149,150].

Calcineurin (CaN) is a CaM-dependent serine/threonine phosphatase widely distributed in mammalian tissues [154,155]. It has been demonstrated that RGN significantly reduce cytosolic and nuclear phosphatase activities in the liver [156,157], while anti-RGN antibody promotes the expected opposite effects [40,156,157]. Also, phosphotyrosine and other phosphatases activities in rat kidney cortex cytosol were significantly inhibited by RGN [158,159]. Cytosolic and nuclear phosphotyrosine and phosphoserine activities were found to be diminished by vanadate, used as a tyrosine phosphatase inhibitor, and cyclosporin A, a CaN inhibitor, even in the presence of anti-RGN antibody [159,160]. Moreover, Ca^{2+} administration elevates cytosolic and nuclear phosphatase activity in rat kidney cortex, an effect abolished by addition of RGN

to the reaction mixture [161]. RGN suppressive effects on phosphatases activity were also demonstrated in rat heart cytosol [162]. RGN also presents a CaM-dependent inhibitory effect on tyrosine, serine and threonine phosphatases in rat brain cytosol [163] and in neuronal cells [164]. RGN suppressive role on phosphotyrosine activity in brain nucleus and microsomes has also been demonstrated, displaying attenuated effects with increasing age [165,166].

RGN effect on Ca^{2+}/CaM-dependent protein kinases has also been evaluated in several reports. In rat liver cytosol, an inhibitory role of RGN in protein kinase activity has been described, which is reverted with anti-RGN antibody [167,168]. Moreover, RGN, which do not have kinase activity, decreased Ca^{2+} or phospholipid-stimulated cytosolic PKC activity [169]. Nuclear PKC activity in the liver was also inhibited by RGN, whereas the use of anti-RGN antibody led to enhancement of PKC activity [46]. These findings demonstrated the regulatory role of RGN in cytosolic and nuclear Ca^{2+}/CaM-dependent PKC activity. Similar results were obtained in rat renal cortex with increased PKC activity in response to Ca^{2+}/CaM, phospholipids (phosphotidylserine or dioctanoygycerol), and PMA; RGN or TFP significantly inhibited enzyme activity [170,171]. Also, in rat brain cytosol and neuronal cells, RGN exerted an inhibitor effect on protein kinase activity by preventing its activation by Ca^{2+}/CaM or dioctanoyglycerol [172,173]. These evidences are indicative of an effective regulatory function of RGN on PKC activity in rat liver, kidney and brain being tightly dependent of Ca^{2+}/CaM pathway.

Calpains are a family of Ca^{2+}-dependent activated neutral cysteine proteases that are ubiquitously expressed or tissue-specific [174]. The ubiquitous μ- and m-calpain isoforms are known to be activated *in vitro* by μM and mM Ca^{2+}-concentrations [175]. Calpains have been described to have important roles in embryogenesis, cell cycle progression, apoptosis, necrosis, proliferation, differentiation, migration, meiosis and mitosis, besides being related with numerous diseases such as muscular dystrophy, cardiac and cerebral ischemia, traumatic brain injury or rheumatoid arthritis [174,175]. Calpain proteolytic activity is enhanced by RGN in rat liver and kidney cortex, even in absence of Ca^{2+}, and prevented by anti-RGN antibody and calpastatin, a calpain specific inhibitor [45,176-178]. RGN-induced proteolitic activity seems to be independent of serine proteases and metaloproteases [176,178]. However, it may be associated with SH groups of cysteinyl-proteases since it is increased by DTT and inhibited by NEM and leupeptin, an SH group inhibitor of proteases [176-178].

Cytoprotective effects of RGN

Alongside its well-recognized function in Ca^{2+} homeostasis and Ca^{2+}-dependent intracellular signaling pathways, RGN has been identified as a gluconolactonase (GNL) [6]. In mammals, GNL activity is involved in the penultimate step of L-ascorbic acid (AA) synthesis in the liver. AA is a well-known antioxidant with free radical scavenger ability and a cofactor in metal-dependent oxygenases [179]. Genetic mutations in the gene, that codify the enzyme required for the last step of AA biosynthesis pathway, oblige human, non-human primates and guinea pigs to obtain it through diet [61,179], while rodents maintain the ability to produce it endogenously. The establishment of RGN knockout (RGN-KO) mice generated an animal model unable to synthesize

vitamin C (VC). These animals develop scurvy symptoms [6] and pulmonary emphysema [180] when feed with a restrained VC diet. The RGN-KO model allowed the confirmation of an alternative AA synthesis pathway *in vivo* throughout *D-glucono-γ-lactone* [6] and demonstrated the antioxidant properties of RGN [31,181,182]. NADPH oxidase enzyme activity, an endogenous source of oxidative stress [183], and anion superoxide levels are increased in the brain of RGN-KO mice [181,182]. Superoxide dismutase (SOD) and catalase activity remained unchanged, while glutathione peroxidase activity was reduced in animals without RGN [181,183].

However, evidences of RGN protective role against oxidative stress is essentially reported in mice lungs. RGN-KO mice exposed to cigarette smoke showed elevated levels of protein carbonyls, an oxidative biomarker, in comparison with wild-type animals, and were the only group in which oxidase glutathione levels were sufficiently elevated to be measured [184].

RGN antioxidative capacity has also been established in other animal models and cell lines. RGN overexpression in mouse embryonic carcinoma P19 cell line increased cell viability, protecting cells from oxidative stress-induced by tert-butyl hydroperoxide [49]. An intracellular favourable redox state has also been demonstrated in HepG2 cells transfected with RGN, which displayed diminished ROS levels both in mitochondria and post-mitochondrial fractions, as well as decreased lipid peroxidation levels and reduced protein levels and activity of glutathione and SOD, respectively [51]. In addition, SOD activity was enhanced in normal rat liver and heart in the presence of exogenous RGN, as well as in RGN knock-in rats [50,52].

NO, produced by the activity of NOS, is involved in NO-dependent signal transduction pathways. However, it is a reactive species influencing cell redox state and being associated with modification of proteins, lipids, DNA and structure of organelles when present in cells at high levels [185]. In rat brain, NOS activity is increased by anti-RGN antibody, while enhancement of RGN in the brain cytosol of young and old female rats reduced the enzyme activity [48]. This suppressor role of RGN in NOS activity is also found in rat liver, kidney and heart cytosol, including in presence of EGTA or TFP [149-151]. Similar results have been described in H4-II-E [56,186], NRK52E [153] and MCT3T3 [92] cells overexpressing RGN.

Neurodegenerative diseases, such as Alzheimer's and Parkinson's, are associated with oxidative stress deregulation. Kainate (KA) is an agonist for a subtype of ionotropic glutamate receptor that increases the ROS levels and disrupts Ca^{2+} homeostasis, leading to neuronal loss mainly in the hippocampus [187,188], which has been used to generate models of neurodegenerative diseases. The levels of RGN protein in the rat hippocampus were significantly increased in response to KA treatment [32]. A similar effect on RGN expression has also been shown in rat astrocytes CTX TNA2 cells treated with KA, which is mediated by the ERK signaling pathway [32]. Accordingly, RGN-KO mice are more sensitive to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a neurotoxin used to induce Parkinson's disease models, presenting significantly increased ROS levels in the striatum as well as microglial activation in comparison with wild-type counterparts. Moreover, RGN deficiency lead to astrocytes inactivation and decrease of brain-derived neurotrophic factor as result of blockage of ERK signaling pathway [31].

Overall, available studies, and particularly the information from RGN-KO mice, have demonstrated the influence of RGN maintaining physiological levels of oxidative stress and consequently its protective role against oxidative damage.

Role of RGN in cell death and proliferation

Since RGN is a protein involved in the regulation of intracellular Ca^{2+} levels, modulation of several cellular signaling pathways, and also with antioxidant properties, it is not surprising that its role in cell survival and proliferation has been questioned by many investigations.

It is well established that NO overproduction is a condition associated with many pathologies underlying deregulation of cell proliferation in cases of male infertility [189] and cancer [190]. In hypoxic conditions, ROS and Ca^{2+} levels are found to be decreased (~60 %) in cardiomyocytes overexpressing RGN, which presented lower cell death induced by H_2O_2 treatment [191]. Also, mouse embryonic carcinoma P19 RGN-transfected cells presented increased cell viability in response to butylhydroperoxide-induced oxidative stress in comparison with mock-transfected cells [49]. In H4-II-E cells, LPS treatment promoted a decrease of NOS activity and cell number, effects that were reverted in RGN overexpressing cells [192].

Several other reports demonstrated the RGN suppressor effect on cell proliferation [54,55,57]. NRK52E and H4-II-E cells overexpressing RGN presented lower index of proliferation than mock-transfected cells [53-55,57], which was associated with a decrease of DNA synthesis activity [55,193]. In addition, intracellular increase of RGN downregulated mRNA expression of c-myc and H-ras, while upregulated p53 and p21, which suggested that RGN suppresses cell proliferation by modulating the expression of proto-oncogenes and tumor suppressor genes [53,54,57]. Also, the expression of c-Jun and chk2 cell cycle regulators is decreased in RGN-transfected NRK52E cells [57].

However, and contrastingly with the previous information, it has also been described that cells overexpressing RGN do not undergo cell cycle arrest promoted by cell cycle inhibitors or other factors. The cell cycle inhibitors sulforaphane, butyrate and roscovitine diminish proliferation of wild-type cells, though this is not observed in RGN-transfected cells [54,57]. Bay K 8644, genistein, wortmannin, an inhibitor of phosphatidylinositol 3-kinase, PD 98059, an ERK inhibitor, or dibucaine, an inhibitor of Ca^{2+}-dependent protein kinase all hampered cell proliferation, an effect reverted by RGN overexpression [54].

There is also evidence of the involvement of RGN in the regulation of apoptosis. It has been reported that RGN affects rat liver nuclei function by suppressing Ca^{2+}-induced DNA fragmentation in the presence or absence of CaM [194]. In fact, the enhancement of DNA fragmentation in NRK52E or H4-II-E cells, after incubation with Bay K 8644, thapsigargin, LPS, insulin or IGF-I, was suppressed by RGN overexpression in both cell lines [56,195,196]. Thus, accordingly, cell death of H4-II-E or NRK52E wild-type cells promoted by tumor necrosis factor-α (TNF-α) or thapsigargin was prevented in RGN-transfected cells [56,196]. RGN overexpression in hepatocarcinoma HepG2 cells also rescues cell death induced by intracellular Ca^{2+} overload

promoted by A23187 [89]. In MCF-7 cells, the downregulation of RGN expression, achieved by thyroid hormone treatment or silencing of RGN gene, lead to an increase of apoptosis [102].

RGN effects suppressing apoptosis may be related to the Akt survival signaling pathway. NRK52 RGN-transfected cells displayed increased levels of both Bcl-2 and Akt-1 mRNAs [196], while an activation of Akt was observed in HepG2 cells overexpressing RGN [58]. TFP attenuated apoptosis of HepG2 RGN-transfected cells and inhibited Akt activation [58]. Thus, enhancement of cell survival by RGN seems to depend on the interplay with CaM and activation of Akt pathway.

RGN anti-apoptotic effects are also evident on the basis of studies using knockout animals. Primary cell cultures of hepatocytes from RGN-KO mice are highly susceptible to apoptosis-induced by TNF-α and actinomycin D [60]. Accordingly, caspase 8 activity was two-fold greater in the hepatocytes of RGN-KO mice whereas no differences were observed in NF-kB activation [60].

Anti-Fas antibody administration to mice has been previously shown to induce severe damage of the liver by apoptosis [197]. RGN-KO mice presented a markedly increase of liver injury by anti-Fas antibody administration, while RGN +/- mice had an intermediate susceptibility between RGN -/- and wild-type animals [60]. Therefore, RGN anti-apoptotic effect seems to be related with Fas activation pathway and not with NF-kB activation. Inhibition of transforming growth factor-β (TGF-β) pathway through deletion of Smad3 gene makes the hepatocytes of Smad3-KO mice more resistant to radiation-induced apoptosis than those of wild-type animals, which is concomitant with significantly increased levels of RGN [59].

Altogether, the existing findings indicate that RGN, despite, apparently having opposite functions, acting as a suppressor of both cell death and proliferation, may have a role in the control of cell cycle, by modulation of cell survival and death pathways (Figure II.3). Testis is one of the tissues where a tight balance between germ cell survival and apoptosis is required, which is the basis for a successful spermatogenesis and thus male fertility. Interestingly, in a recent report, it was shown that RGN expression is augmented in cases of hypospermatogenesis [34], but further research is needed to determine whether the increased RGN expression is the cause of insufficient production of spermatozoa by blockage in cell proliferation. It is also noteworthy the diminished expression of RGN found in both rodent and human cancer tissues [11,37,93,198,199], which is also correlated with the degree of cellular differentiation of breast, prostate and liver carcinomas [11,37]. In a near future it will be essential to determine whether down-regulation of RGN is a selective event for malignant transformation or if it is a consequence of the cancer status. Nevertheless, dual distinctive roles over control of cell proliferation and malignancy have also been reported for other proteins, for example the Ski-novel protein (SnoN). SnoN is a member of the Ski family proteins that is ubiquitously expressed in embryonic and adult tissues possessing, within tumorigenesis, both pro-oncogenic and anti-oncogenic activities [200]. SnoN overexpression in mice mammary gland leads to an increase of adenocarcinoma formation, although heterozygous mice that lack an extra copy of the gene are more susceptible to carcinogen-induce tumors [200]. At the same time, and as anti-

oncogenic, SNO functions negatively regulated the TGF-ß pathway while stabilizing p53 conformation and inducing premature senescence [200,201]. There are also examples of proteins with a dual role controlling both apoptosis and cell cycle. This is the case of Survivin which belongs to the inhibitor of apoptosis protein family. It is localized both outside and inside the cell with pools at cytoplasmic, nuclear and mitochondrial compartments. When present at mitochondria, Survivin protect cells from apoptosis while its nuclear translocation facilitates cell cycle entry and progression [202].

In summary, it is likely that RGN plays an important role in cell physiology by maintaining a tight balance between cell proliferation and apoptosis (Figure II.3).

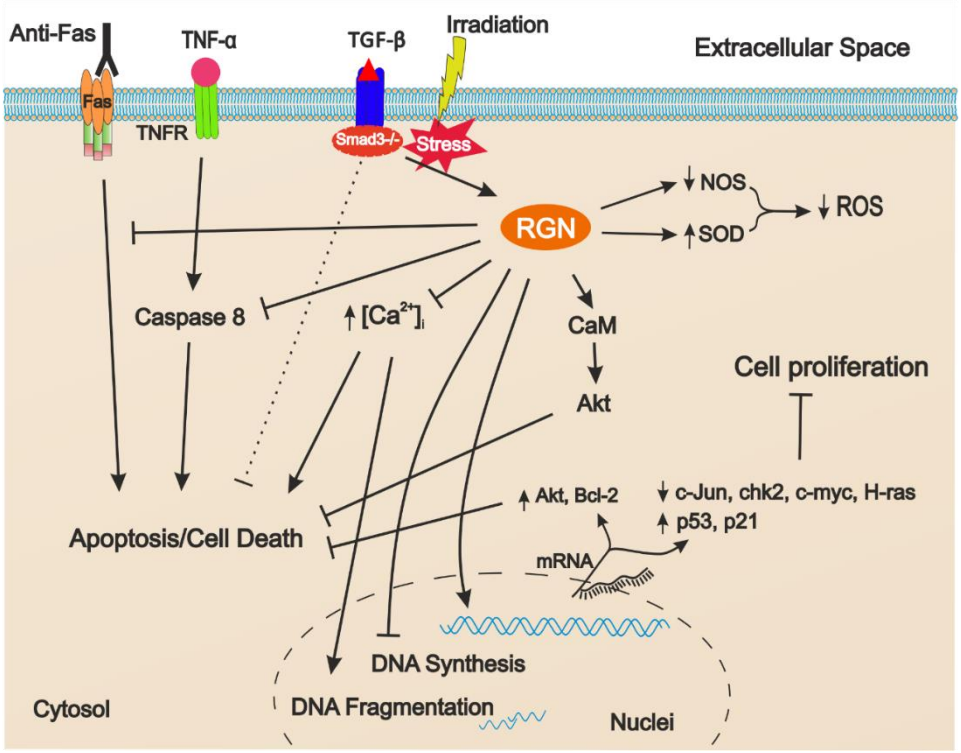

Figure II.3. Schematic representation of the mechanisms involved in the regucalcin (RGN) role controlling cell proliferation and apoptosis. *Arrows* indicate activation and *bar-headed arrows* represent inhibition. RGN diminishes the production of ROS, blocks increases of intracellular calcium, inhibits caspase 8 activity, enhances activity of Akt pathway and increases the expression of apoptosis inhibitors Akt-1 and Bcl2 leading to inhibition of apoptosis. RGN also blocks apoptosis induced by Fas system. *Dashed bar-headed arrow* indicates the inhibition of apoptosis in Smad 3 knockout animals concomitant with increased levels of RGN. In turn, RGN increases the expression of p53 and p21 proteins while repressing the expression of c-Jun, chk2, c-myc and H-ras genes, thus blocking cell proliferation. Legend: TNF-α, tumor necrosis factor; TGF-ß; tumor growth factor; NOS, nitric oxide synthase; SOD, superoxide dismutase; ROS, reactive oxygen species; CaM, calmodulin

Final remarks

In the last years it has been demonstrated that RGN is a protein highly conserved throughout evolutive line, from vertebrates to invertebrate species, which indicates its relevant role in basic cell biologic processes. This particular and unique protein has a preponderant role in Ca^{2+} homeostasis, which is extensive to the control of cell signaling pathways, as well as regulation of cell apoptosis and proliferation, and also of oxidative stress levels. The involvement of RGN

in those processes has also been evaluated in pathological conditions, becoming evident its association with several human diseases that range from muscular dystrophy and infertility to neurodegenerative diseases and carcinomas. Moreover, RGN is a protein present in patients' serum which has been correlated with stages of disease, highlighting its usefulness as a potential biomarker for monitoring disease onset and progression.

At the present moment, research efforts are needed to disclose the role of RGN over the control of cell cycle and intracellular signaling mechanisms. Moreover, since RGN protein is detected in the nuclear compartment, the identification of putative partners for RGN actions in the nucleus also is clearly warranted. Thoroughly deciphering the RGN actions in cell physiology will be a research challenge in next years, which will also contribute to a better understanding of the biology of several human diseases.

Acknowledgments

This work was partially supported by Portuguese Foundation for Science and Technology (FCT) under Program COMPETE (PEst-C/SAU/UI0709/2011). Ricardo Marques, Cátia Vaz and Sara Correia were funded by FCT fellowships (SFRH/BD/66875/2009, SFRH/BD/70316/2010 and SFRH/BD/60945/2009, respectively).

References

1. Yamaguchi M, Yamamoto T (1978) Purification of calcium binding substance from soluble fraction of normal rat liver. Chem Pharm Bull (Tokyo) 26 (6):1915-1918

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

3. Kondo Y, Ishigami A, Kubo S, Handa S, Gomi K, Hirokawa K, Kajiyama N, Chiba T, Shimokado K, Maruyama N (2004) Senescence marker protein-30 is a unique enzyme that hydrolyzes diisopropyl phosphorofluoridate in the liver. FEBS Lett 570 (1-3):57-62

4. Chakraborti S, Bahnson BJ (2010) Crystal structure of human senescence marker protein 30: insights linking structural, enzymatic, and physiological functions. Biochemistry 49 (16):3436-3444

5. Yamaguchi M (2000) Role of regucalcin in calcium signaling. Life Sci 66 (19):1769-1780

6. Kondo Y, Inai Y, Sato Y, Handa S, Kubo S, Shimokado K, Goto S, Nishikimi M, Maruyama N, Ishigami A (2006) Senescence marker protein 30 functions as gluconolactonase in L-ascorbic acid biosynthesis, and its knockout mice are prone to scurvy. Proc Natl Acad Sci U S A 103 (15):5723-5728

7. Fujita T, Mandel JL, Shirasawa T, Hino O, Shirai T, Maruyama N (1995) Isolation of cDNA clone encoding human homologue of senescence marker protein-30 (SMP30) and its location on the X chromosome. Biochim Biophys Acta 1263 (3):249-252

8. Shimokawa N, Matsuda Y, Yamaguchi M (1995) Genomic cloning and chromosomal assignment of rat regucalcin gene. Mol Cell Biochem 151 (2):157-163

9. Yamaguchi M, Makino R, Shimokawa N (1996) The 5' end sequences and exon organization in rat regucalcin gene. Mol Cell Biochem 165 (2):145-150

10. Fujita T, Shirasawa T, Maruyama N (1996) Isolation and characterization of genomic and cDNA clones encoding mouse senescence marker protein-30 (SMP30). Biochim Biophys Acta 1308 (1):49-57

11. Maia C, Santos C, Schmitt F, Socorro S (2009) Regucalcin is under-expressed in human breast and prostate cancers: Effect of sex steroid hormones. J Cell Biochem 107 (4):667-676

12. Fujita T, Shirasawa T, Uchida K, Maruyama N (1992) Isolation of cDNA clone encoding rat senescence marker protein-30 (SMP30) and its tissue distribution. Biochim Biophys Acta 1132 (3):297-305

13. Fujita T, Uchida K, Maruyama N (1992) Purification of senescence marker protein-30 (SMP30) and its androgen-independent decrease with age in the rat liver. Biochim Biophys Acta 1116 (2):122-128

14. Fujita T, Shirasawa T, Uchida K, Maruyama N (1996) Gene regulation of senescence marker protein-30 (SMP30): coordinated up-regulation with tissue maturation and gradual down-regulation with aging. Mech Ageing Dev 87 (3):219-229

15. Shimokawa N, Yamaguchi M (1992) Calcium administration stimulates the expression of calcium-binding protein regucalcin mRNA in rat liver. FEBS Lett 305 (2):151-154

16. Yamaguchi M, Isogai M (1993) Tissue concentration of calcium-binding protein regucalcin in rats by enzyme-linked immunoadsorbent assay. Mol Cell Biochem 122 (1):65-68

17. Yamaguchi M, Isogai M, Kato S, Mori S (1991) Immunohistochemical demonstration of calcium-binding protein regucalcin in the tissues of rats: the protein localizes in liver and brain. Chem Pharm Bull (Tokyo) 39 (6):1601-1603

18. Shimokawa N, Isogai M, Yamaguchi M (1995) Specific species and tissue differences for the gene expression of calcium-binding protein regucalcin. Mol Cell Biochem 143 (1):67-71

19. Misawa H, Yamaguchi M (2000) The gene of Ca2+-binding protein regucalcin is highly conserved in vertebrate species. Int J Mol Med 6 (2):191-196

20. Goto SG (2000) Expression of Drosophila homologue of senescence marker protein-30 during cold acclimation. J Insect Physiol 46 (7):1111-1120

21. Yamaguchi M (2011) The transcriptional regulation of regucalcin gene expression. Mol Cell Biochem 346 (1-2):147-171

22. Yamaguchi M, Kurota H (1995) Expression of calcium-binding protein regucalcin mRNA in the kidney cortex of rats: the stimulation by calcium administration. Mol Cell Biochem 146 (1):71-77

23. Shimokawa N, Yamaguchi M (1993) Expression of hepatic calcium-binding protein regucalcin mRNA is mediated through Ca2+/calmodulin in rat liver. FEBS Lett 316 (1):79-84

24. Nakajima M, Murata T, Yamaguchi M (1999) Expression of calcium-binding protein regucalcin mRNA in the cloned rat hepatoma cells (H4-II-E) is stimulated through Ca2+ signaling factors: involvement of protein kinase C. Mol Cell Biochem 198 (1-2):101-107

25. Yamaguchi M, Nakajima M (1999) Involvement of intracellular signaling factors in the serum-enhanced Ca2+-binding protein regucalcin mRNA expression in the cloned rat hepatoma cells (H4-II-E). J Cell Biochem 74 (1):81-89

26. Maia CJ, Santos CR, Schmitt F, Socorro S (2008) Regucalcin is expressed in rat mammary gland and prostate and down-regulated by 17beta-estradiol. Mol Cell Biochem 311 (1-2):81-86

27. Kurota H, Yamaguchi M (1996) Steroid hormonal regulation of calcium-binding protein regucalcin mRNA expression in the kidney cortex of rats. Mol Cell Biochem 155 (2):105-111

28. Sar P, Rath B, Subudhi U, Chainy GB, Supakar PC (2007) Alterations in expression of senescence marker protein-30 gene by 3,3',5-triiodo-L-thyronine (T3). Mol Cell Biochem 303 (1-2):239-242

29. Yamaguchi M, Kanayama Y, Shimokawa N (1994) Expression of calcium-binding protein regucalcin mRNA in rat liver is stimulated by calcitonin: the hormonal effect is mediated through calcium. Mol Cell Biochem 136 (1):43-48

30. Nakagawa T, Yamaguchi M (2008) Nuclear localization of regucalcin is enhanced in culture with protein kinase C activation in cloned normal rat kidney proximal tubular epithelial NRK52E cells. Int J Mol Med 21 (5):605-610

31. Kim HS, Son TG, Park HR, Lee Y, Jung Y, Ishigami A, Lee J (2012) Senescence marker protein 30 deficiency increases Parkinson's pathology by impairing astrocyte activation. Neurobiol Aging doi:10.1016/j.neurobiolaging.2012.10.008

32. Son TG, Park HR, Kim SJ, Kim K, Kim MS, Ishigami A, Handa S, Maruyama N, Chung HY, Lee J (2009) Senescence marker protein 30 is up-regulated in kainate-induced hippocampal damage through ERK-mediated astrocytosis. J Neurosci Res 87 (13):2890-2897

33. Sun L, Wang L, Sun Y, Tang SW, Hu Y (2006) Protective effects of EUK4010 on beta-amyloid(1-42) induced degeneration of neuronal cells. Eur J Neurosci 24 (4):1011-1019

34. Laurentino SS, Correia S, Cavaco JE, Oliveira PF, de Sousa M, Barros A, Socorro S (2012) Regucalcin, a calcium-binding protein with a role in male reproduction? Mol Hum Reprod 18 (4):161-170

35. Lv S, Wang JH, Liu F, Gao Y, Fei R, Du SC, Wei L (2008) Senescence marker protein 30 in acute liver failure: validation of a mass spectrometry proteomics assay. BMC Gastroenterol 8:17

36. Yamaguchi M, Tsurusaki Y, Misawa H, Inagaki S, Ma ZJ, Takahashi H (2002) Potential role of regucalcin as a specific biochemical marker of chronic liver injury with carbon tetrachloride administration in rats. Mol Cell Biochem 241 (1-2):61-67

37. Zhou SF, Mo FR, Bin YH, Hou GQ, Xie XX, Luo GR (2011) Serum immunoreactivity of SMP30 and its tissues expression in hepatocellular carcinoma. Clin Biochem 44 (4):331-336

38. Doran P, Dowling P, Donoghue P, Buffini M, Ohlendieck K (2006) Reduced expression of regucalcin in young and aged mdx diaphragm indicates abnormal cytosolic calcium handling in dystrophin-deficient muscle. Biochim Biophys Acta 1764 (4):773-785

39. Park H, Ishigami A, Shima T, Mizuno M, Maruyama N, Yamaguchi K, Mitsuyoshi H, Minami M, Yasui K, Itoh Y, Yoshikawa T, Fukui M, Hasegawa G, Nakamura N, Ohta M, Obayashi H, Okanoue T (2009) Hepatic senescence marker protein-30 is involved in the progression of nonalcoholic fatty liver disease. J Gastroenterol 45 (4):426-434

40. Tsurusaki Y, Misawa H, Yamaguchi M (2000) Translocation of regucalcin to rat liver nucleus: involvement of nuclear protein kinase and protein phosphatase regulation. Int J Mol Med 6 (6):655-660

41. Ishigami A, Handa S, Maruyama N, Supakar PC (2003) Nuclear localization of senescence marker protein-30, SMP30, in cultured mouse hepatocytes and its similarity to RNA polymerase. Biosci Biotechnol Biochem 67 (1):158-160

42. Arun P, Aleti V, Parikh K, Manne V, Chilukuri N (2011) Senescence Marker Protein 30 (SMP30) Expression in Eukaryotic Cells: Existence of Multiple Species and Membrane Localization. PLoS One 6 (2):e16545

43. Yamaguchi M (2005) Role of regucalcin in maintaining cell homeostasis and function (review). Int J Mol Med 15 (3):371-389

