UNIVERSIDADE DA BEIRA INTERIOR

Ciências

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A connection with calcium homeostasis?

Carina Sofia Gonçalves Peres

Master degree thesis in
Biochemistry
(2nd cycle of studies)

Supervisor: Sílvia Socorro, PhD
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"Imagination is more important than knowledge. For while, knowledge defines all we currently know and understand, imagination points to all we might yet discover and create."

Albert Einstein

Acknowledgements

Conception of a dissertation requires a personal work of reflection, research and inspiration. It is not merely an individualistic work, it results from several contributions to the construction of incentive, will and support for its development. For this reason, I want to thank and dedicate to all that allowed and helped me on this project.

First of all, I would like to express my deepest gratitude to my supervisor, Professor Sílvia Socorro, for the opportunity she gave me to develop this study and for the unconditional support, guidance and help during my academic journey.

I also acknowledge to my co-supervisor, Professor Cláudio Maia, for all his availability and help.

I would also like to thank to Professor Ignacio Verde for providing us L-type calcium channel (α_{1C} subunit) primers.

Moreover, I would like to thank to all my group colleagues, especially to Ricardo Marques, Inês Gomes, Sara Correia, Cátia Vaz, Luís Rato and Daniel Rodrigues, for all the advices, teaching and support. Principally, their friendship and joy was truly important to face all the difficulties during the development of this project.

I would also like to thank all my friends, especially to Ana Chegão, Andreia Ramos, Joana Varge, Carlos Gaspar and Ana Rita Magalhães, for all the good times shared, advices and patience during my academic life.

Queria agradecer, do fundo do meu coração, aos meus pais por todo o amor, apoio, carinho e confiança incondicionais que sempre me deram. Agradeço-lhes por tudo o que sou hoje! Sem eles nada disto seria possível.

Um especial obrigado à minha irmã, Patrícia, por todo o amor, carinho e amizade que sempre me demonstrou.

I could not end these acknowledgements without a special thanks to my boyfriend, Joel, for all his love, unconditional support, advices, care, patience and understanding during the good and the bad days.

To all of you… Thank you!

Abstract

Several evidences suggest that androgenic actions and calcium (Ca^{2+}) homeostasis alterations may contribute to the development of breast cancer. The androgen receptor is detected in the majority of human breast cancer cases, including those that are oestrogen and progesterone receptor negatives. It has also been shown that androgens play an important role regulating breast cells proliferation and death. On the other hand, it is known that intracellular Ca^{2+} is a ubiquitous second messenger involved in the regulation of several biological processes in the cell such as proliferation and apoptosis. In this way, deregulation of intracellular Ca^{2+} concentration via altered expression and/or function of Ca^{2+} transporters, Ca^{2+} channels and/or Ca^{2+} binding proteins may have implications breast pathophysiology. Recently, studies have demonstrated that androgens regulate the expression and/or activity of several Ca^{2+} regulator proteins, namely the Ca^{2+}-binding protein regucalcin and voltage-dependent L-type Ca^{2+} channel in distinct cell types. The present project aims to investigate the effect of androgen 5α-dihydrotestosterone (DHT) on the expression of regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) in human breast cancer cells (MCF-7). The presence of regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) in these cells was confirmed by means of RT-PCR and Western Blot. The effect of androgens on the mRNA expression of regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) was evaluated by real-time PCR. DHT down-regulated the expression of regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) in MCF-7 cells. In both cases, this effect was reverted in presence of androgen inhibitor flutamide and oestrogen inhibitor ICI 182,780, suggesting that DHT effects regulating regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) expression are mediated by the androgen receptor, but also by the oestrogen receptor due to the metabolization of DHT to oestrogenic products. This study first demonstrated the presence of L-type Ca^{2+} channel (α_{1C} subunit) in human breast cancer cells and showed that androgens modulate expression of Ca^{2+} regulator proteins in these cells. These findings suggest that androgenic actions regulating cell death and proliferation of breast cancer cells may be associated with the control of Ca^{2+} homeostasis.