44. Yamaguchi M (2012) Role of regucalcin in brain calcium signaling: involvement in aging. Integr Biol (Camb) 4 (8):825-837

45. Baba T, Yamaguchi M (2000) Stimulatory effect of regucalcin on proteolytic activity is impaired in the kidney cortex cytosol of rats with saline ingestion. Mol Cell Biochem 206 (1-2):1-6

46. Katsumata T, Yamaguchi M (1998) Inhibitory effect of calcium-binding protein regucalcin on protein kinase activity in the nuclei of regenerating rat liver. J Cell Biochem 71 (4):569-576

47. Fukaya Y, Yamaguchi M (2004) Characterization of protein tyrosine phosphatase activity in rat liver microsomes: suppressive effect of endogenous regucalcin in transgenic rats. Int J Mol Med 14 (3):427-432

48. Tobisawa M, Yamaguchi M (2003) Inhibitory role of regucalcin in the regulation of nitric oxide synthase activity in rat brain cytosol: involvement of aging. J Neurol Sci 209 (1-2):47-54

49. Son TG, Kim SJ, Kim K, Kim MS, Chung HY, Lee J (2008) Cytoprotective roles of senescence marker protein 30 against intracellular calcium elevation and oxidative stress. Arch Pharm Res 31 (7):872-877

50. Fukaya Y, Yamaguchi M (2004) Regucalcin increases superoxide dismutase activity in rat liver cytosol. Biol Pharm Bull 27 (9):1444-1446

51. Handa S, Maruyama N, Ishigami A (2009) Over-expression of Senescence Marker Protein-30 decreases reactive oxygen species in human hepatic carcinoma Hep G2 cells. Biol Pharm Bull 32 (10):1645-1648

52. Ichikawa E, Yamaguchi M (2004) Regucalcin increases superoxide dismutase activity in the heart cytosol of normal and regucalcin transgenic rats. Int J Mol Med 14 (4):691-695

53. Tsurusaki Y, Yamaguchi M (2003) Overexpression of regucalcin modulates tumor-related gene expression in cloned rat hepatoma H4-II-E cells. J Cell Biochem 90 (3):619-626

54. Yamaguchi M, Daimon Y (2005) Overexpression of regucalcin suppresses cell proliferation in cloned rat hepatoma H4-II-E cells: involvement of intracellular signaling factors and cell cycle-related genes. J Cell Biochem 95 (6):1169-1177

55. Misawa H, Inagaki S, Yamaguchi M (2002) Suppression of cell proliferation and deoxyribonucleic acid synthesis in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin. J Cell Biochem 84 (1):143-149

56. Izumi T, Yamaguchi M (2004) Overexpression of regucalcin suppresses cell death in cloned rat hepatoma H4-II-E cells induced by tumor necrosis factor-alpha or thapsigargin. J Cell Biochem 92 (2):296-306

57. Nakagawa T, Sawada N, Yamaguchi M (2005) Overexpression of regucalcin suppresses cell proliferation of cloned normal rat kidney proximal tubular epithelial NRK52E cells. Int J Mol Med 16 (4):637-643

58. Matsuyama S, Kitamura T, Enomoto N, Fujita T, Ishigami A, Handa S, Maruyama N, Zheng D, Ikejima K, Takei Y, Sato N (2004) Senescence marker protein-30 regulates Akt activity and contributes to cell survival in Hep G2 cells. Biochem Biophys Res Commun 321 (2):386-390

59. Jeong DH, Goo MJ, Hong IH, Yang HJ, Ki MR, Do SH, Ha JH, Lee SS, Park JK, Jeong KS (2008) Inhibition of radiation-induced apoptosis via overexpression of SMP30 in Smad3-knockout mice liver. J Radiat Res (Tokyo) 49 (6):653-660

60. Ishigami A, Fujita T, Handa S, Shirasawa T, Koseki H, Kitamura T, Enomoto N, Sato N, Shimosawa T, Maruyama N (2002) Senescence marker protein-30 knockout mouse liver is highly susceptible to tumor necrosis factor-alpha- and Fas-mediated apoptosis. Am J Pathol 161 (4):1273-1281

61. Maruyama N, Ishigami A, Kondo Y (2010) Pathophysiological significance of senescence marker protein-30. Geriatr Gerontol Int 10 Suppl 1:S88-98

62. Nikapitiya C, De Zoysa M, Kang HS, Oh C, Whang I, Lee J (2008) Molecular characterization and expression analysis of regucalcin in disk abalone (Haliotis discus discus): intramuscular calcium administration stimulates the regucalcin mRNA expression. Comp Biochem Physiol B Biochem Mol Biol 150 (1):117-124

63. Wu YD, Jiang L, Zhou Z, Zheng MH, Zhang J, Liang Y (2008) CYP1A/regucalcin gene expression and edema formation in zebrafish embryos exposed to 2,3,7,8-Tetrachlorodibenzo-p-dioxin. Bull Environ Contam Toxicol 80 (6):482-486

64. Nakajima Y, Natori S (2000) Identification and characterization of an anterior fat body protein in an insect. J Biochem 127 (5):901-908

65. Gomi K, Hirokawa K, Kajiyama N (2002) Molecular cloning and expression of the cDNAs encoding luciferin-regenerating enzyme from Luciola cruciata and Luciola lateralis. Gene 294 (1-2):157-166

66. Laurentino SS, Correia S, Cavaco JE, Oliveira PF, Rato L, Sousa M, Barros A, Socorro S (2011) Regucalcin is broadly expressed in male reproductive tissues and is a new androgen-target gene in mammalian testis. Reproduction 142 (3):447-456

67. Nakagawa T, Yamaguchi M (2005) Hormonal regulation on regucalcin mRNA expression in cloned normal rat kidney proximal tubular epithelial NRK52E cells. J Cell Biochem 95 (3):589-597

68. Jung KJ, Maruyama N, Ishigami A, Yu BP, Chung HY (2006) The redox-sensitive DNA binding sites responsible for age-related downregulation of SMP30 by ERK pathway and reversal by calorie restriction. Antioxid Redox Signal 8 (3-4):671-680

69. Fujita T, Maruyama N (1998) [Expression and structure of senescence marker protein-30 (SMP30) and its physiological function]. Nippon Ronen Igakkai Zasshi 35 (9):654-657

70. Yamaguchi M, Misawa H, Uchiyama S, Morooka Y, Tsurusaki Y (2002) Role of endogenous regucalcin in bone metabolism: bone loss is induced in regucalcin transgenic rats. Int J Mol Med 10 (4):377-383

71. Yamaguchi M, Morooka Y, Misawa H, Tsurusaki Y, Nakajima R (2002) Role of endogenous regucalcin in transgenic rats: suppression of kidney cortex cytosolic protein phosphatase activity and enhancement of heart muscle microsomal Ca2+-ATPase activity. J Cell Biochem 86 (3):520-529

72. Yamaguchi M, Sawada N, Uchiyama S, Misawa H, Ma ZJ (2004) Expression of regucalcin in rat bone marrow cells: involvement of osteoclastic bone resorption in regucalcin transgenic rats. Int J Mol Med 13 (3):437-443

73. Ishii K, Tsubaki T, Fujita K, Ishigami A, Maruyama N, Akita M (2005) Immunohistochemical localization of senescence marker protein-30 (SMP30) in the submandibular gland and ultrastructural changes of the granular duct cells in SMP30 knockout mice. Histol Histopathol 20 (3):761-768

74. Yamaguchi M, Nakajima R (2002) Role of regucalcin as an activator of sarcoplasmic reticulum Ca2+-ATPase activity in rat heart muscle. J Cell Biochem 86 (1):184-193

75. van Dijk KD, Berendse HW, Drukarch B, Fratantoni SA, Pham TV, Piersma SR, Huisman E, Breve JJ, Groenewegen HJ, Jimenez CR, van de Berg WD (2012) The proteome of the locus ceruleus in Parkinson's disease: relevance to pathogenesis. Brain Pathol 22 (4):485-498

76. Isogai M, Shimokawa N, Yamaguchi M (1994) Hepatic calcium-binding protein regucalcin in released into the serum of rats administered orally carbon tetrachloride. Mol Cell Biochem 131 (2):173-179

77. Isogai M, Oishi K, Yamaguchi M (1994) Serum release of hepatic calcium-binding protein regucalcin by liver injury with galactosamine administration in rats. Mol Cell Biochem 136 (1):85-90

78. Lv S, Wei L, Wang JH, Wang JY, Liu F (2007) Identification of novel molecular candidates for acute liver failure in plasma of BALB/c murine model. J Proteome Res 6 (7):2746-2752

79. Carolan JC, Fitzroy CI, Ashton PD, Douglas AE, Wilkinson TL (2009) The secreted salivary proteome of the pea aphid Acyrthosiphon pisum characterised by mass spectrometry. Proteomics 9 (9):2457-2467

80. Inoue H, Fujita T, Kitamura T, Shimosawa T, Nagasawa R, Inoue R, Maruyama N, Nagasawa T (1999) Senescence marker protein-30 (SMP30) enhances the calcium efflux from renal tubular epithelial cells. Clin Exp Nephrol 3 (4):261-267

81. Ueoka S, Yamaguchi M (1998) Sexual difference of hepatic calcium-binding protein regucalcin mRNA expression in rats with different ages: effect of ovarian hormone. Biol Pharm Bull 21 (4):405-407

82. Murata T, Yamaguchi M (1997) Molecular cloning of the cDNA coding for regucalcin and its mRNA expression in mouse liver: the expression is stimulated by calcium administration. Mol Cell Biochem 173 (1-2):127-133

83. Fujita T, Shirasawa T, Maruyama N (1999) Expression and structure of senescence marker protein-30 (SMP30) and its biological significance. Mech Ageing Dev 107 (3):271-280

84. Makino R, Yamaguchi M (1996) Expression of calcium-binding protein regucalcin mRNA in hepatoma cells. Mol Cell Biochem 155 (1):85-90

85. Yamaguchi M (1998) Role of calcium-binding protein regucalcin in regenerating rat liver. J Gastroenterol Hepatol 13 Suppl:S106-112

86. Murata T, Yamaguchi M (1998) Ca2+ administration stimulates the binding of AP-1 factor to the 5'-flanking region of the rat gene for the Ca2+-binding protein regucalcin. Biochem J 329 (Pt 1):157-163

87. Inagaki S, Misawa H, Yamaguchi M (2000) Role of endogenous regucalcin in protein tyrosine phosphatase regulation in the cloned rat hepatoma cells (H4-II-E). Mol Cell Biochem 213 (1-2):43-50

88. Ishigami A, Fujita T, Inoue H, Handa S, Kubo S, Kondo Y, Maruyama N (2005) Senescence marker protein-30 (SMP30) induces formation of microvilli and bile canaliculi in Hep G2 cells. Cell Tissue Res 320 (2):243-249

89. Fujita T, Inoue H, Kitamura T, Sato N, Shimosawa T, Maruyama N (1998) Senescence marker protein-30 (SMP30) rescues cell death by enhancing plasma membrane Ca(2+)-pumping activity in Hep G2 cells. Biochem Biophys Res Commun 250 (2):374-380

90. Murata T, Shinya N, Yamaguchi M (1997) Expression of calcium-binding protein regucalcin mRNA in the cloned human hepatoma cells (HepG2): stimulation by insulin. Mol Cell Biochem 175 (1-2):163-168

91. Misawa H, Yamaguchi M (2000) Transcript heterogeneity of the human gene for Ca2+-binding protein regucalcin. Int J Mol Med 5 (3):283-287

92. Yamaguchi M, Kobayashi M, Uchiyama S (2005) Suppressive effect of regucalcin on cell differentiation and mineralization in osteoblastic MC3T3-E1 cells. J Cell Biochem 96 (3):543-554

93. Suzuki S, Asamoto M, Tsujimura K, Shirai T (2004) Specific differences in gene expression profile revealed by cDNA microarray analysis of glutathione S-transferase placental form (GST-P) immunohistochemically positive rat liver foci and surrounding tissue. Carcinogenesis 25 (3):439-443

94. Yamaguchi M, Isogai M, Shimada N (1997) Potential sensitivity of hepatic specific protein regucalcin as a marker of chronic liver injury. Mol Cell Biochem 167 (1-2):187-190

95. Yamaguchi M, Oishi K, Isogai M (1995) Expression of hepatic calcium-binding protein regucalcin mRNA is elevated by refeeding of fasted rats: involvement of glucose, insulin and calcium as stimulating factors. Mol Cell Biochem 142 (1):35-41

96. Shinya N, Yamaguchi M (1998) Stimulatory effect of calcium administration on regucalcin mRNA expression is attenuated in the kidney cortex of rats ingested with saline. Mol Cell Biochem 178 (1-2):275-281

97. Ramasamy I (2006) Recent advances in physiological calcium homeostasis. Clin Chem Lab Med 44 (3):237-273

98. Yamaguchi M (2010) Regucalcin and metabolic disorders: osteoporosis and hyperlipidemia are induced in regucalcin transgenic rats. Mol Cell Biochem 341 (1-2):119-133

99. Yamaguchi M, Otomo Y, Uchiyama S, Nakagawa T (2008) Hormonal regulation of regucalcin mRNA expression in osteoblastic MC3T3-E1 cells. Int J Mol Med 21 (6):771-775

100. Yamaguchi M, Weitzmann MN, Murata T (2012) Exogenous regucalcin stimulates osteoclastogenesis and suppresses osteoblastogenesis through NF-kappaB activation. Mol Cell Biochem 359 (1-2):193-203

101. Yamaguchi M, Oishi K (1995) 17 beta-Estradiol stimulates the expression of hepatic calcium-binding protein regucalcin mRNA in rats. Mol Cell Biochem 143 (2):137-141

102. Sar P, Peter R, Rath B, Mohapatra AD, Mishra SK (2011) 3, 3'5 Triiodo L Thyronine Induces Apoptosis in Human Breast Cancer MCF-7cells, Repressing SMP30 Expression through Negative Thyroid Response Elements. PLoS One 6 (6):e20861

103. Shinya N, Kurota H, Yamaguchi M (1996) Calcium-binding protein regucalcin mRNA expression in the kidney cortex is suppressed by saline ingestion in rats. Mol Cell Biochem 162 (2):139-144

104. Fukui M, Senmaru T, Hasegawa G, Yamazaki M, Asano M, Kagami Y, Ishigami A, Maruyama N, Iwasa K, Kitawaki J, Itoh Y, Okanoue T, Ohta M, Obayashi H, Nakamura N (2011) 17beta-Estradiol attenuates saturated fatty acid diet-induced liver injury in ovariectomized mice by up-regulating hepatic senescence marker protein-30. Biochem Biophys Res Commun 415 (2):252-257

105. Speakman JR, Mitchell SE (2011) Caloric restriction. Mol Aspects Med 32 (3):159-221

106. Jung KJ, Ishigami A, Maruyama N, Takahashi R, Goto S, Yu BP, Chung HY (2004) Modulation of gene expression of SMP-30 by LPS and calorie restriction during aging process. Exp Gerontol 39 (8):1169-1177

107. Ishigami T, Fujita T, Simbula G, Columbano A, Kikuchi K, Ishigami A, Shimosawa T, Arakawa Y, Maruyama N (2001) Regulatory effects of senescence marker protein 30 on the proliferation of hepatocytes. Pathol Int 51 (7):491-497

108. Lee WJ, Monteith GR, Roberts-Thomson SJ (2006) Calcium transport and signaling in the mammary gland: Targets for breast cancer. BBA-Rev Cancer 1765 (2):235-255

109. Cheng HP, Wei S, Wei LP, Verkhratsky A (2006) Calcium signaling in physiology and pathophysiology. Acta Pharmacol Sin 27 (7):767-772

110. Berridge MJ, Bootman MD, Roderick HL (2003) Calcium signalling: Dynamics, homeostasis and remodelling. Nat Rev Mol Cell Bio 4 (7):517-529

111. Birnbaumer L (2009) The TRPC class of ion channels: a critical review of their roles in slow, sustained increases in intracellular Ca(2+) concentrations. Annu Rev Pharmacol Toxicol 49:395-426

112. Nakagawa T, Yamaguchi M (2006) Overexpression of regucalcin enhances its nuclear localization and suppresses L-type Ca2+ channel and calcium-sensing receptor mRNA expressions in cloned normal rat kidney proximal tubular epithelial NRK52E cells. J Cell Biochem 99 (4):1064-1077

113. Yamaguchi M, Mori S, Kato S (1988) Calcium-binding protein regucalcin is an activator of (Ca2+-Mg2+)-adenosine triphosphatase in the plasma membranes of rat liver. Chem Pharm Bull 36 (9):3532-3539

114. Takahashi H, Yamaguchi M (1993) Regucalcin modulates hormonal effect on (Ca(2+)-Mg2+)-ATPase activity in rat liver plasma membranes. Mol Cell Biochem 125 (2):171-177

115. Takahashi H, Yamaguchi M (1997) Stimulatory effect of regucalcin on ATP-dependent calcium transport in rat liver plasma membranes. Mol Cell Biochem 168 (1-2):149-153

116. Takahashi H, Yamaguchi M (1994) Activating effect of regucalcin on (Ca(2+)-Mg2+)-ATPase in rat liver plasma membranes: relation to sulfhydryl group. Mol Cell Biochem 136 (1):71-76

117. Takahasi H, Yamaguchi M (1996) Enhancement of plasma membrane (Ca(2+)-Mg2+)-ATPase activity in regenerating rat liver: involvement of endogenous activating protein regucalcin. Mol Cell Biochem 162 (2):133-138

118. Takahashi H, Yamaguchi M (1995) Increase of (Ca(2+)-Mg2+)-ATPase activity in hepatic plasma membranes of rats administered orally calcium: the endogenous role of regucalcin. Mol Cell Biochem 144 (1):1-6

119. Di Leva F, Domi T, Fedrizzi L, Lim D, Carafoli E (2008) The plasma membrane Ca2+ ATPase of animal cells: structure, function and regulation. Arch Biochem Biophys 476 (1):65-74

120. Takahashi H, Yamaguchi M (1996) Activatory effect of regucalcin on hepatic plasma membrane (Ca(2+)-Mg2+)-ATPase is impaired by liver injury with carbon tetrachloride administration in rats. Mol Cell Biochem 158 (1):9-16

121. Kurota H, Yamaguchi M (1997) Activatory effect of calcium-binding protein regucalcin on ATP-dependent calcium transport in the basolateral membranes of rat kidney cortex. Mol Cell Biochem 169 (1-2):149-156

122. Yamaguchi M, Mori S (1989) Activation of hepatic microsomal Ca2+-adenosine triphosphatase by calcium-binding protein regucalcin. Chem Pharm Bull 37 (4):1031-1034

123. Takahashi H, Yamaguchi M (1999) Role of regucalcin as an activator of Ca(2+)-ATPase activity in rat liver microsomes. J Cell Biochem 74 (4):663-669

124. Lai P, Yip NC, Michelangeli F (2011) Regucalcin (RGN/SMP30) alters agonist- and thapsigargin-induced cytosolic [Ca(2+)] transients in cells by increasing SERCA Ca(2+)ATPase levels. FEBS Lett 585 (14):2291-2294

125. Yamaguchi M, Hanahisa Y, Murata T (1999) Expression of calcium-binding protein regucalcin and microsomal Ca2+-ATPase regulation in rat brain: attenuation with increasing age. Mol Cell Biochem 200 (1-2):43-49

126. Omura M, Yamaguchi M (1999) Regulation of protein phosphatase activity by regucalcin localization in rat liver nuclei. J Cell Biochem 75 (3):437-445

127. Brini M, Carafoli E (2009) Calcium pumps in health and disease. Physiol Rev 89 (4):1341-1378

128. Yamaguchi M (1992) Effect of calcium-binding protein regucalcin on Ca2+ transport system in rat liver nuclei: stimulation of Ca2+ release. Mol Cell Biochem 113 (1):63-70

129. Tsurusaki Y, Yamaguchi M (2000) Role of endogenous regucalcin in the regulation of Ca(2+)-ATPase activity in rat liver nuclei. J Cell Biochem 78 (4):541-549

130. Mori S, Yamaguchi M (1991) Calcium-binding protein regucalcin stimulates the uptake of Ca2+ by rat liver mitochondria. Chem Pharm Bull 39 (1):224-226

131. Takahashi H, Yamaguchi M (2000) Stimulatory effect of regucalcin on ATP-dependent Ca(2+) uptake activity in rat liver mitochondria. J Cell Biochem 78 (1):121-130

132. Xue JH, Takahashi H, Yamaguchi M (2000) Stimulatory effect of regucalcin on mitochondrial ATP-dependent calcium uptake activity in rat kidney cortex. J Cell Biochem 80 (2):285-292

133. Akhter T, Sawada N, Yamaguchi M (2006) Regucalcin increases Ca2+-ATPase activity in the heart mitochondria of normal and regucalcin transgenic rats. Int J Mol Med 18 (1):171-176

134. Yamaguchi M, Takakura Y, Nakagawa T (2008) Regucalcin increases Ca2+-ATPase activity in the mitochondria of brain tissues of normal and transgenic rats. J Cell Biochem 104 (3):795-804

135. Yamaguchi M, Mori S (1988) Effect of Ca2+ and Zn2+ on 5'-nucleotidase activity in rat liver plasma membranes: hepatic calcium-binding protein (regucalcin) reverses the Ca2+ effect. Chem Pharm Bull 36 (1):321-325

136. Yamaguchi M, Shibano H (1987) Reversible effect of calcium-binding protein on the Ca2+-induced activation of succinate dehydrogenase in rat liver mitochondria. Chem Pharm Bull 35 (9):3766-3700

137. Yamaguchi M, Shibano H (1987) Effect of calcium-binding protein on the activation of phosphorylase a in rat hepatic particulate glycogen by Ca2+. Chem Pharm Bull 35 (6):2581-2584

138. Yamaguchi M, Shibano H (1987) Calcium-binding protein isolated from rat liver cytosol reverses activation of pyruvate kinase by Ca2+. Chem Pharm Bull (Tokyo) 35 (5):2025-2029

139. Yamaguchi M, Mori S, Suketa Y (1989) Effects of Ca2+ and V5+ on glucose-6-phosphatase activity in rat liver microsomes: the Ca2+ effect is reversed by regucalcin. Chem Pharm Bull 37 (2):388-390

140. Hanahisa Y, Yamaguchi M (1999) Effect of calcium-binding protein on adenosine 5'-triphosphatase activity in the brain cytosol of rats of different ages: the inhibitory role of regucalcin. Biol Pharm Bull 22 (3):313-316

141. Yamaguchi M, Yoshida H (1985) Regulatory effect of calcium-binding protein isolated from rat liver cytosol on activation of fructose 1,6-diphosphatase by Ca2+-calmodulin. Chem Pharm Bull 33 (10):4489-4493

142. Yamaguchi M, Sakurai T (1992) Reversible effect of calcium-binding protein regucalcin on the Ca(2+)-induced inhibition of deoxyuridine 5'-triphosphatase activity in rat liver cytosol. Mol Cell Biochem 110 (1):25-29

143. Halls ML, Cooper DM (2011) Regulation by Ca2+-signaling pathways of adenylyl cyclases. Cold Spring Harb Perspect Biol 3 (1):a004143

144. Tresguerres M, Levin LR, Buck J (2011) Intracellular cAMP signaling by soluble adenylyl cyclase. Kidney Int 79 (12):1277-1288

145. Levy I, Horvath A, Azevedo M, de Alexandre RB, Stratakis CA (2011) Phosphodiesterase function and endocrine cells: links to human disease and roles in tumor development and treatment. Curr Opin Pharmacol 11 (6):689-697

146. Yamaguchi M, Tai H (1991) Inhibitory effect of calcium-binding protein regucalcin on Ca2+/calmodulin-dependent cyclic nucleotide phosphodiesterase activity in rat liver cytosol. Mol Cell Biochem 106 (1):25-30

147. Yamaguchi M, Kurota H (1997) Inhibitory effect of regucalcin on Ca2+/calmodulin-dependent cyclic AMP phosphodiesterase activity in rat kidney cytosol. Mol Cell Biochem 177 (1-2):209-214

148. Daff S (2010) NO synthase: structures and mechanisms. Nitric Oxide 23 (1):1-11

149. Ma ZJ, Yamaguchi M (2003) Regulatory effect of regucalcin on nitric oxide synthase activity in rat kidney cortex cytosol: Role of endogenous regucalcin in transgenic rats. Int J Mol Med 12 (2):201-206

150. Yamaguchi M, Takahashi H, Tsurusaki Y (2003) Suppressive role of endogenous regucalcin in the enhancement of nitric oxide synthase activity in liver cytosol of normal and regucalcin transgenic rats. J Cell Biochem 88 (6):1226-1234

151. Ma ZJ, Yamaguchi M (2002) Suppressive role of endogenous regucalcin in the regulation of nitric oxide synthase activity in heart muscle cytosol of normal and regucalcin transgenic rats. Int J Mol Med 10 (6):761-766

152. Tobisawa M, Yamaguchi M (2003) Role of endogenous regucalcin in brain function: suppression of cytosolic nitric oxide synthase and nuclear protein tyrosine phosphatase activities in brain tissue of transgenic rats. Int J Mol Med 12 (4):581-585

153. Nakagawa T, Yamaguchi M (2007) Overexpression of regucalcin suppresses cell response for tumor necrosis factor-alpha or transforming growth factor-beta1 in cloned normal rat kidney proximal tubular epithelial NRK52E cells. J Cell Biochem 100 (5):1178-1190

154. Sugiura R, Sio SO, Shuntoh H, Kuno T (2002) Calcineurin phosphatase in signal transduction: lessons from fission yeast. Genes Cells 7 (7):619-627

155. Rusnak F, Mertz P (2000) Calcineurin: form and function. Physiol Rev 80 (4):1483-1521

156. Omura M, Yamaguchi M (1998) Inhibition of Ca2+/calmodulin-dependent phosphatase activity by regucalcin in rat liver cytosol: involvement of calmodulin binding. J Cell Biochem 71 (1):140-148

157. Omura M, Yamaguchi M (1999) Effect of anti-regucalcin antibody on neutral phosphatase activity in rat liver cytosol: involvement of endogenous regucalcin. Mol Cell Biochem 197 (1-2):25-29

158. Omura M, Kurota H, Yamaguchi M (1998) Inhibitory effect of regucalcin on Ca2+/calmodulin-dependent phosphatase activity in rat renal cortex cytosol. Biol Pharm Bull 21 (5):440-443

159. Morooka Y, Yamaguchi M (2001) Suppressive role of endogenous regucalcin in the regulation of protein phosphatase activity in rat renal cortex cytosol. J Cell Biochem 81 (4):639-646

160. Morooka Y, Yamaguchi M (2001) Inhibitory effect of regucalcin on protein phosphatase activity in the nuclei of rat kidney cortex. J Cell Biochem 83 (1):111-120

161. Morooka Y, Yamaguchi M (2002) Endogenous regucalcin suppresses the enhancement of protein phosphatase activity in the cytosol and nucleus of kidney cortex in calcium-administered rats. J Cell Biochem 85 (3):553-560

162. Ichikawa E, Tsurusaki Y, Yamaguchi M (2004) Suppressive effect of regucalcin on protein phosphatase activity in the heart cytosol of normal and regucalcin transgenic rats. Int J Mol Med 13 (2):289-293

163. Hamano T, Yamaguchi M (1999) Inhibitory effect of regucalcin on Ca2+/calmodulin-dependent protein phosphatase activity in rat brain cytosol. Int J Mol Med 3 (6):615-619

164. Yamaguchi M, Hamano T, Misawa H (2000) Expression of Ca(2+)-binding protein regucalcin in rat brain neurons: inhibitory effect on protein phosphatase activity. Brain Res Bull 52 (5):343-348

165. Tobisawa M, Yamaguchi M (2003) Suppressive effect of endogenous regucalcin on protein tyrosine phosphatase activity in the nucleus of rat brain: attenuation with increasing age. Int J Mol Med 11 (2):205-210

166. Tobisawa M, Tsurusaki Y, Yamaguchi M (2003) Decrease in regucalcin level and enhancement of protein tyrosine phosphatase activity in rat brain microsomes with increasing age. Int J Mol Med 12 (4):577-580

167. Yamaguchi M, Katsumata T (1999) Enhancement of protein kinase activity in the cytosol of regenerating rat liver: regulatory role of endogenous regucalcin. Int J Mol Med 3 (5):505-510

168. Mori S, Yamaguchi M (1990) Hepatic calcium-binding protein regucalcin decreases Ca2+/calmodulin-dependent protein kinase activity in rat liver cytosol. Chem Pharm Bull 38 (8):2216-2218

169. Yamaguchi M, Mori S (1990) Inhibitory effect of calcium-binding protein regucalcin on protein kinase C activity in rat liver cytosol. Biochem Med Metab Biol 43 (2):140-146

170. Kurota H, Yamaguchi M (1998) Inhibitory effect of calcium-binding protein regucalcin on protein kinase C activity in rat renal cortex cytosol. Biol Pharm Bull 21 (4):315-318

171. Kurota H, Yamaguchi M (1997) Inhibitory effect of regucalcin on Ca2+/calmodulin-dependent protein kinase activity in rat renal cortex cytosol. Mol Cell Biochem 177 (1-2):239-243

172. Hamano T, Yamaguchi M (2001) Inhibitory role of regucalcin in the regulation of Ca2+ dependent protein kinases activity in rat brain neurons. J Neurol Sci 183 (1):33-38

173. Hamano T, Hanahisa Y, Yamaguchi M (1999) Inhibitory effect of regucalcin on Ca(2+)-dependent protein kinase activity in rat brain cytosol: involvement of endogenous regucalcin. Brain Res Bull 50 (3):187-192

174. Wu HY, Tomizawa K, Matsui H (2007) Calpain-calcineurin signaling in the pathogenesis of calcium-dependent disorder. Acta Med Okayama 61 (3):123-137

175. Liu X, Van Vleet T, Schnellmann RG (2004) The role of calpain in oncotic cell death. Annu Rev Pharmacol Toxicol 44:349-370

176. Baba T, Yamaguchi M (1999) Stimulatory effect of regucalcin on proteolytic activity in rat renal cortex cytosol: involvement of thiol proteases. Mol Cell Biochem 195 (1-2):87-92

177. Yamaguchi M, Tai H (1992) Calcium-binding protein regucalcin increases calcium-independent proteolytic activity in rat liver cytosol. Mol Cell Biochem 112 (1):89-95

178. Yamaguchi M, Nishina N (1995) Characterization of regucalcin effect on proteolytic activity in rat liver cytosol: relation to cysteinyl-proteases. Mol Cell Biochem 148 (1):67-72

179. Linster CL, Van Schaftingen E (2007) Vitamin C. Biosynthesis, recycling and degradation in mammals. FEBS J 274 (1):1-22

180. Koike K, Kondo Y, Sekiya M, Sato Y, Tobino K, Iwakami SI, Goto S, Takahashi K, Maruyama N, Seyama K, Ishigami A (2010) Complete lack of vitamin C intake generates pulmonary emphysema in senescence marker protein-30 knockout mice. Am J Physiol Lung Cell Mol Physiol 298(6):L784-792

181. Kondo Y, Sasaki T, Sato Y, Amano A, Aizawa S, Iwama M, Handa S, Shimada N, Fukuda M, Akita M, Lee J, Jeong KS, Maruyama N, Ishigami A (2008) Vitamin C depletion increases superoxide generation in brains of SMP30/GNL knockout mice. Biochem Biophys Res Commun 377 (1):291-296

182. Sato Y, Kajiyama S, Amano A, Kondo Y, Sasaki T, Handa S, Takahashi R, Fukui M, Hasegawa G, Nakamura N, Fujinawa H, Mori T, Ohta M, Obayashi H, Maruyama N, Ishigami A (2008) Hydrogen-rich pure water prevents superoxide formation in brain slices of vitamin C-depleted SMP30/GNL knockout mice. Biochem Biophys Res Commun 375 (3):346-350

183. Son TG, Zou Y, Jung KJ, Yu BP, Ishigami A, Maruyama N, Lee J (2006) SMP30 deficiency causes increased oxidative stress in brain. Mech Ageing Dev 127 (5):451-457

184. Sato T, Seyama K, Sato Y, Mori H, Souma S, Akiyoshi T, Kodama Y, Mori T, Goto S, Takahashi K, Fukuchi Y, Maruyama N, Ishigami A (2006) Senescence marker protein-30 protects mice lungs from oxidative stress, aging, and smoking. Am J Respir Crit Care Med 174 (5):530-537

185. Ambs S, Glynn SA (2011) Candidate pathways linking inducible nitric oxide synthase to a basal-like transcription pattern and tumor progression in human breast cancer. Cell Cycle 10 (4):619-624

186. Izumi T, Tsurusaki Y, Yamaguchi M (2003) Suppressive effect of endogenous regucalcin on nitric oxide synthase activity in cloned rat hepatoma H4-II-E cells overexpressing regucalcin. J Cell Biochem 89 (4):800-807

187. Ogita K, Takagi R, Oyama N, Okuda H, Ito F, Okui M, Shimizu N, Yoneda Y (2001) Decrease in level of APG-2, a member of the heat shock protein 110 family, in murine brain following systemic administration of kainic acid. Neuropharmacology 41 (3):285-293

188. Zheng XY, Zhang HL, Luo Q, Zhu J (2011) Kainic acid-induced neurodegenerative model: potentials and limitations. J Biomed Biotechnol 2011:457079

189. Makker K, Agarwal A, Sharma R (2009) Oxidative stress & male infertility. Indian J Med Res 129 (4):357-367

190. Crowell JA, Steele VE, Sigman CC, Fay JR (2003) Is inducible nitric oxide synthase a target for chemoprevention? Mol Cancer Ther 2 (8):815-823

191. Lim S, Song BW, Cha MJ, Choi EJ, Ham O, Lee CY, Choi SY, Lee SY, Jang Y, Hwang KC (2009) Differential Expression of Regucalcin (SMP30) and Its Function in Hypoxic Cardiomyocytes. Tissue Eng Regen Med 6 (13):1273-1281

192. Izumi T, Yamaguchi M (2004) Overexpression of regucalcin suppresses cell death and apoptosis in cloned rat hepatoma H4-II-E cells induced by lipopolysaccharide, PD 98059, dibucaine, or Bay K 8644. J Cell Biochem 93 (3):598-608

193. Tsurusaki Y, Yamaguchi M (2002) Suppressive role of endogenous regucalcin in the enhancement of deoxyribonucleic acid synthesis activity in the nucleus of regenerating rat liver. J Cell Biochem 85 (3):516-522

194. Yamaguchi M, Sakurai T (1991) Inhibitory effect of calcium-binding protein regucalcin on Ca2(+)-activated DNA fragmentation in rat liver nuclei. FEBS Lett 279 (2):281-284

195. Fukaya Y, Yamaguchi M (2005) Overexpression of regucalcin suppresses cell death and apoptosis in cloned rat hepatoma H4-II-E cells induced by insulin or insulin-like growth factor-I. J Cell Biochem 96 (1):145-154

196. Nakagawa T, Yamaguchi M (2005) Overexpression of regucalcin suppresses apoptotic cell death in cloned normal rat kidney proximal tubular epithelial NRK52E cells: change in apoptosis-related gene expression. J Cell Biochem 96 (6):1274-1285

197. Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, Nagata S (1993) Lethal effect of the anti-Fas antibody in mice. Nature 364 (6440):806-809

198. Elchuri S, Naeemuddin M, Sharpe O, Robinson WH, Huang TT (2007) Identification of biomarkers associated with the development of hepatocellular carcinoma in CuZn superoxide dismutase deficient mice. Proteomics 7 (12):2121-2129

199. Kim W, Oe Lim S, Kim JS, Ryu YH, Byeon JY, Kim HJ, Kim YI, Heo JS, Park YM, Jung G (2003) Comparison of proteome between hepatitis B virus- and hepatitis C virus-associated hepatocellular carcinoma. Clin Cancer Res 9 (15):5493-5500

200. Jahchan NS, Luo K (2010) SnoN in mammalian development, function and diseases. Curr Opin Pharmacol 10 (6):670-675

201. Lamouille S, Derynck R (2009) Oncogene and tumour suppressor: the two faces of SnoN. Embo J 28 (22):3459-3460

202. Dallaglio K, Marconi A, Pincelli C (2012) Survivin: a dual player in healthy and diseased skin. J Invest Dermatol 132 (1):18-27

Chapter III

Aim and outline of the thesis

Aim and outline of the thesis

Despite the numerous research efforts and the continuous improvements on diagnostic and treatment, breast cancer remains one of the biggest threats for women health compromising their life expectation. Although scientific research has produced substantial advances in the understanding of the molecular mechanisms that underlie breast tumorigenesis, this highly complex pathology continuous to be a challenge for researchers, physicians and women. The accepted hallmarks of cancer were established on the basis of the common molecular mechanisms that are deregulated in non-neoplastic cells leading to the neoplastic transformation. These mechanisms include cell proliferation, apoptosis, glycolytic metabolism and calcium (Ca^{2+}) homeostasis. Regucalcin (RGN) is a Ca^{2+}-binding protein that has been associated with the control of cell proliferation, apoptosis and also cell metabolism. Moreover, RGN was linked to breast cancer. It was found that RGN expression is suppressed in human breast cancer cases and correlated with tumor grade. This led us to hypothesize that the loss of RGN expression may favor the tumorigenic transformation of the mammary gland. On the other hand, it is liable to assume that maintaining RGN expression levels may be protective mechanism against mammary carcinogenesis.

Therefore, the first aim of this thesis was to disclose if RGN has a role protecting against the onset and development of mammary gland tumors. Secondly, we intended to explore the biological mechanisms through which RGN may exert its functions in non-pathologic and pathologic breast tissues. For this purpose, cell proliferation, apoptosis, and metabolic pathways were evaluated. It was also our aim to investigate the factors that regulate RGN expression levels in breast cancer cells.

According to the proposed aims, the thesis was organized in separated complementary chapters. In chapter IV, the protective role of RGN against the carcinogenesis of the mammary gland was investigated by the chemical induction of mammary gland tumors in transgenic rats overexpressing RGN (Tg-RGN) and wild-type (Wt) controls. Also, the influence of RGN overexpression in the proliferative and apoptotic status of the mammary gland and mammary gland tumors was evaluated.

To further dissect the biologic regulatory actions of RGN, the glycolytic metabolism in the mammary gland and non-invasive mammary tumors of Tg-RGN rats and Wt controls was also performed (Chapter V).

Chapter VI explores whether the expression of RGN and another Ca^{2+} regulator, the L-type Ca^{2+} channel, are affected by the treatment with 5α-dihydrotestosterone in MCF-7 breast cancer cells.

Finally, in Chapter VII, an integrative discussion of all the obtained results presented herein is included, depicting future perspectives of research and application of these discoveries into treatment of breast cancer.

Chapter IV

Histopathological and *in vivo* evidence of regucalcin as a protective molecule in mammary gland carcinogenesis

Chapter published in: Ricardo Marques, Cátia V. Vaz, Cláudio J. Maia, Madalena Gomes, Adelina Gama, Gilberto Alves, Cecília R. Santos, Fernando Schmitt, Sílvia Socorro (2015) Histopathological and *in vivo* evidence of regucalcin as a protective molecule in mammary gland carcinogenesis. Exp Cell Res. 330: 325-335.

Histopathological and *in vivo* evidence of regucalcin as a protective molecule in mammary gland carcinogenesis

Abstract

Regucalcin (RGN) is a calcium-binding protein, which has been shown to be underexpressed in cancer cases. This study aimed to determine the association of RGN expression with clinicopathological parameters of human breast cancer. In addition, the role of RGN in malignancy of mammary gland using transgenic rats overexpressing the protein (Tg-RGN) was investigated. Wild-type (Wt) and Tg-RGN rats were treated with 7,12-dimethylbenz[α]anthracene (DMBA). Carcinogen-induced tumors were histologically classified and the Ki67 proliferation index was estimated. Immunohistochemistry analysis showed that RGN immunoreactivity was negatively correlated with the histological grade of breast infiltrating ductal carcinoma suggesting that progression of breast cancer is associated with loss of RGN. Tg-RGN rats displayed lower incidence of carcinogen-induced mammary gland tumors, as well as lower incidence of invasive forms. Moreover, higher proliferation was observed in non-invasive tumors of Wt animals comparatively with Tg-RGN. Overexpression of RGN was associated with diminished expression of cell-cycle inhibitors and increased expression of apoptosis inducers. Augmented activity of apoptosis effector caspase-3 was found in the mammary gland of Tg-RGN. RGN overexpression protected from carcinogen-induced mammary gland tumor development and was linked with reduced proliferation and increased apoptosis. These findings indicated the protective role of RGN in the carcinogenesis of mammary gland.

Keywords: apoptosis / breast cancer / carcinogen / DMBA / mammary gland / regucalcin

Introduction

Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein, without the typical EF-motif, that plays an important role in intracellular Ca^{2+} handling by modulation of Ca^{2+}-pumps activity at the cell membrane and intracellular organelles [1]. The role of RGN in the regulation of multiple intracellular signaling pathways, such as cell proliferation and apoptosis, also has been suggested [2, 3]. Although highly expressed in liver and kidney, RGN is present in a wide variety of reproductive tissues [4, 5], and altered expression patterns of this protein have been associated to pathological conditions [3]. Previous studies conducted by us and others have shown that RGN is underexpressed in human breast, prostate and liver cancer [6, 4, 7]. Moreover, it was observed that loss of RGN expression already occurs in the pre-neoplastic lesions of rat liver and, thus preceding the formation of tumors [8]. This suggests that loss of RGN may be an early event underlying malignant transformation of cells, tumor development and progression. In the present study, we investigated the association of RGN expression with clinicopathological variables of human breast cancer. In addition, to ascertain the role of RGN

in the control of malignant transformation of mammary gland, we made use of a transgenic rat model overexpressing RGN and studied their susceptibility to developing carcinogen-induced tumors.

Material and methods

Chemicals

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

Animals, DMBA treatment and tissue collection

Virgin female Sprague Dawley wild-type (Wt) rats were obtained from Charles River (Barcelona, Spain). Transgenic rats overexpressing RGN (Tg-RGN) originally generated by Yamaguchi et al. [9] by oocyte transgene pronuclear injection were purchased from Japan SLC (Hamamatsu, Japan). 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). They were housed under a 12 h light:12 h darkness cycle, with food and water available *ad libitum* during the experiment. At 3-month of age, both Wt (n = 10) and Tg-RGN (n = 31) rats were treated with the carcinogen 7,12-dimethylbenz[α]anthracene (DMBA, 20 mg/kg in 0.2 ml sesame oil) by a single intragastric administration. Two weeks after DMBA administration animals were weekly palpated for detecting the presence of tumors. When bearing palpable tumors (≥ 2 cm) rats were euthanized by cervical dislocation under anesthesia (100 mg ketamine/8 mg xilazine per Kg). Individual tumors were dissected and fixed in 4 % paraformaldehyde for histological processing.

Breast cancer tissue microarrays

Tissue microarrays (TMAs) containing 158 cases of human breast infiltrating ductal carcinoma were constructed at the Pathology Unit of IPATIMUP (Institute of Molecular Pathology and Immunology of Porto University) using archived formalin-fixed paraffin-embedded resection blocks, as previously described [10]. TMA blocks were cut into 2-4 μm sections, which were processed for immunohistochemical analysis.

RGN immunohistochemistry and staining scores

TMAs sections were deparaffinised with xylene and rehydrated using different grades of ethanol. Heat-induced antigen retrieval was performed in a citrate buffer solution (10 mM, pH 6.0) and endogenous peroxidase was inactivated with 3 % hydrogen peroxide for 10 min. Cells were permeabilized with 0.01 % digitonin for 5 min at room temperature (RT). Non-specific protein binding was prevented by incubating sections with PBS containing 1 % BSA and 0.3 M glycine for 30 min. Sections were incubated overnight at 4°C with rabbit anti-RGN antibody (Cosmo Bio Co., LTD) diluted 1:50 in PBS with 1 % BSA, followed by 1 h incubation with goat

anti-rabbit biotinylated antibody (1:20) at RT. Antibody binding was detected using Extravidin Peroxidase reagent (1:20) and 3,3'-diaminobenzidine hydrochloride (Dako, Glostrup, Denmark) as chromogen. TMAs sections were counterstained with Mayer's hematoxylin, dehydrated, cleared and mounted. Specificity of the immunostaining was assessed by the omission of primary antibody.

A scoring system including intensity and percentage of stained cells was used to assess RGN immunoreactivity in breast tumors [4]. Staining intensity was divided into moderate (score 1) and strong (score 2), while percentage of staining was divided into, up to 1/3 (score 1), up to 2/3 (score 2) and almost all cells stained (score 3). Low RGN immunoreactivity with a score 1 (1x1) indicated that up to 1/3 of cells were moderately stained; moderate immunoreactivity was considered for scores 2-4 (1x2, 1x3, 2x1 or 2x2); high immunoreactivity represented a score of 6 (2x3) indicating that almost all cells were strongly stained. RGN immunoreactivity was associated with clinicopathological parameters of patients/tumors available at the IPATIMUP archive, namely, age, tumor grade and tumor molecular profile subtype. Also, RGN expression was associated with tumors status for estrogen receptor (ER), progesterone receptor (PR), HER-2/neu oncoprotein (HER-2), epidermal growth factor receptor (EGFR), cytokeratin 5 (CK5), P-cadherin (P-Cad), cytokeratin 14 (CK14), p63 and p53.

Ki67 fluorescence immunohistochemistry

Non-invasive tumors of both Tg-RGN and Wt animals were stained with the nuclear proliferation marker Ki67. Briefly, after deparaffinization tissue sections were rehydrated and antigen retrieval was performed in a heated citrate bath (pH 6.0). Following permeabilization with 0.2 % Triton X-100 for 10 min and blocking of non-specific protein binding with PBS containing 1 % BSA and 0.3 M glycine for 30 min at RT, sections were incubated overnight at 4°C with a rabbit anti-Ki67 antibody (1:50, Abcam, ab16667). Incubation with the Alexa fluor 546 goat anti-rabbit IgG secondary antibody (1:500, Invitrogen) was carried out for 1 h at RT. Specificity of immunostaining was assessed by omission of primary antibody. Cell nuclei were stained with Hoechst 33342 (10 µg/mL, Invitrogen) for 5 min at RT. Sections were mounted in Dako fluorescent mounting medium (Dako, Lisbon, Portugal). Slides were observed in the Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Germany) and proliferation index was estimated by counting the number of Ki67 positive cells and Hoechst stained nuclei in thirty randomly selected 40 x magnification fields for each section. The ratio between Ki67 stained cells and the total number of nuclei was calculated.

RNA isolation and cDNA synthesis

Total RNA was extracted from mammary gland tissues of 3-month old Wt and Tg-RGN rats using the TRI reagent (Ambion) following manufacturer's instructions. Total RNA was reverse-transcribed using the First-strand cDNA synthesis kit (MB12502, NZYTech, Lisboa, Portugal). In brief, 1 µg of total RNA was added to NZY Reverse Transcriptase enzyme mix. cDNA synthesis reaction was initiated by a 10 min incubation at 25°C, followed by 30 min at 50°C and an

inactivation step at 85°C for 5 min. 1 µL of RNase H was added and incubation proceed at 37°C for 20 min. The reaction was stopped by heating at 85°C for 5 min and synthesized cDNA was stored at -20°C until further use.

Real-time-PCR (qPCR)

Analysis of expression of cell-cycle and apoptosis related genes (Table IV.1) in the mammary gland of Tg-RGN and Wt rats was performed by qPCR using indicated specific primers and cycling conditions. β_2-Microglobulin (B2M, sense: 5´ATGGTGGGTATGGGTCAG3´; antisense: 5´CAATGCCGTGTTCAATGG3´) and GAPDH (sense: 5´GTTCAACGGCACAGTCAA3´; antisense: 5´CTCAGCACCAGCATCACC3´) genes were used as internal controls for normalization of expression of interest genes. Reactions were carried out in an iQ5 system (Bio-Rad, Hercules, USA) and the efficiency of amplifications was determined for all primer sets using serial dilutions of cDNA (1, 1:3 and 1:9). 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 (Thermo Scientific from Fermentas), sense and antisense primers (300 nM for H-ras, SCF, and B2M; 200 nM for all other genes) and 1-3 µl of cDNA in a final volume of 20 µl. PCR reaction comprised 5 min denaturation at 95°C, followed by 40 cycles at 95°C for 10 s, a specific annealing temperature for each gene (Table IV.1) for 30 s and 72°C for 10 s. Samples were run in triplicate in each PCR assay. Normalized expression values were calculated following the mathematical model proposed by Vandesompele et al. [11].

Western blot

Total proteins were extracted from mammary glands of 3-month old Tg-RGN and Wt rats 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 80 µg 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-cleaved caspase-8 (1:1000, no. 5263, Santa Cruz Biotechnology), rabbit anti-Bcl-2 (1:1000 no. 2876, Cell Signaling Technology) and rabbit anti-Bax (1:500 no. 2772, Cell Signaling Technology). A mouse anti-ß-actin antibody (1:5000, A5441, Sigma-Aldrich) was used for normalization of protein expression. 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 5 min and immunoreactive bands visualized using the Molecular Imager FX Pro plus MultiImager (Bio-Rad). Band densities were obtained according to standard methods using the Quantity One Software (Biorad) and normalized by division with the respective ß-actin band density.

Table IV.1. Oligonucleotides sequences, amplicon size and annealing temperature in qPCR reactions.

Gene	Sequence (5'- 3')	AT (°C)	Amplicon Size (bp)
Myc	Sense: AAG AAC AAG ATG ATG AGG AAG Antisense: GTG CTG GTG AGT AGA GAC	53	143
H-ras	Sense: AGA CCC GGC AGG GTG TGG AG Antisense: CCG GGA CGG GCA CAA AGG AC	60	278
p21	Sense: GTT CCT TGC CAC TTC TTA C Antisense: ACT GCT TCA CTG TCA TCC	53	103
p53	Sense: CTG CCC ACC ACA GCG ACA GG Antisense: AGG AGC CAG GCC GTC ACC AT	60	471
Cdk1	Sense: GGT TGA CAT CTG GAG GAT AG Antisense: GCC ACA CTT CGT TGT TAG G	53	115
Chk2	Sense: ATG AAG GAA GAT GGT CTA AGC Antisense: TGG TGG AGG AAC TGG ATG	53	168
SCF	Sense: ATG GCT TGG GAA ATG TCT G Antisense: GCT GAT GCT ACG GAG TTA C	58	193
c-Kit	Sense: CCG TCT CCA TCC ATC C Antisense: TTC GCT CTG CTT ATT CTC AAT CC	60	143
Bax	Sense: CGC GTG GTT GCC CTC TTC TAC TTT Antisense: CAA GCA GCC GCT CAC GGA GGA	60	124
Bcl-2	Sense: GGG CTA CGA GTG GGA TAC Antisense: AGG CTG GAA GGA GAA GAT G	53	63
caspase-9	Sense:TGC AGG GTA CGC CTT GTG CG Antisense: CCT GAT CCC GCC GAG ACC CA	60	130
caspase-3	Sense: AGG CCT GCC GAG GTA CAG AGC Antisense: CCG TGG CCA CCT TCC GCT TA	60	255

Abbreviations: AT, annealing temperature; bp, base pairs

Caspase-3 activity assay

The activity of caspase-3 was assessed through 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 4 h at 37°C. The caspase-3-like activity was determined after cleavage of the labeled substrate and release of the chromophore p-nitroanilide, measured spectrophotometrically at 405 nm. The method was calibrated with known concentrations of p-nitroanilide.

Statistical analysis

The X^2 contingency test was used for categorical variables to determine differences between phenotypes. Student's t-test was used to evaluate quantitative variables. A *p*-value < 0.05 was considered to reflect a significant association. The Graphpad Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis.

Results

Association of RGN expression with clinicopathological parameters of human breast cancers

RGN immunoreactivity was evaluated in human breast infiltrating ductal carcinomas and associated with several clinicopathological parameters, such as age, tumor grade, tumor status for ER, PR, HER2, EGFR, CK5, P-Cad, CK14, p63 and p53 proteins, and tumor molecular profile (Table IV.2).

Table IV.2. Association of regucalcin expression with clinical and histopathological data of breast cancer patients/tumors.

Variable		Total of cases	Regucalcin immunoreactivity			*p*-value*
			Low n (%)	*Moderate* n (%)	*High* n (%)	
Age (y)	≤ 45	42	11 (26.2)	26 (61.9)	5 (11.9)	0.4862
	> 45	116	33 (28.5)	61 (52.6)	22 (20)	
Tumor grade	I	77	23 (29.9)	38 (49.4)	16 (20.8)	0.0498 (0.0347)[a]
	II	56	11 (19.6)	37 (66.1)	8 (14.3)	
	III	20	10 (50)	9 (45)	1 (5)	
ER	(-)	64	22 (34.4)	33 (51.6)	9 (14.1)	0.2921
	(+)	94	22 (23.4)	54 (57.5)	18 (19.1)	
PR	(-)	99	34 (34.3)	49 (50)	16 (16.2)	0.0595 (0.0183)[a]
	(+)	59	10 (17)	38 (64.4)	11 (18.6)	
HER-2	(-)	139	40 (28.8)	73 (52.5)	26 (18.7)	0.1750
	(+)	19	4 (21.1)	14 (73,7)	1 (5.3)	
EGFR	(-)	147	39 (26.5)	82 (55.8)	26 (17.7)	0.3759
	(+)	11	5 (45.5)	5 (45.5)	1 (9.1)	
CK5	(-)	120	31 (25.8)	64 (53.3)	25 (20.8)	0.0784
	(+)	38	13 (34.2)	23 (60.5)	2 (5.3)	
P-Cad	(-)	118	34 (28.8)	65 (55.1)	19 (16.1)	0.8099
	(+)	40	10 (25)	22 (55)	8 (20)	
CK14	(-)	149	40 (26.9)	83 (55.7)	26 (17.5)	0.4594
	(+)	4	2 (50)	1 (25)	1 (25)	
p63	(-)	156	43 (27.56)	86 (55.1)	27 (17.3)	0.7003
	(+)	2	1 (50)	1 (50)	0 (0)	
p53	(-)	123	37 (30.1)	65 (52.9)	21 (17.1)	0.4746
	(+)	35	7 (20)	22 (62.9)	6 (17.1)	
Molecular Prolife	Basal-like	26	11 (42.3)	12 (46.2)	3 (11.5)	0.2256
	HER-2	17	4 (23.6)	12 (70.6)	1 (5.9)	
	Luminal A	93	22 (23.7)	53 (57)	18 (19.4)	
	Luminal B	3	0 (0)	3 (100)	0 (0)	

*- calculated by the X^2 test; [a] *p*-value when grouping cases as low *vs.* (moderate and high)

RGN expression pattern, divided into low, moderate and high (Figure IV.1), was negatively associated with the differentiation grade of tumors (p = 0.0498). Seventy percent of well differentiated (grade I) breast tumors displayed moderate or high RGN immunoreactivity, while 66.1 % of grade II tumors were moderately stained. In contrast, only 5 % of poorly differentiated tumors (grade III) showed high RGN immunoreactivity, and the percentage of cases presenting low RGN levels increased relatively to grade II and grade I tumors (50 % *vs* 19.6 or 29.9 %, respectively).

No other statistically significant associations were found between RGN expression patterns (low, moderate or high) and histopathlogical parameters. Nonetheless, the percentage of ER (34.4 %) and PR (34.3 %) negative tumors with low RGN staining *vs* the cases with high RGN immunoreactivity (14.1 % (p = 0.2921) and 16.2 % (p = 0.0595) respectively, for ER and PR), should be noted. In fact, when grouping cases as low *vs* (moderate and high) a significant association was found between PR tumor status and RGN expression (p = 0.0183).

Also, within HER-2 (p = 0.175) and CK5 (p = 0.0784) positive cases, 21.1 % and 34.2 % of tumors, respectively, displayed low RGN staining contrasting with only 5.3 % of cases with high RGN levels. Although no association was established between RGN immunoreactivity and tumor molecular profile (p = 0.2256), an elevated percentage of basal-like and HER-2 tumors presented low RGN immunoreactivity (42.3 % and 23.6 %, respectively). The percentage of tumors with high RGN staining was 11.5 % for basal-like and 5.9 % for HER-2.