Keywords

Breast cancer, MCF-7 cells, androgens, DHT, regucalcin, L-type calcium-channel

Resumo

Várias evidências sugerem que as ações androgénicas e alterações na homeostasia do cálcio (Ca^{2+}) podem contribuir para o desenvolvimento do cancro de mama. O recetor de androgénios é detetado na maioria dos casos de cancro de mama humano, incluindo os que são considerados negativos para os recetores de estrogénios e de progesterona. Também se tem demonstrado que os androgénios desempenham um papel importante na regulação da proliferação e morte em células da mama. Por outro lado, sabe-se que o Ca^{2+} intracelular é um segundo mensageiro ubíquo envolvido na regulação de diversos processos biológicos da célula, tais como a proliferação e a apoptose. Desta forma, a desregulação da concentração intracelular de Ca^{2+}, através de alterações na expressão e/ou função de transportadores de Ca^{2+}, canais de Ca^{2+} e/ou proteínas de ligação ao Ca^{2+}, pode ter implicações na fisiopatologia da mama. Recentemente, alguns estudos demonstraram que os androgénios regulam a expressão e/ou atividade de várias proteínas reguladoras de Ca^{2+}, nomeadamente a proteína de ligação ao Ca^{2+} regucalcina e o canal de Ca^{2+} dependente de voltagem do tipo L, em tipos celulares distintos. Com o presente projeto pretende-se investigar o efeito do androgénio 5α-dihidrotestosterona (DHT) na expressão da regucalcina e do canal de Ca^{2+} do tipo L (subunidade α_{1C}) em células de cancro da mama humano (MCF-7). A presença da regucalcina e do canal de Ca^{2+} do tipo L (subunidade α_{1C}) nestas células foi confirmada por RT-PCR e Western Blot. O efeito dos androgénios na expressão do mRNA da regucalcina e do canal de Ca^{2+} do tipo L (subunidade α_{1C}) foi avaliado por PCR em tempo real. A DHT diminui a expressão da regucalcina e do canal de Ca^{2+} do tipo L (subunidade α_{1C}) em células MCF-7. Em ambos os casos, este efeito foi revertido em presença do inibidor de androgénio flutamida e do inibidor de estrogénios ICI 182,780, sugerindo que os efeitos da DHT na regulação da expressão da regucalcina e do canal de Ca^{2+} do tipo L (subunidade α_{1C}) são mediados pelo recetor de androgénios, mas também pelo recetor de estrogénios devido à metabolização da DHT em produtos estrogénicos. Este estudo demonstrou primeiramente a presença do canal de Ca^{2+} do tipo L (subunidade α_{1C}) nas células de cancro da mama humano e demonstrou que os androgénios regulam a expressão de proteínas reguladoras de Ca^{2+} nestas células. Estes resultados sugerem que a ação reguladora dos androgénios na proliferação e morte celular nas células de cancro da mama pode estar associada ao controlo da homeostasia do Ca^{2+}.

Palavras-chave

Cancro da mama, células MCF-7, androgénios, DHT, regucalcina, canais de cálcio tipo L

Resumo Alargado

O cancro de mama é atualmente o cancro mais comum e a causa mais frequente de morte induzida por cancro nas mulheres nos países ocidentais. Nos Estados Unidos, uma em cada oito mulheres, e na Europa uma em cada dez, serão afetadas por esta doença durante a sua vida. Estima-se que mais de um milhão de pessoas no mundo sejam anualmente diagnosticadas com a doença. Apesar da recente diminuição na taxa de mortalidade por cancro de mama nalguns países, essencialmente devido à deteção precoce através de rastreios sistemáticos, vias de diagnóstico eficazes e tratamento ideal, esta doença continua a ser um importante problema de saúde pública. Vários fatores de risco têm sido associados ao cancro de mama, no entanto um deles merece especial atenção. Considerando que o cancro de mama surge em tecidos regulados por hormonas, vários estudos sugerem que alterações hormonais, nomeadamente nos androgénios, podem desempenhar um papel importante no surgimento e progressão do tumor. Por outro lado, várias evidências sugerem que, para além das ações androgénicas, também as alterações na homeostasia do cálcio (Ca^{2+}) podem contribuir para o desenvolvimento do cancro de mama.

Na mulher, os androgénios são secretados pelos ovários e pelas glândulas adrenais em resposta às suas hormonas trópicas, no entanto também a glândula mamária tem capacidade de sintetizar estas hormonas. Apesar de o seu papel ser pouco explorado nas mulheres, o recetor de androgénio é detetado na maioria dos casos de cancro de mama humano, incluindo os que são considerados negativos para os recetores de estrogénio e de progesterona, o que sugere um papel importante destas hormonas no carcinoma da mama. Os androgénios medeiam os seus efeitos biológicos através de mecanismos genómicos e não-genómicos. Os efeitos genómicos podem dever-se diretamente à ligação dos androgénios e consequente ativação do recetor de androgénio ou indiretamente, à ativação do recetor de estrogénio devido à conversão dos androgénios em estrogénios. Por outro lado, e embora a 5α-dihidrotestosterona (DHT) seja não-aromatizável, este esteróide pode ser metabolizado em produtos com actividade estrogénica. Os efeitos não-genómicos podem resultar da ativação de recetores membranares acoplados à proteína G, que por sua vez ativam diversas vias de sinalização possivelmente envolvidas na regulação da proliferação e morte celular.