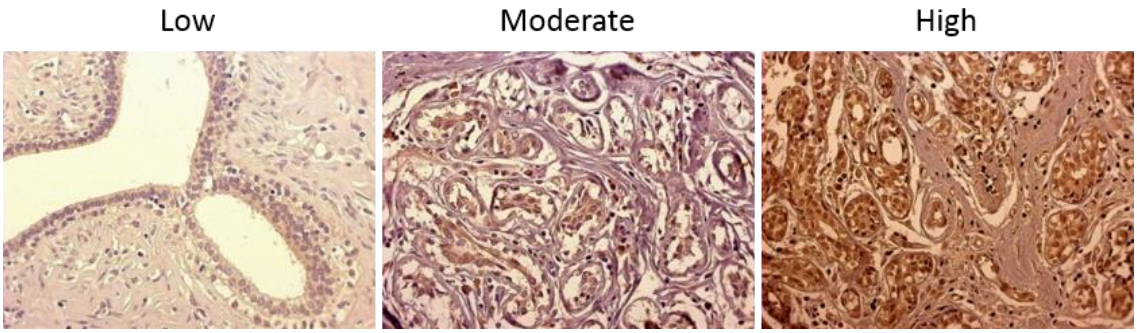

Figure IV.1. Representative images of low, moderate and high RGN immunoreactivity in human breast infiltrating ductal carcinoma. All tissues are shown in an original magnification of 400 x.

Transgenic overexpression of RGN protects from carcinogen-induced mammary gland tumor development

In order to evaluate the role of RGN controlling malignant transformation of mammary gland, Tg-RGN and Wt rats were treated with the DMBA carcinogen, a well-recognized agent inducing rat mammary carcinogenesis [12]. The latency period for tumor development (14 weeks) was identical in both groups; however, tumors emerged quickly in the Wt animals. Thirty-eight and 50 % of Wt animals presented a palpable tumor, respectively, at 18 and 28 weeks after DMBA administration, contrasting with only 6.45 % in Tg-RGN ($p < 0.05$, Figure IV.2). In the Tg-RGN group no other animals developed tumors from 14 weeks to 36 weeks after DMBA administration. At 44 weeks, tumor incidence was 25.8 % in Tg-RGN against 100 % in Wt group ($p < 0.001$, Figure IV.2). Fifty weeks after carcinogen treatment more than half of Tg-RGN animals (62.5 %) had developed a tumor and, thus, the remaining animals were euthanized and the experiment ended.

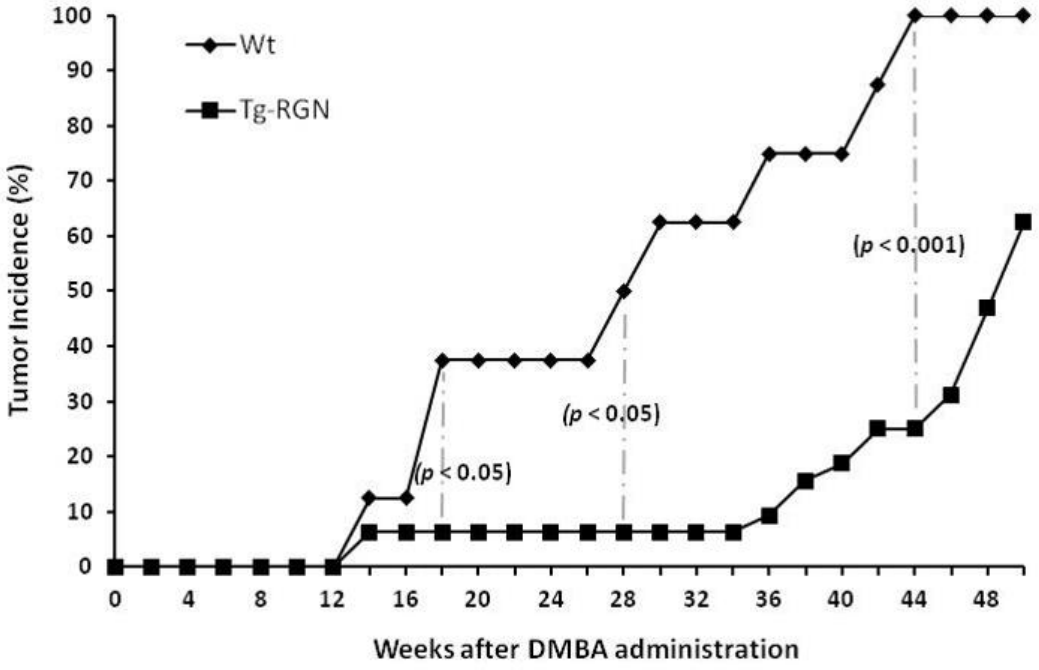

Figure IV.2. Cumulative percentage of bearing a palpable tumor in transgenic rats overexpressing regucalcin (Tg-RGN) and wild-type (Wt) after DMBA administration. Virgin female rats were given DMBA (20mg/kg body weight) at 3 month of age.

Tumors collected from Wt and Tg-RGN animals were histologically classified according to Russo and Russo [13], and the distinct histological types identified are shown in Figure IV.3.

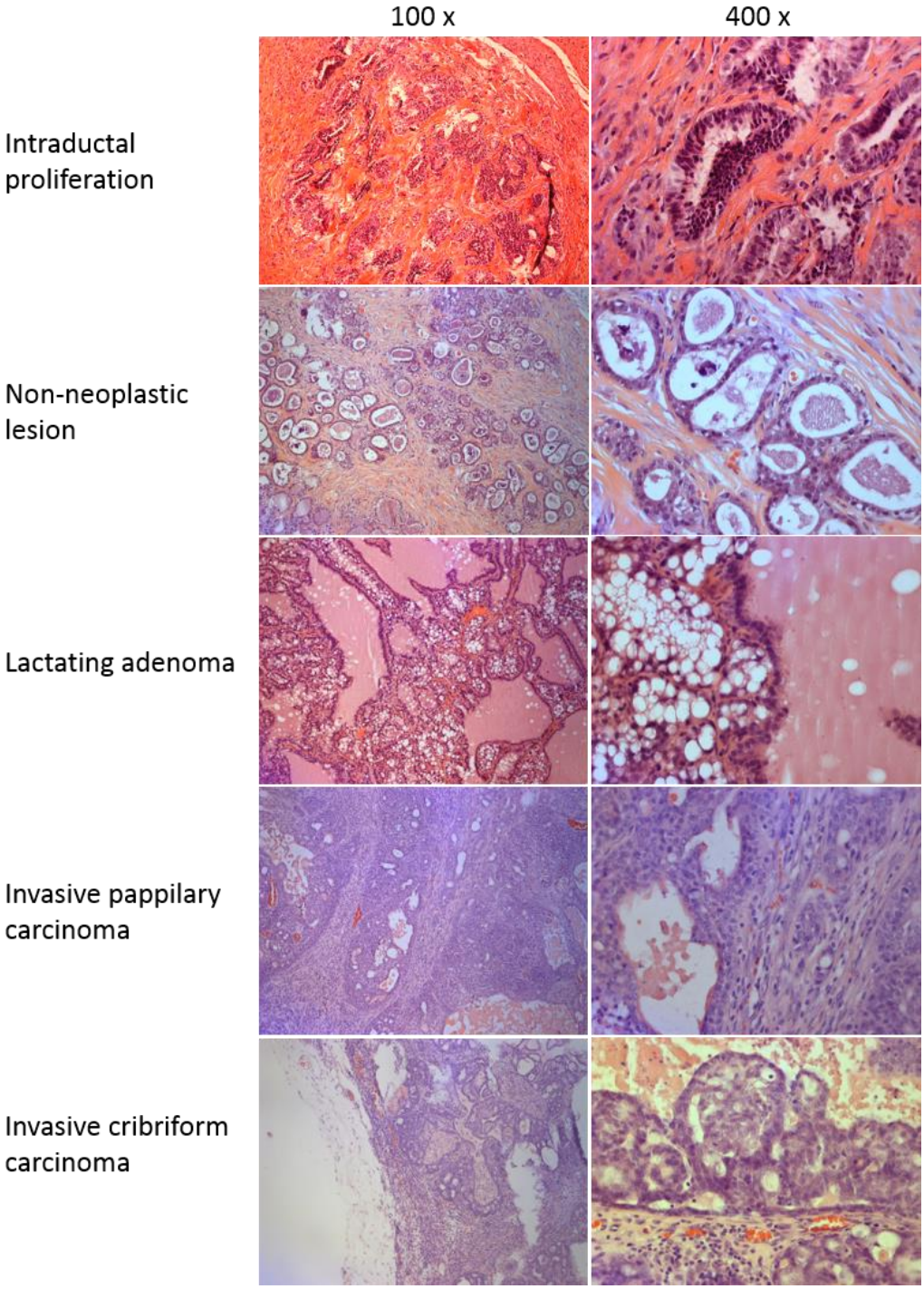

Figure IV.3. Representative images of hematoxilin and eosin stained sections (100x and 400x magnifications) of rat mammary gland tumors developed in response to DMBA treatment (20mg/kg body weight). Tumors were histologically classified and grouped as pre-cancerous lesions (intraductal proliferation), non-invasive tumors (non-neoplastic lesion and lactating adenoma) and invasive tumors (invasive papillary carcinoma and invasive cribriform carcinoma).

Tumors were grouped as pre-cancerous lesions, non-invasive and invasive, showing a significant different incidence between Tg-RGN and Wt animals (Table IV.3, p = 0.0043). Although the majority of tumors both in Tg-RGN (76.9 %) and Wt (54.5 %) groups were non-invasive forms, the percentage of invasive tumors was significantly higher in Wt (45.5 % *vs* 3.8 % in Tg-RGN). Also noteworthy is the fact that pre-cancerous lesions (19.2 %) were only detected in Tg-RGN (Table IV.3).

Table IV.3. Incidence of pre-cancerous lesions, non-invasive and invasive mammary gland tumors in rats overexpressing regucalcin (Tg-RGN) and controls (Wt) 50 weeks after DMBA administration*.

Group	Pre-cancerous lesions		Non-invasive tumors		Invasive tumors		*p*-value
	n	%	n	%	n	%	
Tg-RGN	5 (26)	19.2	20 (26)	76.9	1 (26)	3.8	0.0043
Wt	0 (11)	0	6 (11)	54.5	5 (11)	45.4	

* some animals have developed more than one tumor

Proliferation index in non-invasive mammary gland tumors of RGN transgenic and wild-type rats

Ki67 immunofluorescent staining was used to evaluate the proliferation index in non-invasive mammary gland tumors of Wt and Tg-RGN animals. The number of Ki67-stained cells in tumors of Tg-RGN rats was significantly lower comparatively to tumors of Wt animals (p < 0.05, Figure IV.4).

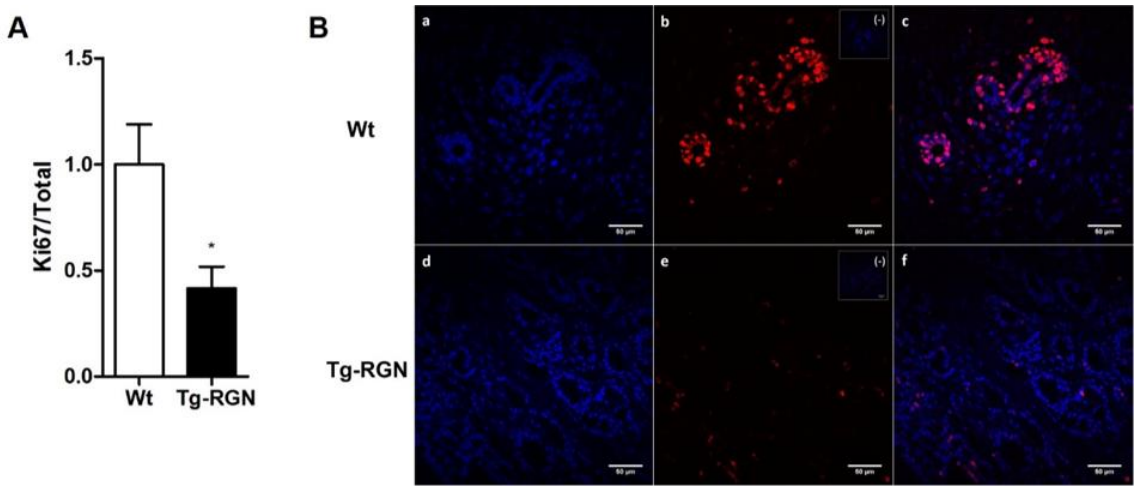

Figure IV.4. Proliferation index in non-invasive mammary gland tumors of transgenic rats overexpressing regucalcin (Tg-RGN) versus wild-type (Wt) counterparts determined by immunofluorescent staining of Ki67. (A) Percentage of Ki67 positive cells relatively to total cells. Results are expressed as fold-variation relatively to the Wt group. Error bars indicate mean ± SEM (n ≥ 4).* p < 0.05. (B) Representative images of Hoechst stained nuclei, Ki67 immunofluorescence and corresponding merged images in Wt (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 (-).

Expression and activity of proliferation and apoptosis regulators in rat mammary gland overexpressing RGN

In order to determine the influence of RGN in regulating cell death and proliferation, the expression of target regulators of cell-cycle and apoptosis pathways was evaluated in the mammary gland of Tg-RGN animals comparatively with their Wt counterparts.

The mRNA expression of Myc oncogene and cell-cycle regulator Cdk1 gene was significantly decreased in Tg-RGN animals ($p < 0.01$, Figure IV.5A). In contrast, mRNA expression of tumor suppressor gene p53 was strongly increased in Tg-RGN animals ($p < 0.05$, Figure IV.5A).

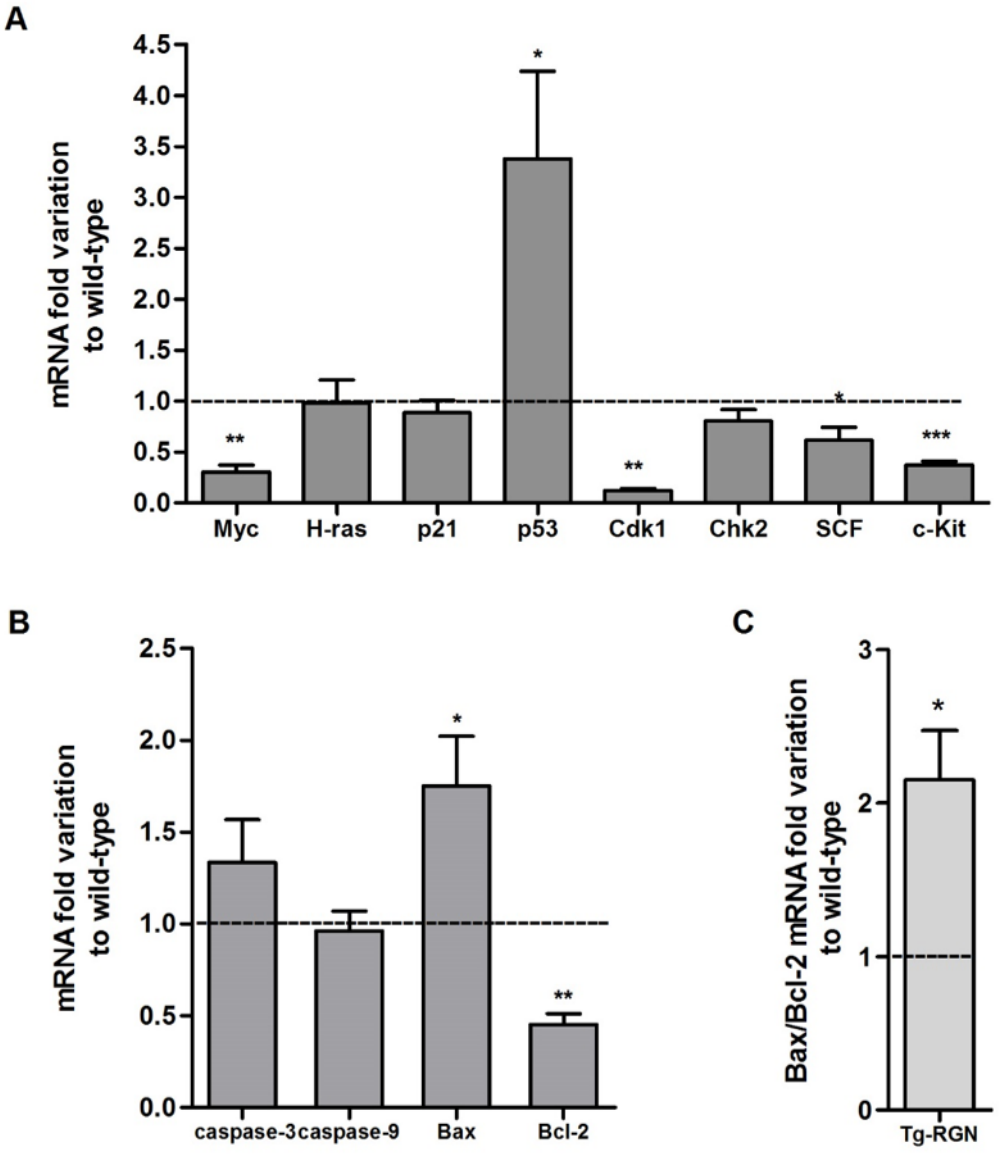

Figure IV.5. mRNA expression of cell cycle (A) and apoptosis (B) regulators in the mammary gland of transgenic rats overexpressing regucalcin (Tg-RGN) comparatively with wild-type (Wt) counterparts determined by qPCR. C) Bax/Bcl-2 mRNA expression ratio of Tg-RGN *vs* Wt. In both groups, animals were 3-month old ($n \geq 5$). Expression was determined after normalization with β_2-Microglobulin and GAPDH housekeeping genes. Results are expressed as fold-variation relatively to the Wt group. Error bars indicate mean ± SEM.* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The stem cell factor (SCF) and its tyrosine kinase receptor, the c-Kit, represent a powerful mechanism in the control of cell survival and proliferation [14]. c-Kit expression has been reported in a variety of human solid tumors including breast carcinoma [15], thus, we decided to analyze the expression of SCF and c-Kit in the mammary gland of Tg-RGN and Wt rats. Both, SCF and c-Kit mRNA expression were diminished in Tg-RGN comparatively to their Wt counterpart (respectively, $p < 0.05$ and $p < 0.01$, Figure IV.5A). mRNA levels of other cell-cycle regulators, namely, p21 and Chk2, as well as the oncogene H-ras were not significantly different between Tg-RGN and Wt (Figure IV.5A).

Considering the expression of apoptosis regulators, the mRNA levels of pro-apoptotic Bax were increased in Tg-RGN relatively to Wt ($p < 0.05$, Figure IV.5B) while anti-apoptotic Bcl-2 was decreased ($p < 0.01$, Figure IV.5B). Consequently, the calculation of Bax/Bcl-2 mRNA ratio revealed an increase ($p < 0.05$, Figure IV.5C) when comparing Tg-RGN and Wt group. Protein levels of Bax and Bcl-2, and the Bax/Bcl-2 protein ratio remained unchanged (Figure IV.6A and B). Also, the mRNA expression of caspase-3 and caspase-9 (Figure IV.5B), as well as the protein levels of caspase-8 (Figure IV.6A) did not display significant differences between the mammary gland of Tg-RGN and Wt animals.

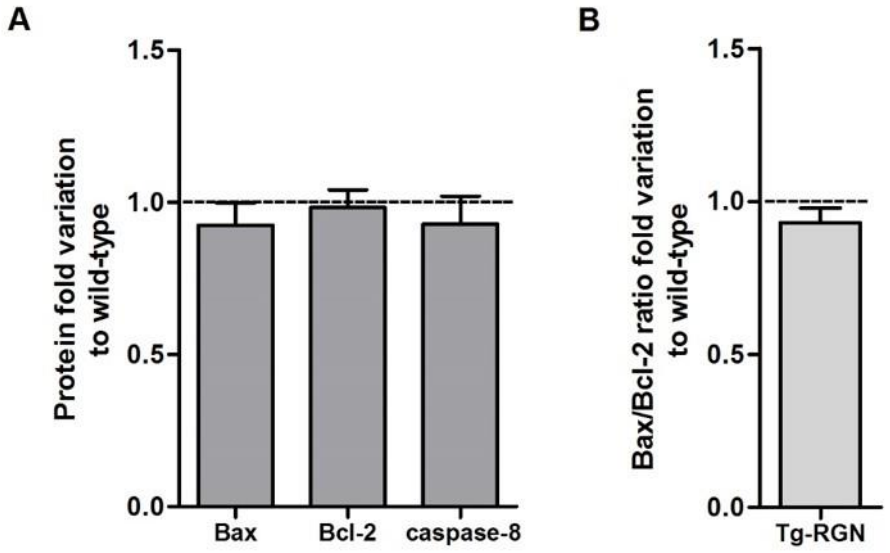

Figure IV.6. Protein expression of apoptosis regulators (A) in the mammary gland of transgenic rats overexpressing regucalcin (Tg-RGN) comparatively with wild-type (Wt) counterparts. B) Bax/Bcl-2 expression ratio of Tg-RGN *vs* Wt. In both groups, animals were 3-month old ($n \geq 8$). Expression was determined after normalization with β-actin. Results are expressed as fold-variation relatively to the Wt group. Error bars indicate mean ± SEM.

The enzymatic activity of the apoptosis effector caspase-3 was 1.4 ± 0.14 fold increased in Tg-RGN comparatively with Wt rats ($p < 0.05$, Figure IV.7).

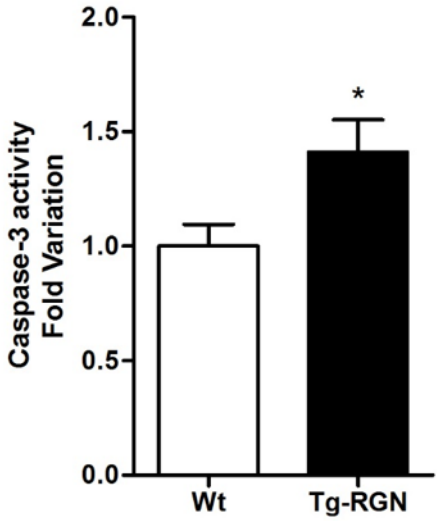

Figure IV.7. Caspase-3 activity in the mammary gland of transgenic rats overexpressing regucalcin (Tg-RGN) relatively to wild-type (Wt) animals. Enzyme activity was measured at 37°C, 4 h after incubation with caspase-3 substrate. Results are presented as a fold-variation to Wt group. Error bars indicate mean ± SEM (n ≥ 6).* $p < 0.05$.

Discussion

In this study we investigated the role of RGN in breast malignancy using both human breast cancer samples and an animal model overexpressing the RGN protein. We found that RGN immunoreactivity in human breast infiltrating ductal carcinoma was negatively correlated with tumor differentiation. The distribution pattern of high RGN immunoreactivity was strongly diminished in poorly differentiated grade III tumors, which mainly displayed low or moderate expression levels of RGN. Accordingly, the large majority of more differentiated tumors of grade I and grade II showed moderate or high RGN immunoexpression. This demonstrated that a decreased expression of RGN accompanies differentiation of breast tumors and suggested that loss of RGN favors tumor progression. The association of RGN down-regulation with tumor development was also suggested in cases of human hepatocellular carcinoma [6]. Moreover, cDNA microarray analysis in rat liver has shown that loss of RGN expression happens in pre-neoplastic foci before the acquisition of a neoplastic phenotype [8]. Therefore, loss of RGN expression may also play a relevant role in the initial steps of breast tumorigenesis. On the other hand, it is liable to suggest that the RGN protein has a protective function against onset and development of breast cancer. We explored this question by analyzing the susceptibility of Tg-RGN animals to develop DMBA-induced mammary gland tumors comparatively with their Wt counterparts. Tg-RGN animals presented a marked resistance to mammary carcinogenesis as shown by the significantly lower incidence of tumors in this group throughout all experiment.
Noteworthy, differences were also found in the histological analysis of mammary gland tumors from the Tg-RGN and Wt. Tg-RGN rats displayed a reduced incidence of invasive tumors

comparatively to Wt (3.8 % *vs* 45.4 %). In addition, the histological identification of pre-cancerous lesions was only possible in the Tg-RGN group, which indicates that RGN overexpression delays tumor onset and progression.

Ki67 nuclear staining is one of the most widespread methods to evaluate cell proliferation in cancer samples [16] and we used it to evaluate proliferation indexes in non-invasive tumors of both Tg-RGN and Wt. Ki67 proliferation index was significantly lower in tumors of Tg-RGN animals, which sustains the less aggressive phenotype and the reduced incidence of invasive tumor forms observed in these animals.

Cancer arises upon deregulation of basic biological processes and unbalanced cell death and proliferation is a known hallmark in the onset of tumorigenesis [17]. In order to investigate the influence of RGN in cell proliferation and apoptotic pathways we analyzed the expression and activity of several cell-cycle and apoptosis regulators in the mammary gland of both Tg-RGN and Wt. The results obtained demonstrated a decreased expression of the cell-cycle regulator Cdk1 in Tg-RGN comparatively with Wt. Cdk1 is a cyclin-dependent kinase essential for the G2/M phase transition and active progression into mitosis [18], which is negatively regulated by other pivotal cell-cycle regulators such as the tumor suppressor p53 [19]. Tg-RGN rats displayed increased levels of p53, which follows previous *in vitro* findings in hepatoma and kidney cells [20, 21], and associates RGN with repression of the cell-cycle at the transition of G2- to M-phase. Also, diminished levels of Myc oncogene were found in the mammary glands of Tg-RGN rats. Myc is a transcription factor overexpressed in human cancer cases and associated with tumor invasiveness and poor prognosis [22], which was also found to be suppressed in hepatoma cells overexpressing RGN [20].

SCF is a membrane-bound cytokine and its binding to c-Kit activates the intrinsic tyrosine kinase activity of receptor [14]. The SCF/c-Kit signaling pathways have been implicated in the regulation of cell proliferation and tumorigenesis in different types of human tissues [14] and recent findings strongly linked c-Kit with breast carcinogenesis. Both *in vivo* and *in vitro* approaches demonstrated that c-Kit levels increase after loss of BRCA1 expression, and that c-Kit contributes for growth and survival of progenitor cells, which seem to be in the origin of BRCA1-associated tumorigenesis [23]. Therefore, the down-regulated expression of both SCF and c-Kit in the mammary gland of Tg-RGN may be looked as an additional mechanism contributing to counteract cell proliferation.

The process of apoptosis involves two major pathways, the extrinsic pathway, mediated by death receptors at plasma membrane, and the mitochondrial or intrinsic pathway, dependent of signals targeting mitochondria [24]. Independently of the activated pathway, the apoptotic process underlies on the action of pro-apoptotic members of the Bcl-2 family and converges to the activation of the apoptosis effector caspase-3, via initiator caspase-8 and caspase-9, respectively, in the extrinsic and mitochondrial pathways [25, 26]. Although the Bax/Bcl-2 protein ratio and expression levels of caspases did not display differences in the mammary gland of Tg-RGN rats relatively to their Wt counterparts, the enzymatic activity of caspase-3 was clearly increased in Tg-RGN. The measurement of caspase-3 activity has been considered

a powerful indicator of the activation of apoptotic pathways [26], which indicates a commitment with apoptosis in Tg-RGN animals.

Besides its role controlling cell proliferation, p53 also acts as a pro-apoptotic factor [27] with a documented cross-talk with Bax/Bcl-2 proteins, which when deregulated is linked with tumor development [28]. p53 induces the transcription of Bax and other mitochondrial pro-apoptotic factors overcoming the anti-apoptotic effects of Bcl-2 [28]. Therefore, the increased expression of p53 observed in the mammary gland of Tg-RGN may explain the increased mRNA expression of Bax and the increased ratio of Bax/Bcl-2. Moreover, p53 may directly interact with Bcl-2 antagonizing its functions and leading to cell death [28]. Although the regulation of apoptosis involves complex and intricate networks, this finding also explains the higher rates of apoptosis in the mammary gland of Tg-RGN, as indicated by the increased enzymatic activity of caspase-3.

In conclusion, we showed that progression of human breast cancer is associated with loss of RGN immunoreactivity. Moreover, RGN overexpression protected from carcinogen-induced mammary gland tumor development, which seems to be related with restricted proliferative activity and fostered apoptotic response. Altogether, our findings represent the first evidence of the protective role of RGN in the development of mammary gland carcinogenesis. Ultimately the present data may also contribute to developing new therapeutic approaches for human breast cancer.

Conflict of interests

The authors declare that they have no conflict of interest.

Funding

This work was supported by Portuguese Foundation for Science and Technology (FCT) under Program COMPETE (PEst-C/SAU/UI0709/2011 and PEst-OE/SAU/UI0709/2014). Ricardo Marques (SFRH/BD/66875/2009) and Cátia V. Vaz (SFRH/BD/70316/2010) are recipient of fellowships from FCT.

References

1. Yamaguchi M (2000) Role of regucalcin in calcium signaling. Life Sci 66(19):1769-1780

2. Yamaguchi M (2011) Regucalcin and cell regulation: role as a suppressor protein in signal transduction. Mol Cell Biochem 353(1-2):101-137

3. Marques R, Maia CJ, Vaz C, Correia S, Socorro S (2014) The diverse roles of calcium-binding protein regucalcin in cell biology: from tissue expression and signalling to disease. Cell Mol Life Sci 71(1):93-111

4. Maia C, Santos C, Schmitt F, Socorro S (2009) Regucalcin is under-expressed in human breast and prostate cancers: Effect of sex steroid hormones. J Cell Biochem 107(4):667-676

5. Laurentino SS, Correia S, Cavaco JE, Oliveira PF, Rato L, Sousa M, Barros A, Socorro S (2011) Regucalcin is broadly expressed in male reproductive tissues and is a new androgen-target gene in mammalian testis. Reproduction 142(3):447-456

6. Zhou SF, Mo FR, Bin YH, Hou GQ, Xie XX, Luo GR (2011) Serum immunoreactivity of SMP30 and its tissues expression in hepatocellular carcinoma. Clin Biochem 44(4):331-336

7. Kim J, Kim SH, Lee SU, Ha GH, Kang DG, Ha NY, Ahn JS, Cho HY, Kang SJ, Lee YJ et al. (2002) Proteome analysis of human liver tumor tissue by two-dimensional gel electrophoresis and matrix assisted laser desorption/ionization-mass spectrometry for identification of disease-related proteins. Electrophoresis 23(24):4142-4156

8. Suzuki S, Asamoto M, Tsujimura K, Shirai T (2004) Specific differences in gene expression profile revealed by cDNA microarray analysis of glutathione S-transferase placental form (GST-P) immunohistochemically positive rat liver foci and surrounding tissue. Carcinogenesis 25(3):439-443

9. Yamaguchi M, Misawa H, Uchiyama S, Morooka Y, Tsurusaki Y (2002) Role of endogenous regucalcin in bone metabolism: bone loss is induced in regucalcin transgenic rats. Int J Mol Med 10(4):377-383

10. Ricardo SA, Milanezi F, Carvalho ST, Leitao DR, Schmitt FC (2007) HER2 evaluation using the novel rabbit monoclonal antibody SP3 and CISH in tissue microarrays of invasive breast carcinomas. J Clin Pathol 60(9):1001-1005

11. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3(7):RESEARCH0034

12. Huggins C, Grand LC, Brillantes FP (1961) Mammary cancer induced by a single feeding of polymucular hydrocarbons, and its suppression. Nature 189:204-207

13. Russo J, Russo IH (2000) Atlas and histologic classification of tumors of the rat mammary gland. J Mammary Gland Biol Neoplasia 5(2):187-200

14. Liang J, Wu YL, Chen BJ, Zhang W, Tanaka Y, Sugiyama H (2013) The C-kit receptor-mediated signal transduction and tumor-related diseases. Int J Biol Sci 9(5):435-443

15. Kashiwagi S, Yashiro M, Takashima T, Aomatsu N, Kawajiri H, Ogawa Y, Onoda N, Ishikawa T, Wakasa K, Hirakawa K (2013) c-Kit expression as a prognostic molecular marker in patients with basal-like breast cancer. Brit J Surg 100(4):490-496

16. Dowsett M, Nielsen TO, A'Hern R, Bartlett J, Coombes RC, Cuzick J, Ellis M, Henry NL, Hugh JC, Lively T et al. (2011) Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in Breast Cancer working group. J Natl Cancer Inst 103(22):1656-1664

17. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5):646-674

18. Wang Y, Ji P, Liu J, Broaddus RR, Xue F, Zhang W (2009) Centrosome-associated regulators of the G(2)/M checkpoint as targets for cancer therapy. Mol Cancer 8:8

19. Yun J, Chae HD, Choy HE, Chung J, Yoo HS, Han MH, Shin DY (1999) p53 negatively regulates cdc2 transcription via the CCAAT-binding NF-Y transcription factor. J Biol Chem 274(42):29677-29682

20. Tsurusaki Y, Yamaguchi M (2003) Overexpression of regucalcin modulates tumor-related gene expression in cloned rat hepatoma H4-II-E cells. J Cell Biochem 90(3):619-626

21. Nakagawa T, Sawada N, Yamaguchi M (2005) Overexpression of regucalcin suppresses cell proliferation of cloned normal rat kidney proximal tubular epithelial NRK52E cells. Int J Mol Med 16(4):637-643

22. Lin CY, Loven J, Rahl PB, Paranal RM, Burge CB, Bradner JE, Lee TI, Young RA (2012) Transcriptional amplification in tumor cells with elevated c-Myc. Cell 151(1):56-67

23. Regan JL, Kendrick H, Magnay FA, Vafaizadeh V, Groner B, Smalley MJ (2012) c-Kit is required for growth and survival of the cells of origin of Brca1-mutation-associated breast cancer. Oncogene 31(7):869-883

24. Lawen A (2003) Apoptosis-an introduction. Bioessays 25(9):888-896

25. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 94(4):481-490

26. Elmore S (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol 35(4):495-516

27. Schmitt CA, Fridman JS, Yang M, Baranov E, Hoffman RM, Lowe SW (2002) Dissecting p53 tumor suppressor functions in vivo. Cancer Cell 1(3):289-298

28. Hemann MT, Lowe SW (2006) The p53-Bcl-2 connection. Cell Death Differ 13(8):1256-1259

Chapter V

Glycolytic metabolism in the mammary gland of transgenic rats overexpressing calcium-binding protein regucalcin: new clues for the protective role against tumor development

Chapter submitted for publication: Ricardo Marques, Cátia V. Vaz, Sílvia Socorro Glycolytic metabolism in the mammary gland of transgenic rats overexpressing calcium-binding protein regucalcin: new clues for the protective role against tumor development. Cellular Oncology

Glycolytic metabolism in the mammary gland of transgenic rats overexpressing calcium-binding protein regucalcin: new clues for the protective role against tumor development

Abstract

Purpose The metabolic reprogramming with enhanced glycolytic metabolism is a common feature of neoplastic transformation. Regucalcin (RGN) is a protein with suppressive effects on mammary gland cell proliferation, and for which an anti-tumor activity has been indicated, but the mechanisms underlying these actions need clarification. This study investigated the glycolytic metabolism in the mammary gland (and chemically-induced tumors) of transgenic rats overexpressing RGN (Tg-RGN) and wild-type (Wt) controls.

Methods The glucose and lactate content in the mammary gland and chemically-induced tumors of Tg-RGN and Wt animals were determined spectrophotometrically. Real-time PCR/Western blot and biochemical assays were used to evaluate, respectively, the expression and activity of glycolytic-associated genes.

Results The glycolytic metabolism was repressed in the mammary gland of Tg-RGN animals comparatively to Wt group, as indicated by the reduced levels of glucose, the decreased expression of glucose transporter, GLUT3 and the diminished activity of phosphofructokinase 1. However, lactate levels and the activity of lactate dehydrogenase (LDH) were enhanced in the mammary gland of Tg-RGN rats. A metabolic switch was observed in the mammary gland tumors overexpressing RGN, which showed reduced activity of LDH comparatively with controls.

Conclusions The suppressed glycolytic flux found in the mammary gland of Tg-RGN animals supports the role of RGN counteracting tumor development. Moreover, the diminished activity of LDH in mammary gland tumors of Tg-RGN rats may be associated with the retardation of tumor progression. The present findings provide new clues to the clarification of the mechanisms by which RGN confers protection against mammary gland carcinogenesis.

Keywords: Glycolysis / Glucose / GLUTs / LDH / regucalcin / mammary gland

Introduction

The calcium (Ca^{2+})-binding protein regucalcin (RGN) is a multifunctional protein that regulates a myriad of cellular functions. Besides its classical role in the maintenance of intracellular Ca^{2+}-homeostasis, RGN has been shown to be involved in the regulation of cell proliferation, apoptosis, metabolism and oxidative stress levels [1], all deregulated processes in the development of human tumors. Interestingly, the down-regulated expression of RGN has been reported in several types of human cancer and cell lines, including breast cancer [2-4]. Furthermore, RGN overexpression *in vivo* conferred protection against the development of

carcinogen-induced mammary gland tumors [2]. Although this anti-tumor activity has been linked with suppressive effects in cell proliferation, the mechanisms by which RGN may counteract tumor development need clarification.

The metabolic reprogramming has become an emergent and widely recognized hallmark in cancer cell transformation [5]. Metabolic adaptation of cancer cells is usually associated with enhanced glycolysis, as well as, with augmented conversion of pyruvate to lactate [6]. On the other hand, RGN has been associated with the control of cell metabolism, being capable of influencing glycolysis, oxidative phosphorylation, glycogenolysis and gluconeogenesis [1, 7].

The present study aims to determine the influence of RGN on the glycolytic metabolism of mammary gland tissues. For this purpose, glucose and lactate contents, as well as, the expression and activity of glycolytic metabolism-associated genes in the mammary gland of transgenic rats overexpressing RGN (Tg-RGN) and wild-type (Wt) controls were investigated. The metabolic features of chemically-induced mammary gland tumors of Tg-RGN and Wt animals were also evaluated.

Materials and methods

Animals

Three-month old Sprague Dawley Wt and Tg-RGN female rats (*Rattus norvegicus*) were obtained from Charles River (Barcelona, Spain) and Japan SLC (Hamamatsu, Japan), respectively. Animals were handled in compliance with the European Union rules for the care and handling of laboratory animals (Directive number 2010/63/EU). Blood was collected by heart puncture under anesthesia (Clorketam 1000, Vetoquinol, Lure, France), mammary glands were dissected and animals were euthanized by cervical dislocation. All samples were immediately frozen in liquid nitrogen and stored at -80 °C until further use. Serum and mammary glands were used for metabolites extraction. In addition, mammary glands were used for RNA and protein extraction.

Mammary gland tumors

Mammary gland tumors were chemically-induced by the administration of the carcinogen 7,12-dimethylbenz[α]anthracene (DMBA, 20 mg/Kg in 0.2 ml sesame oil), and the characterization of tumor incidence in Tg-RGN *vs.* Wt, as well as the tumor histological classification were previously published [2]. Benign mammary gland tumors of Tg-RGN and Wt rats were dissected and used for metabolites and protein extraction.

Glucose and lactate assays

Serum glucose and lactate concentration in Tg-RGN and Wt animals were determined using commercial Kits (Spinreact, Girona, Spain). The assay conditions were established, after verification of linearity, to a final volume of 200 µL, in 96 wells flat bottom microplates. The

absorbance was measured at 505 nm using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, USA).

To determine the glucose and lactate content in the mammary gland and mammary gland tumors of Wt and Tg-RGN rats, 100 mg of tissue were powdered in of liquid nitrogen-cooled mortar. Polar and apolar metabolites were extracted simultaneously using a methanol/chloroform/water extraction [8]. Briefly, 1.5 ml of cold-methanol plus 500 µL cold-water were added to the powdered samples. After homogenization, 1 mL of chloroform was added, samples were vortexed, and sonicated for 15 min at 4 °C. Additional 500 µl chloroform plus 1 mL of ice-cold water were added to each sample and vortexed. Samples were frozen in liquid nitrogen, mixed again for 60 s and centrifuged at 2,300 g for 20 min at 4 ºC. The upper methanol layer containing water-soluble metabolites was collected, lyophilized and subsequently diluted in sterile water for quantification of glucose and lactate using commercial kits (Spinreact).

Real-time PCR (qPCR)

Total RNA was extracted from rat mammary gland using TRI reagent (Ambion, California, USA) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed using the First-Strand cDNA synthesis Kit (NZYtech, Lisboa, Portugal) in a final volume of 20 µL, following the manufacturer's instructions.

Primer sequences and cycling conditions for the amplification of glucose transporters (GLUTs, namely, *GLUT1* and *GLUT3*), *phosphofructokinase 1* (*PFK1*), *monocarboxylate transporter* (*MCT4*) and housekeeping genes are provided in Table 1. Rat *β_2-microglobulin* (β_2M) and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH)* were used as internal controls for normalization of expression of target genes. qPCR reactions were carried out in the CFX Connect™ Real-Time PCR Detection System (Bio-Rad) and the efficiency and specificity of amplification were determined for all primer sets as previously described [9]. 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 ºC, followed by 35 cycles of 95 ºC for 10 s, a specific annealing temperature for each primer set for 30 s and 72 ºC for 10 s. Samples were run in triplicate in each qPCR assay. Normalized expression values were calculated following the mathematical model proposed by Vandesompele et al. [10].

Table V.1. Oligonucleotide sequences, amplicon size and annealing temperature in qPCR

Gene	Sequence (5'-3')	Amplicon size bp)	AT (° C)
GLUT1	Sense: CAG CCA CTC TCC TAT CTC Antisense: AGC CAT TGT TCA GTA TTC G	155	50
GLUT3	Sense: GGT GTT CGC TGT TAC TGT TG Antisense: CTC GCT TGG TAG GTC TTC C	138	56
PFK1	Sense: CGC ACC TTG AGC ATA GAC Antisense: AGA GCA GCA CAG TAG ACC	172	56
MCT4	Sense: ACA CTT AGG AGA CAA CAC Antisense: GGC AAT ATA GGA GAC TGG	131	50
β_2M	Sense: ATGGTGGGTATGGGTCAG Antisense: CAATGCCGTGTTCAATGG	149	56
GAPDH	Sense: GTTCAACGGCACAGTCAA Antisense: CTCAGCACCAGCATCACC	177	56

Western Blot (WB)

Total proteins were extracted using RIPA buffer (1 % Nonidet-P40 substitute, 0.5 % Na-deoxycholate, 0.1 % SDS in PBS) supplemented with protease inhibitors cocktail (Sigma-Aldrich) plus PMSF, and protein concentration was determined by the Bradford micro-assay (Bio-Rad). A total of 60 µg of each protein sample were resolved in a 12 % handcast gel by SDS-PAGE. 10 % TGX stain-free™ gels (Bio-Rad) were used for protein analysis in mammary gland tumors to allow total protein determination and normalization avoiding variability in the expression of housekeeping genes proper of tumor samples. Proteins were electrotransferred to a PVDF membrane (GE Healthcare, Buckinghamshire, UK) and membranes were incubated overnight at 4 ºC with, rabbit anti-GLUT1 (1:1000, CBL242, Millipore, CA, USA), rabbit anti-GLUT3 (1:2500, sc-30107, Santa Cruz Biotechnology (SCBT), CA, USA), rabbit anti-PFK1 (1:1000, sc-67028, SCBT), rabbit anti-Lactate dehydrogenase (LDH 1:3000, Ab52488, Abcam, Cambridge, United Kingdom), or rabbit anti-MCT4 (1:1000, sc-50329, SCBT) primary antibodies. Protein expression was normalized using a mouse anti-ß-actin (1:40000, A5441, Sigma-Aldrich) antibody or in case of tumor samples with the total protein quantified using the stain-free™ gels. Goat anti-rabbit IgG-HRP (1:40000, sc-2004, SCBT) or goat anti-mouse IgG-HRP (1:40000, sc-2005, SCBT) were used as secondary antibodies. Membranes were incubated with Clarity™ Western ECL substrate (Bio-Rad) for 5 min and immunoreactive bands were visualized using the ChemiDoc™ MP Imaging System (Bio-Rad). Band densities were obtained using the Image Lab 5.1 Software (Bio-Rad) and normalized to the corresponding ß-actin density or the total protein in stain-free™ gels. Results are presented as fold-variation relatively to the Wt group.

LDH and PFK enzymatic activities

LDH activity was measured 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 decrease of NADH concentration that was measured spectrophotometrically at 340 nm at constant temperature (37°C) using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). PFK activity was determined as previously described [11]. Enzymatic activities were expressed as fold-variation relatively to the Wt group.

Statistical analysis

The statistical significance of differences between experimental groups was assessed by the two-tailed Student´s t-test (GraphPad Software, San Diego, CA, USA). Significant differences were considered when $p < 0.05$ and experimental data are shown as mean ± SEM.

Results

Diminished glucose and elevated lactate levels were found in the mammary gland of Tg-RGN rats

Glucose levels were significantly higher in the serum of Tg-RGN animals (20.13 ± 0.45 *vs.* 17.39 ± 0.94 mmol/L in the Wt group, Figure V.1A). Contrarily, the mammary gland of Tg-RGN rats showed diminished concentration of glucose comparatively with their Wt counterparts (respectively, 78.63 ± 3.84 and 101.5 ± 6.7 nmol/g, $p < 0.01$, Figure V.1B). Lactate content was found to be significantly enhanced both in the serum and mammary gland of Tg-RGN animals comparatively with their Wt counterparts (1.88 ± 0.16 *vs.* 0.99 ± 0.12 mmol/L and 57.25 ± 4.33 *vs.* 31.14 ± 4.05 nmol/g, respectively, Figure V.1C, D).

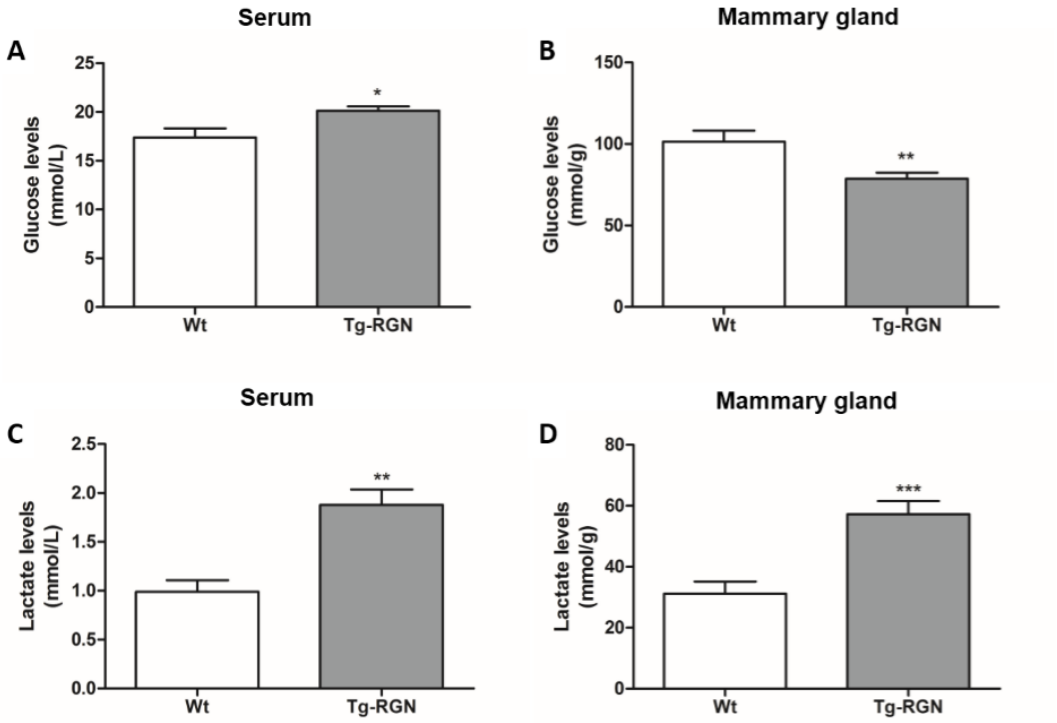

Figure V.1. Concentration of glucose and lactate in serum (**A,C**) and mammary gland (**B,D**) of transgenic rats overexpressing RGN (Tg-RGN) comparatively with their wild-type (Wt) counterparts. Error bars indicate mean ± SEM (n ≥ 5). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relatively to the Wt group.

Mammary gland of Tg-RGN animals presented decreased expression of GLUT3 and reduced activity of PFK

Glycolysis is the metabolic pathway used by cells to obtain energy from glucose, and intimately depends on the uptake of glucose from the extracellular space. This task is mediated by several members of the GLUTs family, which also transport other hexoses [12]. GLUT1 and GLUT3 proteins are devoted to glucose transport [12], and both have been described in mammary gland cells [12-14]. To evaluate the influence of RGN on the regulation of glucose transport in rat mammary gland, the mRNA and protein expression of GLUT1 and GLUT3 was determined by using qPCR and WB, respectively. The mammary gland of Tg-RGN animals displayed significantly lower mRNA and protein expression of GLUT3 (respectively, 0.43 ± 0.11 and 0.74 ± 0.07 fold-variation relatively to Wt, Figure V.2A, B), whereas no significant changes were observed in the expression of GLUT1 in comparison with the Wt control group.

PFK1, an enzyme that controls the glycolytic pathway by catalyzing the first irreversible step of glycolysis with conversion of fructose-6-phosphate to fructose-1-6-bis-phosphate, was evaluated in what concern its expression and activity [15]. The mRNA and protein expression of PFK were significantly decreased in the mammary gland of Tg-RGN rats comparatively with the Wt group (respectively, 0.76 ± 0.03 and 0.64 ± 0.08 fold-variation, Figure V.2A, B). Accordingly, the activity of PFK was also diminished in the mammary gland of Tg-RGN rats (0.57 ± 0.08 fold-variation relatively to Wt control group, $p < 0.05$, Figure V.2D).

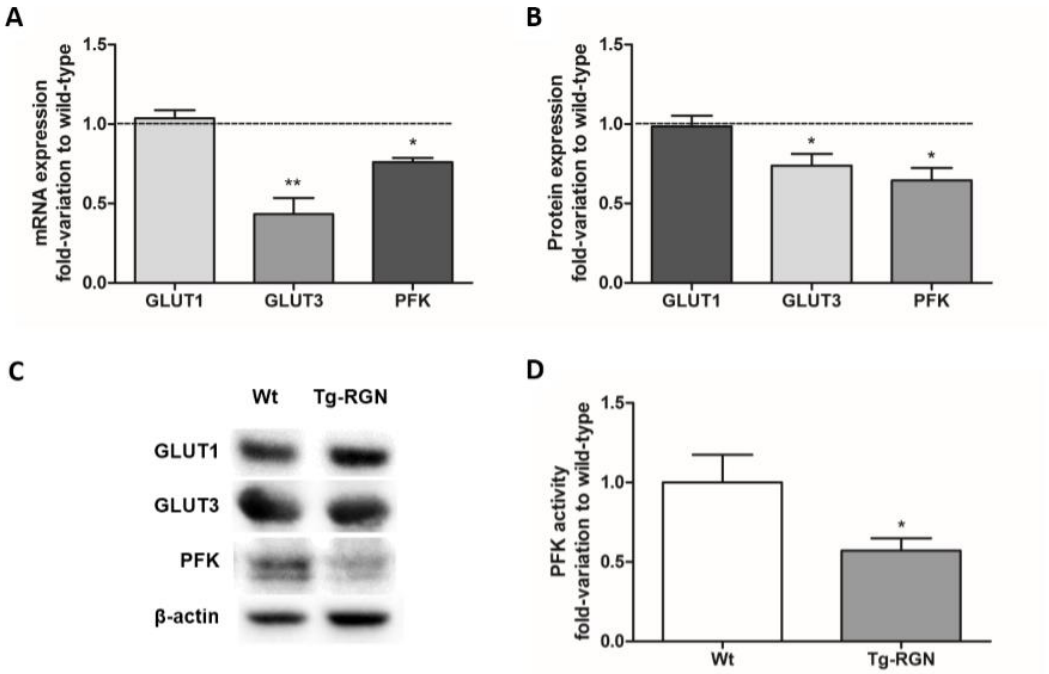

Figure V.2. GLUT1, GLUT3 and PFK expression (activity) in the mammary gland of transgenic rats overexpressing RGN (Tg-RGN) comparatively with their wild-type (Wt) counterparts. **A**) mRNA expression was determined by qPCR after normalization with GADPH and $ß_2$-microglobulin housekeeping genes. **B**) Protein levels determined by WB analysis after normalization with ß-actin. **C**) Representative immunoblots showing protein levels in Tg-RGN and Wt animals. **D**) PFK activity. Results are expressed as fold-variation relatively to the Wt group, represented by the dashed line. Error bars indicate mean ± SEM (n ≥ 5). * $p < 0.05$; ** $p < 0.01$.

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

MCT4 is a monocarboxylate transporter mainly associated with the regulation of cellular lactate efflux [16, 17]. The protein expression of MCT4 was significantly decreased in the mammary gland of Tg-RGN animals relatively to Wt (0.69 ± 0.09 fold-variation, Figure V.3A) though no changes were found on mRNA levels between Tg-RGN and Wt (Figure V.3C).

The LDH enzyme catalyzes the reversible conversion of pyruvate into lactate, with simultaneous oxidation of NADH to NAD^+ [18]. Although LDH protein expression was diminished in the mammary gland of Tg-RGN animals (0.69 ± 0.05 fold-variation relatively to Wt group, $p < 0.01$, Figure V.3A), the enzymatic activity of LDH was significantly enhanced (1.26 ± 0.07 fold-variation relatively to Wt group, Figure V.3D).

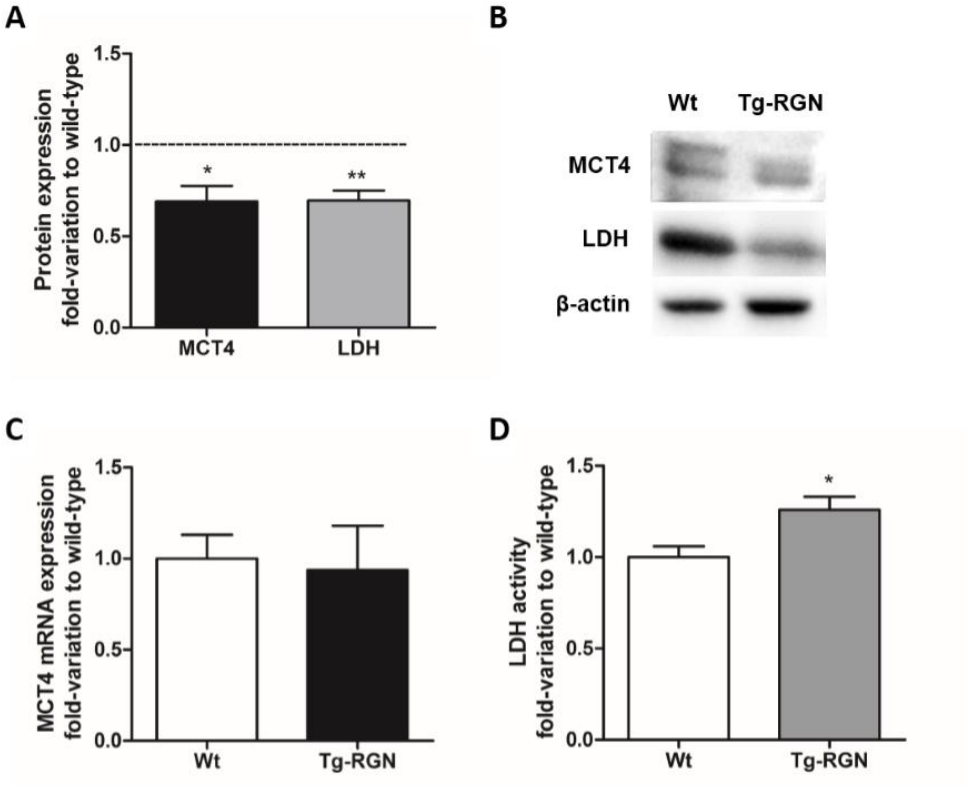

Figure V.3. Expression of MCT4 and LDH, and LDH activity in the mammary gland of transgenic rats overexpressing RGN (Tg-RGN) comparatively with their wild-type (Wt) counterparts. **A**) MCT4 and LDH protein levels determined by WB analysis after normalization with ß-actin. **B**) Representative immunoblots showing protein levels in Tg-RGN and Wt animals. **C**) MCT4 mRNA expression was determined by qPCR after normalization with GADPH and $ß_2M$ housekeeping genes. **D**) LDH activity. Results are expressed as fold-variation relatively to the Wt group, represented by the dashed line. Error bars indicate mean ± SEM (n ≥ 5). * $p < 0.05$; ** $p < 0.01$.

Mammary gland tumors of Tg-RGN animals displayed decreased expression and activity of LDH

The metabolic features of DMBA-induced benign tumors of Tg-RGN and Wt animals were evaluated by determination of the glucose and lactate content and expression (activity) analysis of GLUT1, GLUT3, PFK, LDH and MCT4.

Glucose (Figure V.4A) and lactate (Figure V.4B) measurements showed no differences between benign mammary gland tumors of Tg-RGN and Wt animals. Also, GLUT1 protein expression

remained unaltered comparing both experimental groups (Figure V.4C). However, GLUT3 expression was significantly increased in the mammary gland tumors of Tg-RGN rats (2.36 ± 0.32 fold-variation, Figure V.4C).

PFK protein expression levels were diminished in the mammary gland tumors of Tg-RGN animals (0.65 ± 0.05 fold-variation relatively to the Wt group, Figure V.4C). Notwithstanding, the activity of PFK was highly enhanced in the mammary gland tumors of Tg-RGN comparatively with Wt controls (4.36 ± 0.53 fold-variation, Figure V.4D).

Although no differences were perceived in the lactate content, MCT4 protein expression was shown to be up-regulated in the mammary gland tumors of Tg-RGN animals comparatively with the Wt control group (1.54 ± 0.07 fold-variation, $p < 0.01$, Figure V.4E). Nevertheless, both LDH protein expression and activity were significantly decreased in the mammary gland tumors of Tg-RGN rats (respectively, 0.6 ± 0.07 and 0.6 ± 0.04 fold-variation relatively to the Wt group, Figure V.4E, F).

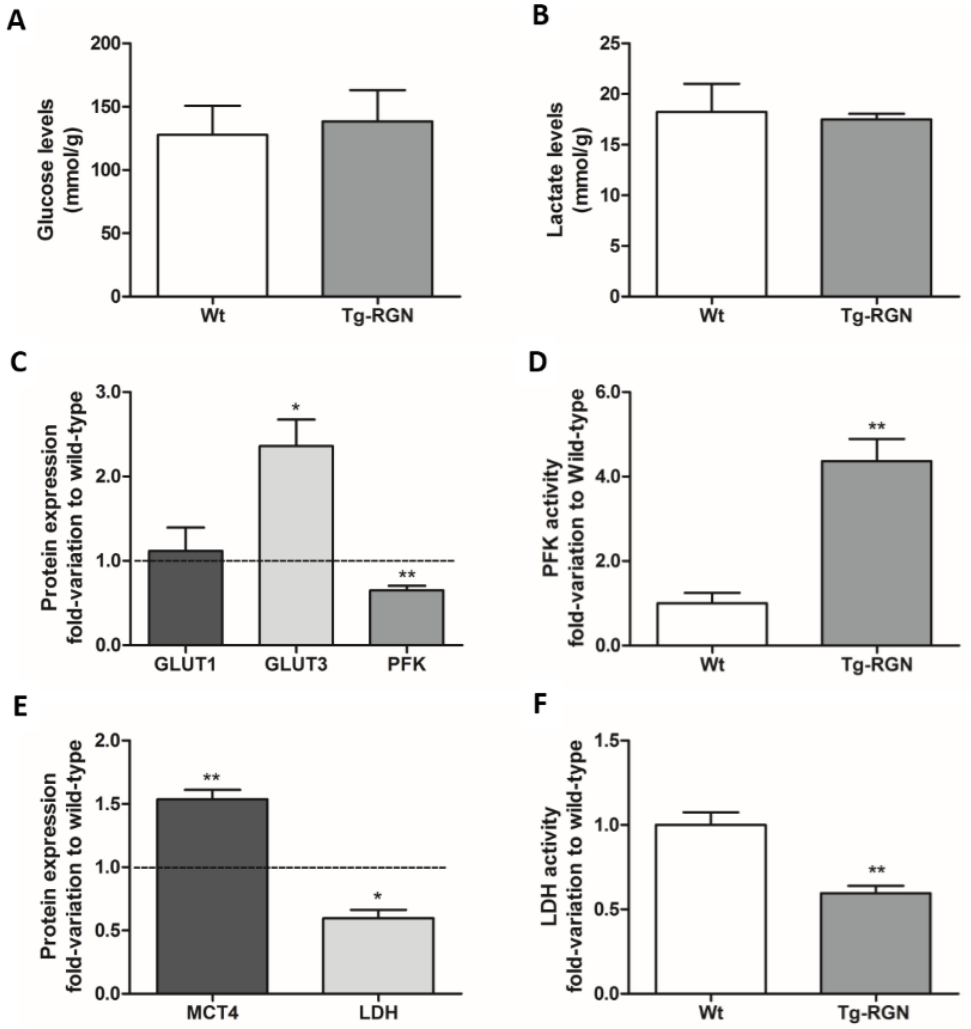

Figure V.4. Glycolytic metabolism in the mammary gland benign tumors of transgenic rats overexpressing RGN (Tg-RGN) comparatively with their wild-type (Wt) counterparts. Glucose (**A**) and lactate (**B**) were determined in tumor tissues, together with the protein expression of GLUT1, GLUT3 and PFK (**C**), and MCT4 and LDH (**E**). The activity of PFK (**D**) and LDH (**F**) was also determined. Error bars indicate mean ± SEM ($n \geq 3$).

Discussion

The present study compared the glycolytic metabolism in the mammary gland of Wt and Tg-RGN rats to determine the influence of RGN in the metabolic phenotype. A decreased glucose content was found in the mammary gland of Tg-RGN rats, despite these animals presented a slight increase in serum glucose levels. This contrasts with a previous report measuring serum glucose in Tg-RGN rats, which showed no significant differences comparatively with Wt controls [19]. Nevertheless, the decreased levels of this hexose even under elevated serum levels suggest that the mammary gland tissues of Tg-RGN animals display a diminished uptake of glucose.

The import of glucose into the cell is mediated by several GLUT family members [12]. However, the GLUT3 has the highest affinity and the maximum turnover for glucose [20, 21]. Herein, we showed that the expression of GLUT3 was decreased in the Tg-RGN group. These findings are indicative of a suppressive role of RGN in the regulation of GLUTs expression, and, thus, of a compromised uptake of glucose, which is concordant with the diminished glucose levels found in the mammary gland of Tg-RGN animals. Moreover, although the expression of GLUT3 has been described in the mammary gland of human and bovine species, for the best of our knowledge this is the first report demonstrating the expression of this GLUT in rat mammary tissue [12, 14]. Noteworthy, the overexpression of RGN enhanced the expression of GLUT2 in rat hepatoma H4-II-E cells [22], which indicates a broad action of RGN as a modulator of GLUTs expression.

Glycogen, a polymer of glucose, is the primary short-term energy storage molecule, which is primarily synthesized in the liver and muscle tissues [23]. Therefore, it cannot be excluded the possibility of glucose being stored as glycogen since the majority of tissues possess this ability, including the mammary gland [24]. However, this hypothesis seems to be quite unlikely, because reports exist demonstrating the reduction of glycogen content in the liver of Tg-RGN rats [25]. This further supports that the decreased glucose content observed in the mammary gland of Tg-RGN animals should be linked to the restrained uptake of glucose, as a consequence of the diminished expression of GLUT3, and not related to the storage of glucose as glycogen.

PFK catalyzes an irreversible reaction and a rate-limiting step of glycolysis playing a preponderant role in the metabolic flux [15]. Ours results demonstrated a decreased expression of PFK in the mammary gland of Tg-RGN animals, which was accompanied by reduced PFK activity. Previous studies also described that in the liver RGN inhibited the activity of pyruvate kinase, an enzyme involved in the last step of glycolysis [26]. Moreover, other metabolism-related enzymes, such as succinate dehydrogenase [27], glucose-6-phosphatase [28] and fructose 1,6-diphosphatase [29], and glycogen phosphorylase a [30] also seem to be negatively regulated by RGN.

Globally, the obtained results indicate that RGN exert a set of restrictive actions in the glycolytic pathway associated with blockage of glucose uptake, in consequence of the diminished expression of GLUT3, and with the slowdown of glycolytic flux due to the decreased activity of PFK.

In cancer cells, the main metabolite of glycolysis, pyruvate, is not directed to the tricarboxylic acid cycle, and it is mainly converted into lactate by LDH in the cytosol [15]. The lactate produced is exported together with a proton by a mechanism of facilitated diffusion mediated by MCT4 [16, 17]. This MCT has been associated with the neoplastic transformation [31], and the overproduction of lactate is a common feature of cancer cells [15, 32, 31]. The efflux of lactate leads to the acidification of the microenvironment, and since cancer cells are resistant to low pH, it provides a survival advantage against attacks of the immune system [6, 32].

MCT4 levels were diminished in the mammary gland of Tg-RGN animals, tough lactate levels were increased both in serum and mammary tissue. The augmented content of lactate is concordant with the enhanced activity of LDH observed in the mammary gland of Tg-RGN rats. Considering the reduction in glycolysis rate indicated by the diminished activity of PFK, it is highly probable that the pyruvate needed for production of lactate came from the metabolization of alanine. Additionally, lactate has been described as a down regulator of glycolytic enzymes, such as hexokinase and PFK [33], which further supports the observed diminished activity of PFK. Therefore, it cannot be excluded that the suppressive effect of RGN over glycolysis also depends on the modulation of lactate handling and its accumulation in the mammary gland. Recently, we have shown that the mammary gland of Tg-RGN rats displayed reduced cell proliferation rates associated with altered expression of cycle regulators and oncogenes [2]. Moreover, Tg-RGN animals were resistant to the development of DMBA-induced mammary gland tumors. After 44 weeks of treatment, only 25.8 % of Tg-RGN rats presented tumors against 100 % in the Wt group [2]. Presently, it is widely accepted that the emergence of a hyperglycolytic phenotype is a common characteristic of cancer cells, which is associated with high proliferation and survival [6]. Thus, it is liable to assume that the RGN actions suppressing the glycolytic metabolism may be a relevant mechanism to counteract cell proliferation and tumor development.

Besides the resistance to tumor development, Tg-RGN rats systematically presented tumors with less aggressive phenotypes and reduced proliferative activity [2]. We decided to evaluate the glycolytic metabolism in benign tumors of Tg-RGN and Wt animals. Interestingly, the obtained results showed that a metabolic switch occurred in mammary gland Tg-RGN rats from healthy tissue to the onset of benign tumors. Contrarily to what was seen in the non-pathological mammary gland, the glycolysis pathway was stimulated in the mammary gland tumors of Tg-RGN rats. These was evident by the increased expression of GLUT3 and the augmented activity of PFK. However, no statistical differences were obtained in glucose and lactate levels in the mammary gland tumors of Tg-RGN and Wt groups. Also in opposition to the results found in healthy tissues, the expression of MCT4 protein was enhanced in tumors of Tg-RGN animals comparatively with Wt controls whereas the expression and activity of LDH were significantly reduced. As mentioned above, cancer cells abnormally use glycolysis as the energy source, and typically show overexpression of LDH and the increased production of lactate [6]. Although lactate levels remained unaltered between Tg-RGN and Wt animals, the diminished expression and activity of LDH suggest the impairment of anaerobic glycolysis (pyruvate to

lactate) in the mammary gland tumors of Tg-RGN rats, which may be regarded as a protective feature for tumor progression into more aggressive phenotypes.

The present findings showed that glycolysis is restrained in the mammary gland of Tg-RGN animals in consequence of diminished glucose uptake and suppressed activity of PFK, which may relevant to slowdown cell proliferation and prevent tumor development. Moreover, the metabolic switch observed during tumor progression in Tg-RGN rats, namely the RGN action limiting the activity of LDH, could be associated with the retardation of tumor progression to more aggressive stages. This work adds additional clues to the clarification of the molecular mechanisms that underpin the RGN actions protecting against mammary gland carcinogenesis.

Acknowledgments

This work was partially supported by Portuguese Foundation for Science and Technology (FCT) under Program COMPETE (UID/Multi/00709/2013 and PEst-OE/SAU/UI0709/2014). Ricardo Marques and Cátia V. Vaz were funded by FCT fellowships (SFRH/BD/66875/2009 and SFRH/BD/70316/2010, respectively).

Conflict of interest

The authors declare no conflict of interest.

References

1. Marques R, Maia CJ, Vaz C, Correia S, Socorro S (2014) The diverse roles of calcium-binding protein regucalcin in cell biology: from tissue expression and signalling to disease. Cell Mol Life Sci 71(1):93-111

2. Marques R, Vaz CV, Maia CJ, Gomes M, Gama A, Alves G, Santos CR, Schmitt F, Socorro S (2015) Histopathological and in vivo evidence of regucalcin as a protective molecule in mammary gland carcinogenesis. Exp Cell Res 330(2):325-335

3. Maia C, Santos C, Schmitt F, Socorro S (2009) Regucalcin is under-expressed in human breast and prostate cancers: Effect of sex steroid hormones. J Cell Biochem 107(4):667-676

4. Murata T, Shinya N, Yamaguchi M (1997) Expression of calcium-binding protein regucalcin mRNA in the cloned human hepatoma cells (HepG2): stimulation by insulin. Mol Cell Biochem 175(1-2):163-168

5. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5):646-674

6. Upadhyay M, Samal J, Kandpal M, Singh OV, Vivekanandan P (2013) The Warburg effect: insights from the past decade. Pharmacol Ther 137(3):318-330

7. Yamaguchi M (2011) Regucalcin and cell regulation: role as a suppressor protein in signal transduction. Mol Cell Biochem 353(1-2):101-137

8. Rudolph MC, McManaman JL, Phang T, Russell T, Kominsky DJ, Serkova NJ, Stein T, Anderson SM, Neville MC (2007) Metabolic regulation in the lactating mammary gland: a lipid synthesizing machine. Physiol Genomics 28(3):323-336

9. Marques R, Peres CG, Vaz CV, Gomes IM, Figueira MI, Cairrao E, Verde I, Maia CJ, Socorro S (2015) 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. Med Oncol 32(9):676

10. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3(7):RESEARCH0034

11. Vaz CV, Alves MG, Marques R, Moreira PI, Oliveira PF, Maia CJ, Socorro S (2012) Androgen-responsive and nonresponsive prostate cancer cells present a distinct glycolytic metabolism profile. Int J Biochem Cell Biol 44(11):2077-2084

12. Zhao FQ (2014) Biology of glucose transport in the mammary gland. J Mammary Gland Biol Neoplasia 19(1):3-17

13. Burnol AF, Leturque A, Loizeau M, Postic C, Girard J (1990) Glucose transporter expression in rat mammary gland. Biochem J 270(1):277-279

14. Krzeslak A, Wojcik-Krowiranda K, Forma E, Jozwiak P, Romanowicz H, Bienkiewicz A, Brys M (2012) Expression of GLUT1 and GLUT3 glucose transporters in endometrial and breast cancers. Pathol Oncol Res 18(3):721-728

15. Lu J, Tan M, Cai Q (2015) The Warburg effect in tumor progression: mitochondrial oxidative metabolism as an anti-metastasis mechanism. Cancer Lett 356:156-164

16. Dimmer KS, Friedrich B, Lang F, Deitmer JW, Broer S (2000) The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. Biochem J 350 (1):219-227

17. Halestrap AP (2013) Monocarboxylic acid transport. Compr Physiol 3(4):1611-1643

18. Everse J, Kaplan NO (1973) Lactate dehydrogenases: structure and function. Ad Enzymol Relat Areas Mol Biol 37:61-133

19. Yamaguchi M, Igarashi A, Uchiyama S, Sawada N (2004) Hyperlipidemia is induced in regucalcin transgenic rats with increasing age. Int J Mol Med 14(4):647-651

20. Mueckler M, Thorens B (2013) The SLC2 (GLUT) family of membrane transporters. Mol Aspects Med 34(2-3):121-138

21. Simpson IA, Dwyer D, Malide D, Moley KH, Travis A, Vannucci SJ (2008) The facilitative glucose transporter GLUT3: 20 years of distinction. Am J Physiol Endocrinol Metab 295(2):E242-253

22. Nakashima C, Yamaguchi M (2006) Overexpression of regucalcin enhances glucose utilization and lipid production in cloned rat hepatoma H4-II-E cells: Involvement of insulin resistance. J Cell Biochem 99(6):1582-1592

23. Zois CE, Favaro E, Harris AL (2014) Glycogen metabolism in cancer. Biochem Pharmacol 92(1):3-11

24. Emerman JT, Bartley JC, Bissell MJ (1980) Interrelationship of glycogen metabolism and lactose synthesis in mammary epithelial cells of mice. Biochem J 192(2):695-702

25. Yamaguchi M, Nakagawa T (2007) Change in lipid components in the adipose and liver tissues of regucalcin transgenic rats with increasing age: suppression of leptin and adiponectin gene expression. Int J Mol Med 20(3):323-328

26. Yamaguchi M, Shibano H (1987) Calcium-binding protein isolated from rat liver cytosol reverses activation of pyruvate kinase by Ca2+. Chem Pharm Bull 35(5):2025-2029

27. Yamaguchi M, Shibano H (1987) Reversible effect of calcium-binding protein on the Ca2+-induced activation of succinate dehydrogenase in rat liver mitochondria. Chem Pharm Bull 35(9):3766-3700

28. Yamaguchi M, Mori S, Suketa Y (1989) Effects of Ca2+ and V5+ on glucose-6-phosphatase activity in rat liver microsomes: the Ca2+ effect is reversed by regucalcin. Chem Pharm Bull 37(2):388-390

29. Yamaguchi M, Yoshida H (1985) Regulatory effect of calcium-binding protein isolated from rat liver cytosol on activation of fructose 1,6-diphosphatase by Ca2+-calmodulin. Chem Pharm Bull 33(10):4489-4493

30. Yamaguchi M, Shibano H (1987) Effect of calcium-binding protein on the activation of phosphorylase a in rat hepatic particulate glycogen by Ca2+. Chem Pharm Bull 35(6):2581-2584

31. Baenke F, Dubuis S, Brault C, Weigelt B, Dankworth B, Griffiths B, Jiang M, Mackay A, Saunders B, Spencer-Dene B et al. (2015) Functional screening identifies MCT4 as a key regulator of breast cancer cell metabolism and survival. J Pathol doi:10.1002/path.4562.

32. Cairns RA (2015) Drivers of the Warburg phenotype. Cancer J 21(2):56-61

33. Leite TC, Coelho RG, Da Silva D, Coelho WS, Marinho-Carvalho MM, Sola-Penna M (2011) Lactate downregulates the glycolytic enzymes hexokinase and phosphofructokinase in diverse tissues from mice. FEBS Lett 585(1):92-98

Chapter VI

5α-dihydrotestosterone regulates the expression of L-type calcium channels and calcium-binding protein regucalcin in human breast cancer cells with suppression of cell growth

Chapter published in: Ricardo Marques, Carina G. Peres, Cátia V. Vaz, Inês M. Gomes, Marília I. Figueira, Elisa Cairrão, Ignacio Verde, Cláudio J. Maia, Sílvia Socorro (2015) 5α-dihydrotestosterone regulates the expression of L-type calcium channels and calcium-binding protein regucalcin in human breast cancer cells with suppression of cell growth. Med Oncol 32:228

5α-dihydrotestosterone regulates the expression of L-type calcium channels and calcium-binding protein regucalcin in human breast cancer cells with suppression of cell growth

Abstract

Androgens have been associated with the development of normal breast, and their role in mammary gland carcinogenesis has also been described. Several studies reported that androgens inhibit breast cancer cell growth, whereas others linked their action with the modulation of calcium (Ca^{2+})-pumps, Ca^{2+} channels and Ca^{2+}-binding proteins. Also, it is known that deregulated Ca^{2+} homeostasis has been implicated in the pathophysiology of breast. The L-type Ca^{2+} channels (LTCCs) were found to be up-regulated in colon, colorectal and prostate cancer but their presence in breast tissues remains uncharacterized. On the other hand, regucalcin (RGN) is a Ca^{2+}-binding protein involved in the control of mammary gland cell proliferation, which has been identified as an androgen target gene in distinct tissues except breast. This study aimed to confirm the expression and activity of LTCCs channels in human breast cancer cells, and investigate the effect of androgens in regulating the expression of α_{1C} subunit ($Ca_v1.2$) of LTCCs channels and Ca^{2+}-binding protein RGN. PCR, Western blot, immunofluorescence and electrophysiological experiments demonstrated the expression and activity of $Ca_v1.2$ subunit in MCF-7 cells. The MCF-7 cells were treated with 1, 10 or 100 nM of 5α-dihydrotestosterone (DHT) for 24 to 72 h. The obtained results showed that 1 nM DHT up-regulated the expression of $Ca_v1.2$ subunit while diminishing RGN protein levels, which was underpinned by reduce cell viability. These findings first confirmed the presence of LTCCs channels in breast cancer cells and opened new perspectives for the development of therapeutic approaches targeting Ca^{2+} signaling.

Keywords: 5α-dihydrotestosterone / $Ca_v1.2$ / DHT / L-type calcium channels / MCF-7 cells / regucalcin.

Introduction

Breast is a hormone-sensitive organ; hence, its normal development and function are largely known to be dependent on the effect of sex steroid hormones, namely estrogens. Although less clearly, the relationship between androgens and breast physiology also has been established [1]. In normal breast, androgens inhibit breast cell growth [1], but their role in the carcinogenesis of mammary gland has remained somewhat controversial. Nevertheless, a substantial amount of studies indicate the growth inhibitory effects of androgens in breast cancer cells [2-4]. Androgenic effects are mediated by the androgen receptor (AR), a transcription factor of nuclear receptor superfamily that regulates gene expression in several biological contexts [5]. Also, the influence of androgens over calcium (Ca^{2+}) homeostasis has been reported, which includes the regulation of Ca^{2+} pumps activity [6], Ca^{2+} influx through several Ca^{2+} channels [7, 8] and gene expression of Ca^{2+} handling proteins [9, 10].

On the other hand, it is widely recognized that Ca^{2+} signaling controls diverse cellular processes depending on its location, concentration, and frequency. Moreover, the deregulation of intracellular Ca^{2+} levels by altered expression and function of Ca^{2+} handling proteins has been implicated in the pathophysiology of mammary gland [11], which renders Ca^{2+} signaling as an important therapeutic target. In this regard, several Ca^{2+} channels seem to play a role in the control of cell proliferation and have been linked to cancer development [12]. Particularly, the L-type Ca^{2+} channels (LTCCs) were found to be up-regulated in colon [13], colorectal [14] and prostate cancer tissues [15]. Moreover, antagonists of LTCCs were shown to inhibit tumor growth *in vivo* [16]. However, the presence of LTCCs in human breast cancer cells remains to be described.

Regucalcin (RGN) is a Ca^{2+}-binding protein that besides its function in Ca^{2+} homeostasis also plays a role in the control of cell proliferation [17]. Moreover, RGN has been identified as an androgen target gene in distinct tissues except breast [18-20], and the loss of RGN expression has been associated with the development of mammary gland tumors [20, 21].

In the present study, we aimed to confirm the expression and activity of LTCCs in human breast cancer cells, and investigate the effect of androgens in regulating the expression of α_{1C} subunit ($Ca_V1.2$) of LTCCs and Ca^{2+}-binding protein RGN.

Material and methods

Cell culture and hormonal stimulation

The human breast cancer epithelial cell line (MCF-7) was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technologies, Paisley, UK) supplemented with 10 % fetal bovine serum (FBS, Biochrom, Berlin, Germany) and 1 % penicillin/streptomycin (Invitrogen, Life Technologies, Karlsruhe, Germany) at pH of 7.4, in an incubator equilibrated with 5 % CO_2 at 37 °C. MCF-7 cells used for electrophysiological experiments were trypsinized with a 0.3 %

trypsin Ca^{2+}-Mg^{2+}-free phosphate solution buffered with EDTA (0.025 %) and kept at 4 °C in FBS-free medium until the beginning of experiments.

For hormonal stimulation experiments, MCF-7 cells were grown up to 60 % confluence and maintained for additional 24 h in phenol red-free DMEM (Gibco) supplemented with 5 % charcoal-stripped FBS (Gibco). Then, cells were exposed to 1, 10 or 100 nM of 5α-dihydrotestosterone (DHT, Sigma- Aldrich, St Louis, USA) for different periods (0, 6, 12, 24, 48, and 72 h). Stimulation with 1 nM DHT was repeated in presence of 1 μM of AR antagonist flutamide (Sigma) or protein synthesis inhibitor cycloheximide (1 μg/mL, Sigma) for 24 h. Inhibitors were added to cell cultures 30 min before hormone stimulation. All assays were carried out in hexaplicate. After treatment, cells were trypsinized and harvested for total RNA and protein extraction.

Rat and human Sertoli cells were cultured as previously described [19] to be used as positive controls for detection of the α_{1C} subunit ($Ca_v1.2$) of LTCC.

RNA extraction and cDNA synthesis

Total RNA was extracted from MCF-7 cells using TRI reagent (Ambion, California, USA) following manufacturer's instructions. The quantity and quality of total RNA was assessed by spectrophotometry at 260 and 280 nm (Pharmacia Biotech, Ultrospec 3000), and agarose gel electrophoresis, respectively. One microgram of total RNA was reverse-transcribed using the First-Strand cDNA synthesis kit (NZYTech, Lisboa, Portugal) in a final volume of 20 μl. Briefly, cDNA synthesis reaction was initiated by a 10 min incubation at 25 °C, followed by 30 min at 50 °C and an inactivation step at 85 °C for 5 min. 1 μL of RNase H was added and incubation proceeded at 37 °C for 20 min. The reaction was stopped by heating at 85 °C for 5 min and synthesized cDNA was stored at -20 °C until further use.

Reverse transcription PCR (RT-PCR)

RT-PCR was performed to confirm the expression of $Ca_v1.2$ channel subunit in human breast cancer MCF-7 cell line. Reactions were carried out using 1 μL of cDNA synthesized from MCF-7 cells in a final volume of 20 μL containing 1x Taq DNA polymerase buffer (Promega, Madison, USA), 500 μM dNTPs (GE Healthcare, Buckinghamshire, UK), 3 mM $MgCl_2$ (Promega), 300 nM specific primers, 1 μL of Platinum Taq DNA polymerase (Promega) and sterile water. Prior to the amplification of $Ca_v1.2$ channel subunit, the integrity of cDNA samples was assessed by amplification of the 18S housekeeping gene. The expression of $Ca_v1.2$ channel subunit in human and rat Sertoli cells was determined and used as positive control. Cycling conditions, primer sequences and corresponding amplicon sizes are indicated in Table VI.1.

Table VI.1. Oligonucleotides sequences, amplicon size and annealing temperature in PCR reactions

Gene	Primer sequence (5'- 3')	AT (ºC)	Amplicon Size (bp)
$Ca_v1.2$ subunit	Sense: AAT GCC TAC CTC CGC AAC GGC TG Antisense: TGA TGC CGT GCT TGG GAC CAT CC	62	469
Regucalcin	Sense: GCA AGT ACA GCG AGT GAC Antisense: TTC CCA TCA TTG AAG CGA TTG	60	177
18S	Sense: AAG ACG AAC CAG AGC GAA AG Antisense: GGC GGG TCA TGG GAA TAA	58	152
GAPDH	Sense: CGC CAG CCG AGC CAC ATC Antisense: CGC CCA ATA CGA CCA AAT CCG	60	75

AT annealing temperature
bp, base pairs

Real-time-PCR (qPCR)

The mRNA expression of $Ca_v1.2$ channel subunit and RGN in DHT-treated MCF-7 cells was determined by qPCR using specific primers (Table VI.1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control to normalize gene expression levels. qPCR reactions were carried out in an iQ5 system (Bio-Rad, Hercules, CA), and efficiency of the amplifications was determined for all primer sets using serial dilutions (1, 1:5 and 1:25) of MCF-7 cDNA. Primer concentration and annealing temperature for each primer set were optimized, and the specificity of amplicons was determined by melting curve analysis. Amplification reactions were carried out using 1 µL of synthesized cDNA in a final volume of 20 µL containing 10 µL Maxima™ SYBR Green/Fluorescein qPCR Master Mix (Fermentas) and 300 nM of sense and antisense primers for each gene. Cycling conditions comprised 5 min denaturation at 95 ºC, followed by 40 cycles at 95 ºC for 10 s, a specific annealing temperature for each gene (Table VI.1) for 30 s, and 72 ºC for 10 s. Samples were run in triplicate in each qPCR assay. Normalized expression values were calculated following the mathematical model proposed by Pfaffl as previously described [18, 21].

Western blot (WB) analysis

Total proteins were extracted from MCF-7 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) supplemented with 1 % protease inhibitors cocktail (Sigma-Aldrich) and 10 % PMSF (Sigma-Aldrich). Protein concentration was determined by the Bradford assay (Bio-Rad) and 60 µg of protein extracts were resolved by SDS-PAGE on 10 % and 12 % gels, respectively, for $Ca_v1.2$ channel subunit and RGN, and electrotransferred to a PVDF membrane (GE Healthcare). Membranes were incubated overnight at 4 ºC with rabbit anti-$Ca_v1.2$ α_{1C} subunit (1:200, C 1603, Sigma-Aldrich), mouse anti-RGN (1:3000, ab67336, Abcam, Cambridge, UK) or rabbit anti-P53 (1:5000, sc-6243, Santa Cruz Biotechnology, Heidelberg, Germany). A mouse anti-ß-actin antibody (1:40000, A5441, Sigma-Aldrich) was used for normalization of protein expression. Goat anti-rabbit IgG-HRP (1:40000, sc-2004, Santa Cruz Biotechnology) or goat anti-mouse IgG-

HRP (1:40000, sc-2005, Santa Cruz Biotechnology) were used as secondary antibodies. Membranes were incubated with Clarity™ Western ECL substrate (Bio-Rad) for 5 min and immunoreactive bands visualized using the ChemiDoc™ MP Imaging System (Bio-Rad). Band densities were obtained according to standard methods using the Image Lab 5.1 Software (Bio-Rad) and normalized by division with the respective ß-actin band density.

Fluorescent immunocytochemistry

MCF-7 cells were fixed with 4 % paraformaldehyde for 10 min at 37 °C and permeabilized with 0.1 %Triton X-100 for 5 min. Then, a blocking step was performed by incubating cells with 20 % FBS in phosphate buffer saline containing 0.1 % tween®-20 (PBST) for 1 h at room temperature. After washing, cells were incubated overnight at 4 °C with anti-$Ca_v1.2$ antibody (1:50, C 1603, Sigma-Aldrich). The Alexa fluor 594-conjugated goat anti-mouse IgG (1:500, Invitrogen) was used as secondary antibody. The specificity of staining was accessed by omission of the primary antibody. Cell nuclei were stained with Hoechst 33342 (10 µg/mL, Invitrogen) for 5 min. Lamellae were mounted onto microscope slides with Dako fluorescent mounting medium (Dako, Glostrup, Denmark) and images acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany).

Electrophysiological experiments

The whole-cell configuration of patch clamp technique was used to analyse the current generated by voltage dependent Ca^{2+} channels (I_{Ca}) in MCF-7 cells. Patch electrodes (2-3 MΩ) were filled with an internal solution containing 58 mM CsCl, 1.2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM EGTA, 15 mM HEPES, 65 mM N-methyl-D-glucamine and 5 mM NaCl, pH 7.4 adjusted with CsOH. The control external solution contained 30 mM $BaCl_2$, 10 mM HEPES, 110 mM tetraethylammonium sodium salt, 10 mM CsCl, 40 mM sucrose and 1 mM 4-aminopyridine, pH 7.3 adjusted with NaOH. Cells were maintained at a holding potential of -80 mV and depolarised every 8 s to +10 mV test potential for 500 ms to measure I_{Ca}. Basal I_{Ca} was measured 3-5 min after patch break to allow the equilibrium between pipette and intracellular solutions. Currents were not compensated for capacitance and leak currents. All experiments were carried out at room temperature (21-25 ºC), and the temperature did not vary by more than 1 ºC in a given experiment. Cells were voltage-clamped using the patch-clamp amplifier Axopatch 200B (Axon instruments, USA), and currents were recorded at a frequency of 10 kHz and filtered at 0.1 kHz using the analog-digital interface Digidata 1322A (Axon Instruments, USA) connected to a compatible computer with the Pclamp8 software (Axon Instruments, USA). The external solution was applied to the proximity of cell membrane by placing the cell at the opening of a 250 µm inner diameter capillary tube with a flow rate of 20 µL/min. I_{Ca} amplitudes were automatically calculated between the maximum current peak and the stable current plateau near the final of the every 8 s pulse. I_{Ca} amplitude was analysed in basal conditions and also in the presence of LTCC inhibitor nifedipine (1 µM), T-type calcium channel (TTCC) inhibitor

mibefradil (10 μM) and nifedipine plus mibefradil dissolved in the external solution. Changes in basal I_{Ca} induced by the different inhibitors were expressed as a percentage of the basal I_{Ca}.

Cell viability assay

MCF-7 cells (4000/well) were grown in 96-well plates and cell viability was determined by the colorimetric CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega) at 24, 48 and 72 h after treatment with DHT. The conversion of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2M) tetrazolium compound to the colored formazan product was detected at 490 nm in the a xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). The cell viability in each experimental condition was estimated by normalizing the absorbance to that of the corresponding control.

Statistical analysis

The statistical significance of differences among experimental groups was assessed by Student´s t-test or 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 ± SEM, and expressed as fold-variation relatively to the control group.

Results

Identification of $Ca_v1.2$ channel subunit in MCF-7 cells

RT-PCR, WB and fluorescent immunocytochemistry were used to evaluate the expression of $Ca_v1.2$ channel subunit in human breast cancer MCF-7 cells. RT-PCR analysis using specific primers confirmed the mRNA expression of $Ca_v1.2$ channel subunit in MCF-7 cells. An amplicon of the expected size was identified in MCF-7 cells and in human and rat Sertoli cells, which were used as positive control (Figure VI.1A). Also, an immunoreactive band of approximately 135 kDa was identified in the WB for MCF-7 and rat Sertoli cells proteins (Figure VI.1B), and the intensity of obtained bands followed the increase in the total amount of loaded protein. The presence of $Ca_v1.2$ channel subunit protein in MCF-7 cells was also confirmed by immunofluorescence staining (Figure VI.1C), which showed intense membrane and cytoplasm labeling.

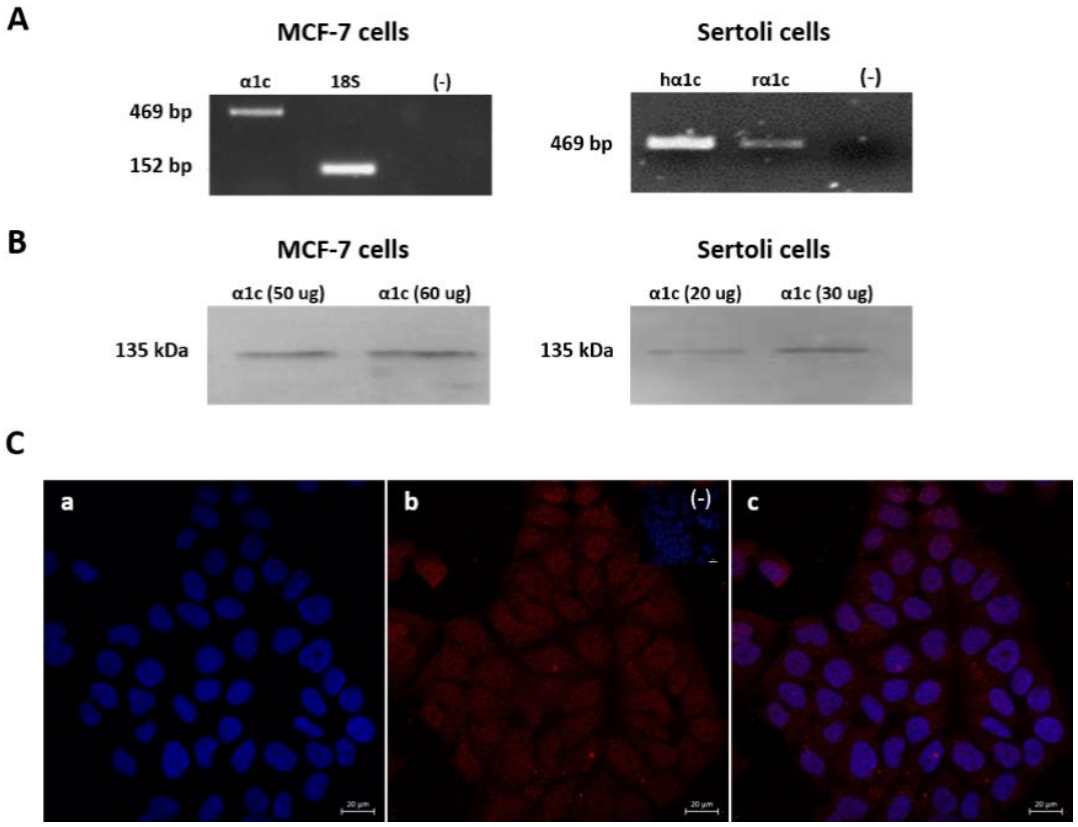

Figure VI.1. Expression of $Ca_v1.2$ channel subunit in human breast cancer MCF-7 cells. (A) RT-PCR analysis of $Ca_v1.2$ α_{1C} channel subunit in MCF-7 cells. Amplification of 18S housekeeping gene was used as a control of the cDNA synthesis. (-) Negative control, without cDNA. Human and rat Sertoli cells were used as positive control for the amplification of $Ca_v1.2$ subunit, respectively, $h\alpha_{1C}$ and $r\alpha_{1C}$. (B) Western blot analysis of $Ca_v1.2$ subunit in MCF-7 cells using different amounts of loaded protein. Rat Sertoli cells were used as positive control. DNA base pair (bp) and protein molecular weight are indicated on the left side of each corresponding panel. (C) Representative images of $Ca_v1.2$ subunit immunofluorescent staining in MCF-7 cells (red, panel b). Nucleus were stained with Hoechst 33342 (blue, panel a) and merged images are shown in panel c. Negative control obtained by omission of $Ca_v1.2$ subunit primary antibody is shown as insert panel (-).

Voltage-dependent Ca^{2+} channels in MCF-7 cells

To analyze the existence of voltage dependent Ca^{2+} channels, we measured the maximal amplitude of the current peak elicited after each depolarization pulse (I_{Ca}). The I_{Ca} was recorded using a high concentration of Ba^{2+} (30 mM) in the extracellular solution, which is commonly used to increase the magnitude of LTCC I_{Ca}. The induced I_{Ca} were slowly inactivated, while the basal I_{Ca} amplitude and cell capacitance were on average 29.2 ± 5.0 pA (n=16) and 67.6 ± 8.5 pF (n=16), respectively. As presented in the time-course experiment shown in Figure VI.2A, both nifedipine, an inhibitor of LTCC, and mibefradil, an inhibitor of TTCC, were able to reversibly inhibit the I_{Ca} in MCF-7 cells. Nifedipine inhibited 26 % of the basal $I_{Ca,}$ whereas mibefradil inhibited 63 % of the basal I_{Ca} (Figure VI.2B). The simultaneous application of nifedipine and mibefradil inhibited 80 % of the I_{Ca} (Figure VI.2B).

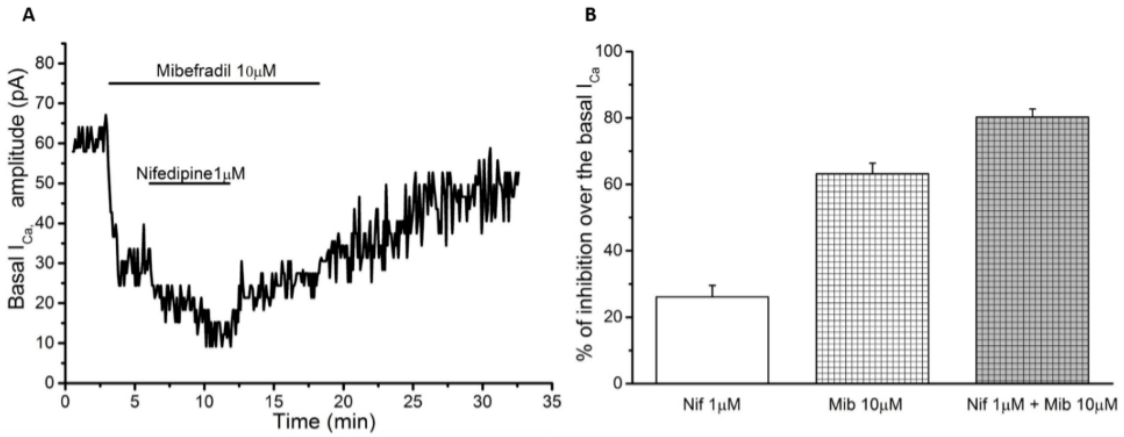

Figure VI.2. Voltage Ca^{2+} currents in MCF-7 cells. (A) original records showing the effect of nifedipine (1 μM) plus mibefradil (10 μM) on basal I_{Ca} amplitudes measured in patch-clamp experiments. (B) bar graph illustrating the percentage of basal I_{Ca} inhibition elicited by nifedipine (Nif), mibefradil (Mib) and Nif plus Mib ($n \geq 6$).

DHT regulates the expression of $Ca_v1.2$ subunit and regucalcin in MCF-7 cells

In the last years, several studies have demonstrated the role of androgens as regulators of intracellular Ca^{2+} levels and cell proliferation [2-4, 6-8]. Moreover, reports exist linking the LTCC and the Ca^{2+}-binding protein RGN with carcinogenesis [13-16, 20, 21]. Therefore, we decided to investigate the effect of DHT on the expression of these Ca^{2+} modulators in the androgen-responsive MCF-7 cells. A time-course experiment was performed for 6 to 24 h with 1, 10 and 100 nM DHT (Figure VI.3). All tested concentrations of DHT significantly down-regulated the mRNA expression of $Ca_v1.2$ channel subunit in MCF-7 cells at 24 h of stimulation (0.36 ± 0.04, 0.31 ± 0.06 and 0.17 ± 0.03 fold, respectively for 1, 10 and 100 nM, Figure VI.3A). The same response pattern was observed for the mRNA expression of RGN in DHT-treated MCF-7 cells (0.34 ± 0.07, 0.36 ± 0.09 and 0.48 ± 0.05 fold, respectively for 1, 10 and 100 nM, Figure VI.3D).

The 1 nM DHT concentration was chosen for subsequent analysis because it induced changes on the mRNA expression of $Ca_v1.2$ channel subunit and RGN, and it is in the range of reported serum physiological concentrations in pre (0.05 nM) and postmenopausal (1.26 nM) women [22]. Concerning the expression of $Ca_v1.2$ channel subunit and RGN proteins, a distinct response was observed upon treatment with DHT. MCF-7 cells treated with 1 nM DHT displayed increased expression of $Ca_v1.2$ protein at 24 h (1.84 ± 0.15 fold, $p < 0.01$, Figure VI.3B). Inversely, DHT treatment for 24 h diminished the protein expression of RGN in MCF-7 cells (0.75 ± 0.05 fold, $p < 0.01$, Figure VI.3E). At 48 h the protein expression levels of both $Ca_v1.2$ channel subunit and RGN returned to that of the control (Figure VI.3B and 3E).

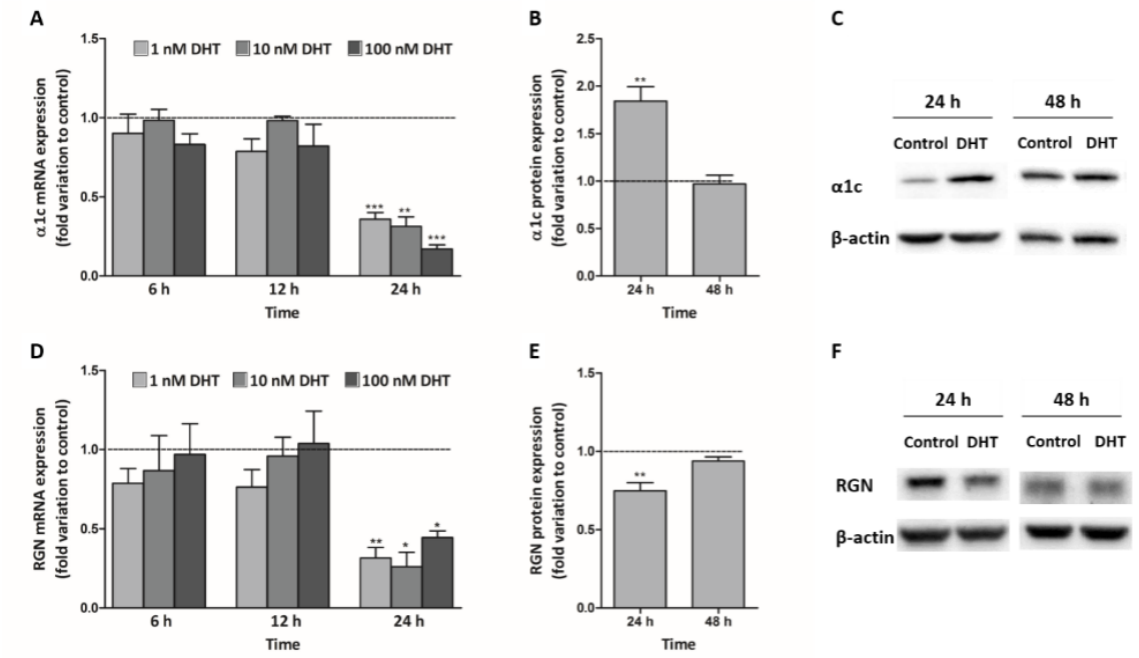

Figure VI.3. Effect of DHT on $Ca_v1.2$ channel subunits and RGN expression in MCF-7 cells. MCF-7 cells were either exposed to vehicle (control) or 1, 10 and 100 nM of DHT for 6, 12, 24 h and 48 h. mRNA expression of $Ca_v1.2$ channel subunits (A) and RGN (D) was determined by qPCR after normalization with the GAPDH housekeeping gene. Protein levels of $Ca_v1.2$ subunits (B) and RGN (E) were determined by WB after normalization with β-actin. Representative immunoblots for $Ca_v1.2$ channel protein and RGN are provided in panels C and F, respectively. Results are expressed as fold variation relatively to control (*dashed line*). Error bars indicate mean ± S.E.M (n ≥ 5). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ comparatively with the control group.

DHT effects regulating the expression of $Ca_v1.2$ channel subunit and regucalcin are mediated by the androgen receptor

It is widely known that DHT actions are mediated by the classical intracellular AR, which acts as a transcription factor regulating the expression of target genes [5]. In order to explore the mechanisms underlying the effect of DHT in regulating the expression of $Ca_v1.2$ channels subunits and RGN, MCF-7 cells were exposed to 1 nM DHT for 24 h in presence or absence of AR antagonist flutamide or protein synthesis inhibitor cycloheximide. Flutamide abrogated the effect of DHT down-regulating the mRNA expression of $Ca_v1.2$ channel subunits and RGN (Figure VI.4A and 4B). Cycloheximide, however, only reverted the effect of DHT regulating the expression of $Ca_v1.2$ channel subunits (Figure VI.4A); no effect was observed abrogating the down-regulation of RGN expression by DHT (Figure VI.4B). In addition, flutamide alone significantly up-regulated the expression of $Ca_v1.2$ channel subunit relatively to the control group (1.96 ± 0.21 fold, Figure VI.4A).

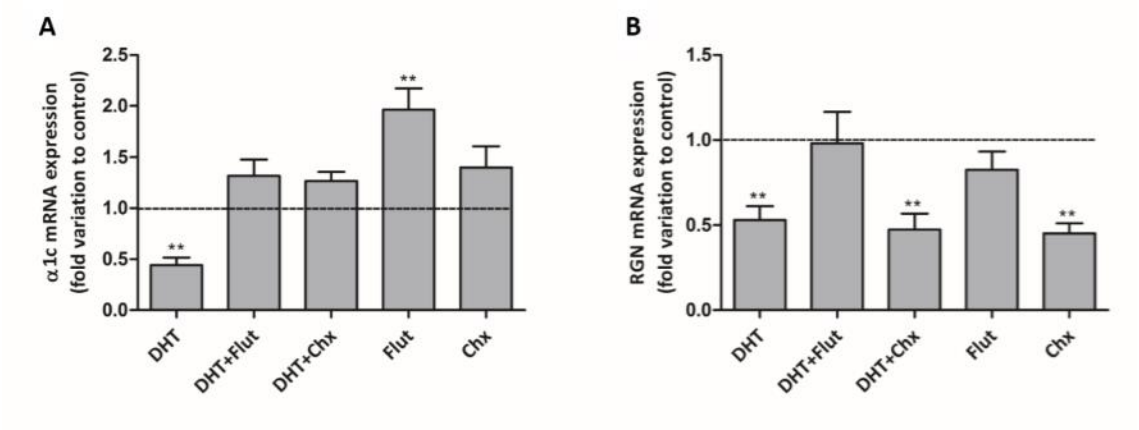

Figure VI.4. Effect of androgen receptor inhibitor flutamide (Flut) and protein synthesis inhibitor cycloheximide (Chx) on DHT regulation of $Ca_V1.2$ subunit (A) and RGN (B) mRNA expression in MCF-7 cells. MCF-7 cells were exposed to DHT (1 nM), DHT plus Flut (1 μM), DHT plus Chx (1 μg/mL), Flut or Chx for 24 h. Results are represented as mean ± S.E.M (n ≥ 5) $^{**}p < 0.01$ comparatively with the control group.

Effect of DHT on the viability of MCF-7 cells

The viability of MCF-7 cells treated with 1 nM DHT significantly decreased at the different experimental time points (81.79 ± 4.74, 71.99 ± 10.14 and 61.97 ± 4.67 % of control for 24, 48 and 72 h of stimulation, respectively, Figure VI.5A).

The tumor suppressor P53 is a major cell death regulator that also controls cycle arrest [23]; thus, in parallel with the assessment of cell viability, we investigated the expression of P53 in response to DHT treatment. Although no significant difference was observed on the expression of P53 for 24 h of treatment, at 48 h its expression was enhanced (1.13 ± 0.05 fold, $p < 0.05$, Figure VI.5B).

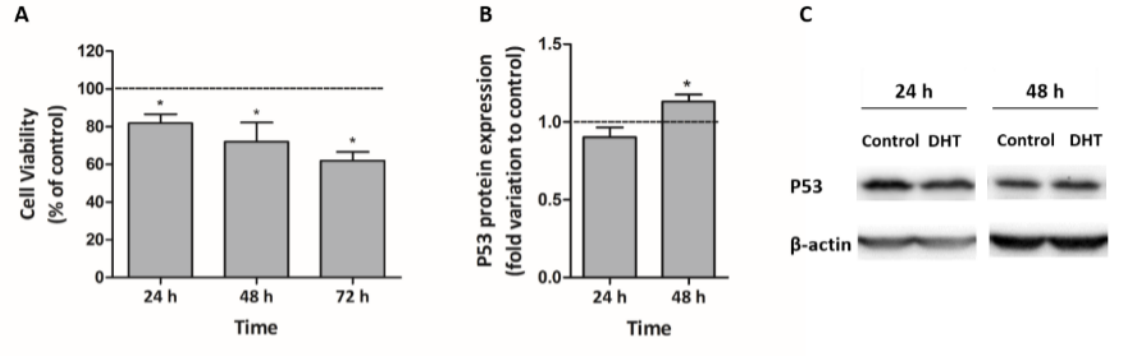

Figure VI.5. Effect of DHT on the viability of MCF-7 cells (A) and expression of P53 (B). Cells were either exposed to vehicle (control) or 1 nM of DHT for 24, 48 and 72 h. Results of cell viability are expressed as % of control (*dashed line*). The expression of P53 was determined by WB after normalization with β-actin. Representative immunoblots for P53 are provided in panel C. Results are expressed as fold variation relatively to control (*dashed line*). Error bars indicate mean ± S.E.M (n ≥ 5). * $p < 0.05$ when compared with the control group.

Discussion

The present study investigated the effect of non-aromatizable androgen DHT in regulating the expression of Ca^{2+} handling proteins, namely the $Ca_v1.2$ channel subunits and the Ca^{2+}-binding protein RGN, in MCF-7 cells.

The $Ca_v1.2$ channel subunits are products of the CACNA1C gene and belong to the LTCC subfamily ($Ca_v1.1$-$Ca_v1.4$) of voltage-gated Ca^{2+} channels (VGCC), which are characterized by high-voltage activation and long-lasting activity [24]. The LTCC were mostly associated with cardiac and neuronal cells [24], and its activation requires a strong depolarization of cell membrane that generates elevated currents. Nevertheless, $Ca_v1.2$ channel subunits have been identified in several non-excitable cell types, such as osteoblasts, osteoclasts, monocytes, macrophages and stem cell hair follicles [25-27]. Moreover, $Ca_v1.1$ and $Ca_v1.2$ isoforms were detected and up-regulated in the colon and colorectal cancer cells [13, 14]. Also, the presence of $Ca_v1.2$ and $Ca_v1.3$ channel subunits in prostate cancer tissues was shown [15]. On the other hand in breast cancers cells, different isoforms of the TTCC channels ($Ca_v3.1$ and $Ca_v3.2$ subunits) have been detected and associated with enhanced proliferation [28]. However, the expression of the LTTC has remained uncharacterized in breast cells. On the contrary, previous work of our research team had already identified the Ca^{2+}-binding protein RGN in human breast cancer tissues and MCF-7 breast cancer cell line [20, 21]. Therefore, we started by describing the presence of $Ca_v1.2$ channel subunits in human MCF-7 breast cancer cells. Specifically, it was detected the subunit α_1, which is the core subunit responsible for pore-forming and that contains the regulatory sites for dihydropyridine blockers [24]. WB analysis detected an immunoreactive band of ~135 kDa, which is slightly inferior to the most common molecular weight indicated for $Ca_v1.2$ proteins. The molecular weight for subunit α_1 ranges from 165 to 240 kDa [24, 29], but peptides with different size also have been described dependently on the reducing conditions in WB [29], which supports the present findings. Using an antibody that, as our, recognizes an epitope in the II-III cytoplasmic loop of $Ca_v1.2$ subunit others also detected a smaller protein of 150 KDa in rat brain cortex [30]. Also, it has been established that the human CACNA1C gene encompasses 55 exons and has, at least, 19 alternative splicing loci that may generate diverse transcripts with predicted structural and functional diversity [24, 31]. A recent report identified multiple transcriptional start sites at CACNA1C gene producing new transcripts in rat brain [31]. One of these transcripts is capable to produce a membrane-bound 120 kDa protein both *in vitro* and *in vivo* [31], which further indicates that a tissue-specific variability exists concerning the size of $Ca_v1.2$ proteins.

Considering the subcellular location $Ca_v1.2$ channel subunits in MCF-7 cells, these were detected in the plasma membrane and cytoplasm. It was recently demonstrated that $Ca_v1.2$ proteins are also localized to endoplasmic reticulum (ER)-plasma membrane junctions in association with the ER Ca^{2+} sensing protein STIM1, and that $Ca_v1.2$ labeling is beyond the localization of STIM1 in junctional areas [32], which is consistent with the observed cytoplasm staining in MCF-7 cells.

The electrophysiological experiments allowed to measure I_{Ca} in MCF-7 cells, which can be a consequence of LTCC and TTCC channels activity because both nifedipine and mibefradil had inhibitory effects on the I_{Ca} relatively to basal levels. Indeed, previous studies have demonstrated the activity of TTCC channels in MCF-7 cells [28], but, at least to our knowledge, this is the first report showing the presence and activity of L-type I_{Ca} in breast cancer cells.

Concerning the hormonal stimulation of MCF-7 cells, we showed that 1 nM DHT upregulated the expression of $Ca_v1.2$ channel subunits while diminishing RGN protein levels. The effect of DHT suppressing RGN expression was also described in rat prostate *in vivo* [18] and LNCaP prostate cancer cells [20]. However, in rat seminiferous tubules cultured *ex vivo* in presence of DHT the RGN levels were augmented [19], which may be related to the peculiarities and specific requirements of germ cells. Androgens have been indicated as important regulators of Ca^{2+} homeostasis in different cell types [6-10], and following our results, it was reported that DHT increases $Ca_v1.2$ subunit levels in coronary smooth muscle [9]. No other studies exist characterizing the androgenic regulation of VGCC in breast cells.

The fact that $Ca_v1.2$ mRNA is diminished in MCF-7 cells treated with DHT cannot be excluded from the discussion. This discordance between mRNA and protein levels may arise due to the great complexity of gene expression regulatory mechanisms in eukaryotes, and is something described in other human cell lines (e.g. [33]). Moreover, it suggests an increased stability and half-life time of mRNA and that a post-transcriptional control may be occurring. Nevertheless, we demonstrated that the DHT regulation of $Ca_v1.2$ and RGN expression is dependent on AR. However, the mechanism involved seems to be distinct since the DHT effect mediated by AR in regulating the expression of $Ca_v1.2$ channels requires *de novo* protein synthesis in opposition with what was observed for RGN. The involvement of AR in the modulation of RGN levels was also reported in LNCaP human prostate cancer cells and rat seminiferous tubules [19, 20], and androgen-responsive elements were identified by *in silico* analysis in the RGN gene promoter [20].

Concomitantly with the altered expression of Ca^{2+} regulators, DHT significantly decreased the viability of MCF-7 cells after 24 h of treatment. Although some controversy exists, the majority of studies has described the growth-inhibitory effects of DHT in MCF-7 cells [2-4]. Noteworthy, DHT seems to induce cell cycle arrest in MCF-7 cells at G0/G1 [2, 34], a cell cycle phase shown to be associated with enhanced activity of LTCC [35]. Very recently, it was also shown that increased levels of DHT, upon inhibition of 17β-hydroxysteroid dehydrogenase type 7, are associated with cell cycle arrest in MCF-7 cells [36]. Although no changes were detected in the expression of RGN and $Ca_v1.2$ in response to DHT at other experimental time points, a diminished viability of MCF-7 cells was also observed for 48 and 72 h of treatment. This indicates that other factors may be involved, as seems to be the case of cell cycle suppressor protein P53. The increased expression of P53 in response to DHT supports the diminished viability observed for MCF-7 cells under androgenic stimulation.

Nevertheless, the present findings indicate that DHT effects counteracting the proliferation of MCF-7 cells may depend on the regulation of Ca^{2+} modulators and modulation of cytosolic Ca^{2+}

levels. Indeed, it has been suggested that altered activity of VGCC may influence cell proliferation, differentiation and migration [12]. Moreover, breast cancer cells display depolarized membrane potential comparatively with non-neoplastic conditions [37], which is associated with a high proliferative ability [12]. However, the role of intracellular Ca^{2+} homeostasis in the control of cell proliferation in breast cancer cells has been producing conflicting reports. Antagonists for the three major classes of LTCCs, namely, amlodipine, diltiazem, and verapamil inhibit the proliferation of human HT-39 breast cancer cells and tumor growth in vivo [16]. Also, the pharmacologic inhibition of LTCCs with Ni^{2+} blocked the growth of mouse 4T1 [38] and human MDA-MB-231 breast tumor cells [39]. Beyond that, increased intracellular Ca^{2+} levels have been linked with the pro-proliferative behavior of MCF-7 cells [12, 40]. On the other hand, the inhibition of capacitative Ca^{2+} entry and Ca^{2+} release from ER stores reduced EGF-induced proliferation of mouse mammary gland epithelial cells [41]. However, and contrary to these findings, the anti-estrogen tamoxifen increased the intracellular Ca^{2+} concentration in ZR-75-1 breast cancer cells and reduced cell viability, by releasing Ca^{2+} from the ER and inducing Ca^{2+} entry from the extracellular space [42]. These divergent results highlight a duality in the functional consequences of Ca^{2+} handling and indicate that Ca^{2+} signaling pathways controlling cell growth may depend on the cancer cell type and cell status. This matter was elegantly reviewed by Prevarskaya et al. [12] in the context of determining the relevance of ion channels as targets for cancer diagnosis and treatment.

In what concerns RGN, it plays a role in intracellular Ca^{2+} homeostasis, by enhancing the activity of Ca^{2+}-ATPase at plasma membrane and ER and down-regulating the expression levels of LTCCs and Ca^{2+} sensing receptor [43, 44]. Nevertheless, RGN protein seems to have a broad spectrum of action in cell physiology [17] and has been associated with the control of cell proliferation [18, 21]. RGN overexpression *in vivo* was protective against the development of rat mammary gland tumors and effective in inhibiting cell proliferation [21]. The apparently contradictory results of reduced expression of RGN and diminished viability of MCF-7 cells indicate that the increase in intracellular Ca^{2+} levels in consequence of altered expression of RGN and LTCC is the determinant for suppressing cell growth (Figure VI.6). Since it is also known that augmented intracellular Ca^{2+} levels stimulate the expression of RGN in different cell types [45], this assumption also explains the recovery of RGN expression to control levels at 48 h.

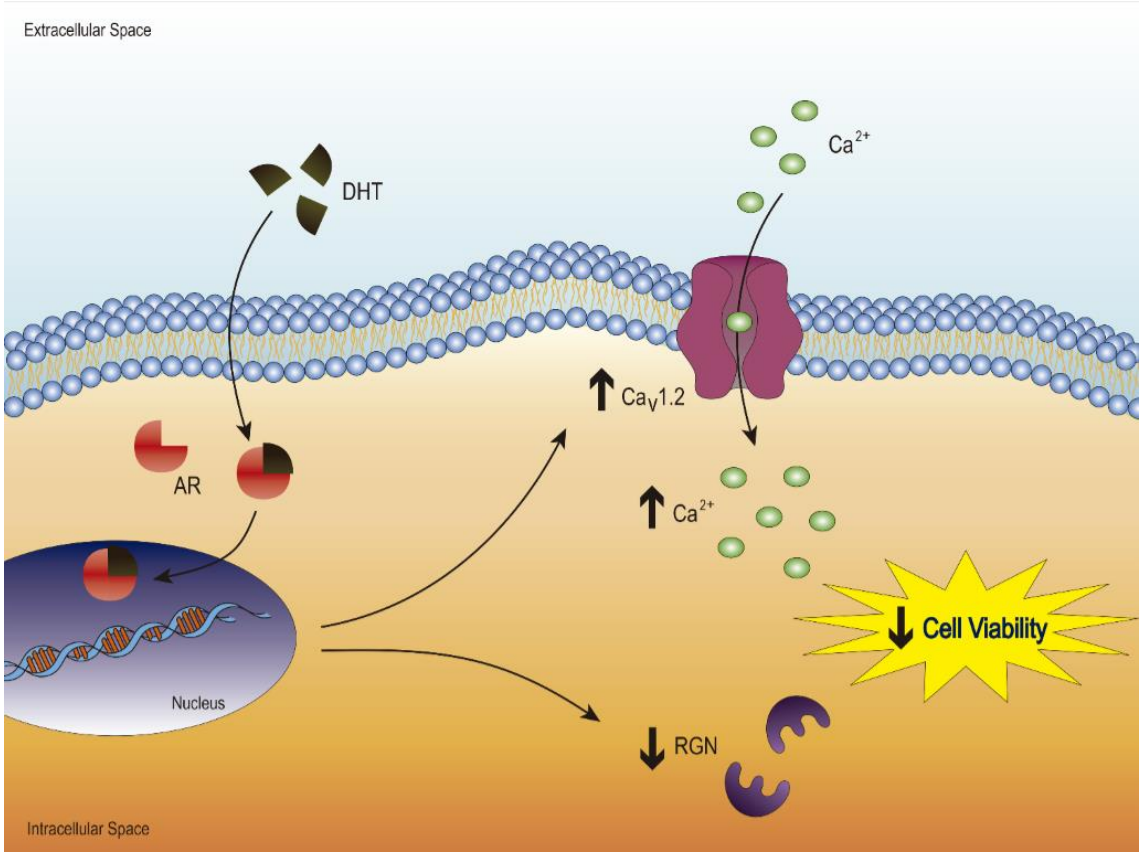

Figure VI.6. Schematic representation of DHT effects on Ca^{2+} homeostasis and cell viability in MCF-7 breast cancer cells. After entering the cell, DHT binds the classical androgen receptor (AR). After translocation to cell nucleus, the hormone receptor complex regulates the expression of α_{1C} subunit ($Ca_v1.2$) of L-type Ca^{2+} channels and regucalcin (RGN), which contributes to reduced cell proliferation.

In sum, this work first characterized the presence of $Ca_v1.2$ channel subunits in MCF-7 cells. Also, it was confirmed that the expression of both $Ca_v1.2$ and RGN in MCF-7 cells is regulated by DHT, which was related to diminished cell proliferation. Although further studies are needed to fully address the role of LTCC in the proliferative activity of breast cancer cells, these findings represent an important step towards the development of new therapeutic approaches targeting Ca^{2+} signaling.

Acknowledgments

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

References

1. Dimitrakakis C, Bondy C (2009) Androgens and the breast. Breast Cancer Res 11(5):212

2. Greeve MA, Allan RK, Harvey JM, Bentel JM (2004) Inhibition of MCF-7 breast cancer cell proliferation by 5alpha-dihydrotestosterone; a role for p21(Cip1/Waf1). J Mol Endocrinol 32(3):793-810

3. Chottanapund S, Van Duursen MB, Navasumrit P, Hunsonti P, Timtavorn S, Ruchirawat M, Van den Berg M (2013) Effect of androgens on different breast cancer cells co-cultured with or without breast adipose fibroblasts. J Steroid Biochem 138:54-62

4. Ortmann J, Prifti S, Bohlmann MK, Rehberger-Schneider S, Strowitzki T, Rabe T (2002) Testosterone and 5 alpha-dihydrotestosterone inhibit in vitro growth of human breast cancer cell lines. Gynecol Endocrinol 16(2):113-120

5. Li J, Al-Azzawi F (2009) Mechanism of androgen receptor action. Maturitas 63(2):142-148

6. Dick IM, Liu J, Glendenning P, Prince RL (2003) Estrogen and androgen regulation of plasma membrane calcium pump activity in immortalized distal tubule kidney cells. Mol Cell Endocrinol 212(1-2):11-18

7. Zhang L, Barritt GJ (2004) Evidence that TRPM8 is an androgen-dependent Ca2+ channel required for the survival of prostate cancer cells. Cancer Res 64(22):8365-8373

8. Oliver VL, Anderson C, Ventura S, Haynes JM (2010) Androgens regulate adenylate cyclase activity and intracellular calcium in stromal cells derived from human prostate. Prostate 70(11):1222-1232

9. Bowles DK, Maddali KK, Ganjam VK, Rubin LJ, Tharp DL, Turk JR, Heaps CL (2004) Endogenous testosterone increases L-type Ca2+ channel expression in porcine coronary smooth muscle. Am J Physiol Heart Circ Physiol 287(5):H2091-2098

10. Golden KL, Marsh JD, Jiang Y (2004) Testosterone regulates mRNA levels of calcium regulatory proteins in cardiac myocytes. Horm Metab Res 36(4):197-202

11. Lee WJ, Monteith GR, Roberts-Thomson SJ (2006) Calcium transport and signaling in the mammary gland: Targets for breast cancer. BBA-Rev Cancer 1765(2):235-255

12. Prevarskaya N, Skryma R, Shuba Y (2010) Ion channels and the hallmarks of cancer. Trends Mol Med 16(3):107-121

13. Wang XT, Nagaba Y, Cross HS, Wrba F, Zhang L, Guggino SE (2000) The mRNA of L-type calcium channel elevated in colon cancer: protein distribution in normal and cancerous colon. Am J Pathol 157(5):1549-1562

14. Zhang L, Zhou W, Velculescu VE, Kern SE, Hruban RH, Hamilton SR, Vogelstein B, Kinzler KW (1997) Gene expression profiles in normal and cancer cells. Science 276(5316):1268-1272

15. Chen R, Zeng X, Zhang R, Huang J, Kuang X, Yang J, Liu J, Tawfik O, Thrasher JB, Li B (2014) Cav1.3 channel alpha1D protein is overexpressed and modulates androgen receptor transactivation in prostate cancers. Urol Oncol 32(5):524-536

16. Taylor JM, Simpson RU (1992) Inhibition of Cancer Cell-Growth by Calcium-Channel Antagonists in the Athymic Mouse. Cancer Res 52(9):2413-2418

17. Marques R, Maia CJ, Vaz C, Correia S, Socorro S (2014) The diverse roles of calcium-binding protein regucalcin in cell biology: from tissue expression and signalling to disease. Cell Mol Life Sci 71(1):93-111

18. Vaz CV, Maia CJ, Marques R, Gomes IM, Correia S, Alves MG, Cavaco JE, Oliveira PF, Socorro S (2014) Regucalcin is an androgen-target gene in the rat prostate modulating cell-cycle and apoptotic pathways. Prostate 74(12):1189-1198

19. Laurentino SS, Correia S, Cavaco JE, Oliveira PF, Rato L, Sousa M, Barros A, Socorro S (2011) Regucalcin is broadly expressed in male reproductive tissues and is a new androgen-target gene in mammalian testis. Reproduction 142(3):447-456

20. Maia C, Santos C, Schmitt F, Socorro S (2009) Regucalcin is under-expressed in human breast and prostate cancers: Effect of sex steroid hormones. J Cell Biochem 107(4):667-676

21. Marques R, Vaz CV, Maia CJ, Gomes M, Gama A, Alves G, Santos CR, Schmitt F, Socorro S (2015) Histopathological and in vivo evidence of regucalcin as a protective molecule in mammary gland carcinogenesis. Exp Cell Res 330(2):325-335

22. Esfahani A, Kendall CW, Bashyam B, Archer MC, Jenkins DJ (2010) The effect of physiological concentrations of sex hormones, insulin, and glucagon on growth of breast and prostate cells supplemented with unmodified human serum. Vitro Cell Dev Biol Anim 46(10):856-862

23. Vousden KH, Lane DP (2007) p53 in health and disease. Nat Rev Mol Cell Biol 8(4):275-283

24. Hofmann F, Flockerzi V, Kahl S, Wegener JW (2014) L-type CaV1.2 calcium channels: from in vitro findings to in vivo function. Physiol Rev 94(1):303-326

25. Li F, Wang W, Gu M, Gyoneva S, Zhang J, Huang S, Traynelis SF, Cai H, Guggino SE, Zhang X (2011) L-type calcium channel activity in osteoblast cells is regulated by the actin cytoskeleton independent of protein trafficking. J Bone Miner Metab 29(5):515-525

26. Yucel G, Altindag B, Gomez-Ospina N, Rana A, Panagiotakos G, Lara MF, Dolmetsch R, Oro AE (2013) State-dependent signaling by Cav1.2 regulates hair follicle stem cell function. Genes Dev 27(11):1217-1222

27. Das R, Burke T, Van Wagoner DR, Plow EF (2009) L-type calcium channel blockers exert an antiinflammatory effect by suppressing expression of plasminogen receptors on macrophages. Circ Res 105(2):167-175

28. Taylor JT, Huang L, Pottle JE, Liu K, Yang Y, Zeng X, Keyser BM, Agrawal KC, Hansen JB, Li M (2008) Selective blockade of T-type Ca2+ channels suppresses human breast cancer cell proliferation. Cancer Lett 267(1):116-124

29. Sieber M, Nastainczyk W, Zubor V, Wernet W, Hofmann F (1987) The 165-kDa peptide of the purified skeletal muscle dihydropyridine receptor contains the known regulatory sites of the calcium channel. Eur J Biochem 167(1):117-122

30. Gomez-Ospina N, Tsuruta F, Barreto-Chang O, Hu L, Dolmetsch R (2006) The C terminus of the L-type voltage-gated calcium channel Ca(V)1.2 encodes a transcription factor. Cell 127(3):591-606

31. Gomez-Ospina N, Panagiotakos G, Portmann T, Pasca SP, Rabah D, Budzillo A, Kinet JP, Dolmetsch RE (2013) A promoter in the coding region of the calcium channel gene CACNA1C generates the transcription factor CCAT. PLoS One 8(4):e60526

32. Wang Y, Deng X, Mancarella S, Hendron E, Eguchi S, Soboloff J, Tang XD, Gill DL (2010) The calcium store sensor, STIM1, reciprocally controls Orai and CaV1.2 channels. Science 330(6000):105-109

33. Gry M, Rimini R, Stromberg S, Asplund A, Ponten F, Uhlen M, Nilsson P (2009) Correlations between RNA and protein expression profiles in 23 human cell lines. BMC Genom 10:365

34. Ando S, De Amicis F, Rago V, Carpino A, Maggiolini M, Panno ML, Lanzino M (2002) Breast cancer: from estrogen to androgen receptor. Mol Cell Endocrinol 193(1-2):121-128

35. Capiod T (2013) The need for calcium channels in cell proliferation. Recent Pat Anticancer Drug Discov 8(1):4-17

36. Wang X, Gerard C, Theriault JF, Poirier D, Doillon CJ, Lin SX (2015) Synergistic control of sex hormones by 17beta-HSD type 7: a novel target for estrogen-dependent breast cancer. Journal of molecular cell biology doi:10.1093/jmcb/mjv028.

37. Marino AA, Iliev IG, Schwalke MA, Gonzalez E, Marler KC, Flanagan CA (1994) Association between cell membrane potential and breast cancer. Tumour Biol 15(2):82-89

38. Doering CJ, Zamponi GW (2003) Molecular pharmacology of high voltage-activated calcium channels. J Bioenerg Biomembr 35(6):491-505

39. Yang S, Huang XY (2005) Ca2+ influx through L-type Ca2+ channels controls the trailing tail contraction in growth factor-induced fibroblast cell migration. J Biol Chem 280(29):27130-27137

40. Dixon CJ, Bowler WB, Fleetwood P, Ginty AF, Gallagher JA, Carron JA (1997) Extracellular nucleotides stimulate proliferation in MCF-7 breast cancer cells via P2-purinoceptors. Br J Cancer 75(1):34-39

41. Ichikawa J, Kiyohara T (2001) Suppression of EGF-induced cell proliferation by the blockade of Ca2+ mobilization and capacitative Ca2+ entry in mouse mammary epithelial cells. Cell Biochem Funct 19(3):213-219

42. Chang HT, Huang JK, Wang JL, Cheng JS, Lee KC, Lo YK, Liu CP, Chou KJ, Chen WC, Su W et al. (2002) Tamoxifen-induced increases in cytoplasmic free Ca2+ levels in human breast cancer cells. Breast Cancer Res Treat 71(2):125-131

43. Vaz CV, Rodrigues DB, Socorro S, Maia CJ (2015) Effect of extracellular calcium on regucalcin expression and cell viability in neoplastic and non-neoplastic human prostate cells. Biochim Biophys Acta 1853: 2621-2628

44. Nakagawa T, Yamaguchi M (2006) Overexpression of regucalcin enhances its nuclear localization and suppresses L-type Ca2+ channel and calcium-sensing receptor mRNA expressions in cloned normal rat kidney proximal tubular epithelial NRK52E cells. J Cell Biochem 99(4):1064-1077

45. Yamaguchi M, Kurota H (1995) Expression of calcium-binding protein regucalcin mRNA in the kidney cortex of rats: the stimulation by calcium administration. Mol Cell Biochem 146(1):71-77

Chapter VII

Summarizing discussion and conclusion

Summarizing discussion and conclusion

The mammary gland is a complex tissue composed by a multiplicity of cellular components, which are affected by the specific and unique characteristics of female physiology. Innumerous studies regarding mammary carcinogenesis have tried to decipher the initiator factors that lead to cancer onset, and also the drivers promoting the escape of the neoplastic mass to therapeutic approaches. Despite all the efforts developed until now, that have been translated into improved diagnosis, clinical responses and prognosis of patients life, breast cancer is still a disease that affects millions of women every year and sometimes with an uncertain outcome.

Cancer thrives from the alteration of the basic biologic processes that maintain normal cell physiology. The intracellular player RGN has an active role in a variety of process within cell biology, namely cell proliferation, apoptosis, calcium (Ca^{2+}) homeostasis, gene expression, metabolism and signaling pathways. Moreover, the RGN protein was associated with breast carcinogenesis. Human breast cancer cases displayed loss of RGN immunoreactivity, which was correlated with clinicopathological parameters and negatively associated with tumor grade. These were remarkable findings suggesting that the loss of RGN expression favors progression of tumors into more aggressive phenotypes. Nevertheless, much remains to be understood about the involvement of RGN in cancer mechanisms, particularly in breast cancer.

The present thesis aimed to fulfill some of the existent gaps and clarify the RGN actions that may protect against the development of breast cancer.

We started by hypothesizing that RGN overexpression may confer protection against chemically-induced tumorigenesis of the mammary gland. In the Chapter IV of this thesis the development of tumors in wild-type (Wt) and transgenic rats overexpressing RGN (Tg-RGN) treated with the carcinogen 7,12-dimethylbenz[α]anthracene (DMBA) was evaluated. This approach demonstrated that RGN conferred a marked resistance to mammary carcinogenesis by slowing down tumor onset, as seen by the lower incidence of tumors (25.8 % in Tg-RGN *vs* 100 % in Wt group), as well as, delayed progression of tumors into more aggressive phenotypes in Tg-RGN rats. Tumor histological classification showed that Tg-RGN animals displayed less tumors of invasive type (3.8 %) comparatively to Wt counterparts (45.4 %), whereas pre-cancerous lesions (19.2 %) were only present in Tg-RGN animals. The non-invasive features of mammary gland tumors of Tg-RGN were underpinned by the decreased Ki67 expression. These findings clearly established the protective role of RGN in mammary carcinogenesis.

To get into the mechanist of RGN actions restricting development of mammary tumors we compared the proliferative status of mammary gland tissues in Wt and Tg-RGN animals (Chapter IV). We found that Tg-RGN animals presented lower cell proliferation accompanied by altered expression of target cell cycle regulators. The obtained results have allowed us to confirm that RGN exerted a suppressive role in mammary cells growth by down-regulating the expression of oncogene Myc, cell cycle regulator Cdk1, and the SCF/c-Kit system, which is associated with proliferation and tumorigenesis. Additionally, p53 expression was enhanced in

Tg-RGN rats, which may have contributed to counteract cell proliferation, and further supports the role of RGN preventing tumorigenesis of mammary gland.

Metabolic reprogramming is a common feature of cancer cells characterized by the increased glycolytic flux and augmented production of lactate even under aerobic conditions, which represents a survival advantage and favors the malignant transformation. Considering the existent knowledge linking RGN actions with the control of cell metabolism, in Chapter V of this thesis we compared the glycolytic metabolism in the mammary gland of Tg-RGN and Wt animals, and investigated the alterations in the glycolytic metabolism occurring during development of DMBA-induced mammary gland tumors. Despite the observed enhancement of glycolysis, tumors of Tg-RGN rats showed diminished expression and activity of lactate dehydrogenase (LDH), which may be considered as a protective mechanism. The increased lactate production and the acidification of the environment are well-known alterations in tumor invasion and aggressiveness; thus, RGN could restrain breast cancer progression into more aggressive stages by controlling the lactate production by cancer cells. This is strongly supported by the findings described above confirming that Tg-RGN animals are resistant to develop mammary tumors including tumors with invasive features. Curiously, in non-neoplastic mammary gland an inhibition of the glycolytic metabolism was observed in Tg-RGN rats, put in evidence by the: i) reduced content of glucose as result of the diminished expression of GLUT3; ii) suppressed activity of PFK; iv) increased levels of lactate reflecting the decreased expression of MCT4; and v) augmented activity of LDH. Thus, RGN seems to reduce energy production that consequently, contributes to restricting cell proliferation and supports its role in counteracting tumor initiation.

Indeed, fulfill of the energetic needs is mandatory to sustain the high proliferative rates of tumor cells, and not surprisingly, several regulators of the cell cycle have been indicated as regulators of cell metabolism, as it is the case of Myc and P53 proteins. As previously described Myc and P53 were found to be down- and up-regulated in the mammary tissue of Tg-RGN, and are likely involved both in the proliferative and metabolic features observed in these animals.

In fact, the increase of P53 suppresses the glycolytic metabolism and looks as an additional tumor suppressor mechanism by which P53 limits the malignant transformation. This effect of P53 is thought to be achieved by the inhibition of glucose transporters expression (GLUT1, GLUT3, and GLUT4), glycolytic enzymes, and even the pentose phosphate pathway (for a review [1, 2]). Additionally, the proto-oncogene Myc promotes the synthesis of almost all glycolytic enzymes, stimulates the mitochondrial metabolism and glutamine catabolism, and enhances LDH expression (reviewed in [3-6]). Therefore, we can hypothesize that the downregulated expression of Myc and the enhancement of P53 in the mammary gland of Tg-RGN animals may mediate the observed effects of RGN in the regulation of cell proliferation and metabolism related with its protective role in cancer initiation. Further research is warranted to clarify the cross-talk between these pathways and the regulatory actions of RGN over Myc and P53 proteins.

The multifunctional roles of RGN have also been associated with the control of apoptosis. So, in Chapter IV, we also evaluated the expression of target regulators of this process in the mammary gland of Tg-RGN rats. Overexpression of RGN augmented apoptosis of mammary gland cells, demonstrated by the increased activity of caspase-3 and the elevated expression of P53. These findings also account for the protective role of RGN in the prevention of mammary cancer, by favoring apoptosis, which may be also considered to act via P53 up-regulation. Although, our results support an anti-apoptotic activity of RGN, several reports have described that it may act to suppress apoptosis in response to noxious stimuli [7]. Since RGN is a cytoplasmic protein that can be translocated to the nucleus, it will be crucial to disclose whether the particular subcellular location is related to its different functions.

The sex steroid androgens and Ca^{2+} homeostasis and signaling have been described as important players in the physiology of mammary gland. Moreover, it is known that androgens inhibit proliferation of breast cancer cells and control intracellular Ca^{2+} levels by modulating the expression (activity) of several Ca^{2+} modulators. RGN is known to be involved in Ca^{2+} homeostasis and was also identified as an androgen-target gene in several tissues, but these features have not been assessed in breast cancer cells yet. In Chapter VI, we have evaluated whether the effects of 5α-dihydrotestosterone (DHT) in MCF-7 breast cancer cells may involve the regulation of RGN and $Ca_v1.2$ channel, another Ca^{2+} player that belongs to the L-type Ca^{2+} channel subfamily of voltage-dependent Ca^{2+} channels. Relatively to the $Ca_v1.2$ channel, this was the first study demonstrating its presence in breast cells. Hormonal stimulation experiments demonstrated that DHT regulates the expression of RGN and $Ca_v1.2$ channels by a mechanism dependent of androgen receptor. However, distinct effects were observed at the protein level, with a decrease of RGN and an increase of $Ca_v1.2$ channels. Nevertheless, the concomitant increase in P53 expression and the decreased cell viability led us to suggest that the modulation of intracellular Ca^{2+} by RGN and $Ca_v1.2$ channels may be on the basis of suppression of cell growth. Nevertheless, further studies are needed to determine the interaction of RGN and $Ca_v1.2$ channels in the maintenance of Ca^{2+} homeostasis and Ca^{2+} signaling in the regulation of cell proliferation.

Taken together the overall results of this thesis it is liable to suggest that the RGN protein has a preponderant and protective function in the development and progression of breast cancer. The present findings also allowed a better understanding of the biologic mechanisms of RGN action in the control of mammary gland physiology and tumorigenesis namely, cell proliferation, Ca^{2+} homeostasis, metabolism and apoptosis. This biological evidence probably encompasses the molecular basis by which RGN protein exerts its role in delaying the acquisition of tumor phenotype and the progression into more aggressive stages. Finally, the present thesis puts forward new challenges and provided additional clues for future breast cancer research, which doubtless should include the RGN actions. Further efforts are also required to definitely establish the potential of RGN as a biomarker in the management of disease or as a therapeutic target in the prevention or treatment of breast cancer.

References

1. Liang Y, Liu J, Feng Z (2013) The regulation of cellular metabolism by tumor suppressor p53. Cell & bioscience 3(1):9

2. Vousden KH, Ryan KM (2009) p53 and metabolism. Nat Rev Cancer 9(10):691-700

3. DeBerardinis RJ, Cheng T (2010) Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. Oncogene 29(3):313-324

4. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB (2008) The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metab 7(1):11-20

5. Chen JQ, Russo J (2012) Dysregulation of glucose transport, glycolysis, TCA cycle and glutaminolysis by oncogenes and tumor suppressors in cancer cells. Biochim Biophys Acta 1826(2):370-384

6. Dang CV (2013) MYC, metabolism, cell growth, and tumorigenesis. Cold Spring Harbor Perspect Med 3(8) pii: a014217

7. Correia S, Alves MG, Oliveira PF, Alves MR, van Pelt AM, Cavaco JE, Socorro S (2014) Transgenic overexpression of regucalcin leads to suppression of thapsigargin- and actinomycin D-induced apoptosis in the testis by modulation of apoptotic pathways. Andrology 2(2):290-298