O Ca^{2+} intracelular é um segundo mensageiro ubíquo envolvido na regulação de diversos processos biológicos essenciais na célula, tais como a proliferação e a apoptose. A homeostasia do Ca^{2+} é um estado de equilíbrio entre o influxo, efluxo e armazenamento de Ca^{2+} essencialmente regulado por transportadores de Ca^{2+}, canais de Ca^{2+} e proteínas de ligação ao Ca^{2+} (incluindo tampões citosólicos de Ca^{2+}). A desregulação da concentração intracelular de Ca^{2+}, nomeadamente devido a alterações na expressão e/ou função de transportadores de Ca^{2+}, canais de Ca^{2+} e proteínas de ligação ao Ca^{2+} pode também contribuir para o desenvolvimento do cancro de mama. Vários estudos têm demonstrado que os

andrógenos têm a capacidade de alterar a concentração intracelular de Ca^{2+}, em diferentes sistemas celulares, podendo modificar a expressão e/ou função das várias proteínas reguladoras de Ca^{2+}, como a proteína de ligação ao Ca^{2+} regucalcina e o canal de Ca^{2+} dependente de voltagem do tipo L, e consequentemente afetar a homeostasia do Ca^{2+}.

A regucalcina, também designada de proteína marcadora de senescência 30, é uma proteína de ligação ao Ca^{2+} que não contém o motivo *EF-hand* no domínio de ligação ao Ca^{2+}. Esta proteína está envolvida em diversas funções tais como a manutenção da homeostasia intracelular do Ca^{2+}, devido à ativação de diversas enzimas de bombeamento de Ca^{2+} na membrana plasmática, no retículo endoplasmático e na mitocôndria de várias células, mas também se encontra envolvida na regulação de diversas enzimas dependentes de Ca^{2+} e vias de sinalização, na regulação de funções nucleares, proliferação e apoptose.

Os canais de Ca^{2+} do tipo L são canais dependentes de voltagem existentes em células excitáveis e não-excitáveis que têm como função a manutenção da homeostasia do Ca^{2+}, regulando o influxo de Ca^{2+} do espaço extracelular para o citoplasma.

Com o presente projecto pretendeu-se investigar o efeito da DHT na expressão da regucalcina e do canal de Ca^{2+} do tipo L (subunidade α_{1C}) em células de cancro da mama humano (MCF-7). Primeiramente, demonstrou-se a presença da regucalcina e do canal de Ca^{2+} do tipo L (subunidade α_{1C}) nestas células através de RT-PCR e Western blot, usando iniciadores e anticorpos específicos, respetivamente. A análise por Western blot, permitiu a deteção de uma proteína imunorreativa com o peso esperado de aproximadamente 33 kDa correspondente à regucalcina, e uma outra de aproximadamente 190 kDa, correspondente ao canal de Ca^{2+} do tipo L (subunidade α_{1C}). Posteriormente, através de PCR em tempo real, demonstrou-se que os androgénios diminuem a expressão do mRNA de ambas as proteínas após 24h de estímulo com a DHT. De modo a determinar o mecanismo envolvido na diminuição de expressão da regucalcina e do canal de Ca^{2+} do tipo L (subunidade α_{1C}) pelos androgénios, as células MCF-7 foram estimuladas com DHT na presença de inibidores do recetor de androgénios (flutamida) e do recetor de estrogénios (ICI 182,780). Verificou-se que a diminuição da expressão de mRNA da regucalcina e do canal de Ca^{2+} do tipo L (subunidade α_{1C}) causada nas MCF-7 pelo tratamento com DHT é revertida em presença dos inibidores, sugerindo que os efeitos da DHT na regulação da expressão de ambas as proteínas poderão ser mediados pelo recetor de androgénios, mas também pelo recetor de estrogénios devido à metabolização da DHT em produtos com actividade estrogénica. Este estudo demonstrou primeiramente a presença do canal de Ca^{2+} do tipo L (subunidade α_{1C}) nas células de cancro da mama humano e demonstrou que os androgénios regulam a expressão de proteínas reguladoras de Ca^{2+} nestas células. Estes resultados sugerem que a ação reguladora dos androgénios na proliferação e morte celular pode estar associada ao controlo da homeostasia do Ca^{2+}.

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List of Acronyms

Δ4	Regucalcin exon 4-deleted variant
Δ4,5	Regucalcin exon 4- and exon 5-deleted variant
3ß-diol	5α-androstane-3ß-17ß-diol
aa	Amino acid
AC	Adenylyl cyclase
AMP	Adenosine monophosphate
AP-1	Activator protein-1
AP-2	Activator protein-2
AR	Androgen receptor
ATP	Adenosine triphosphate
ßAR	B-adrenergic receptor
bp	Base pair
Ca^{2+}	Calcium ion
$[Ca^{2+}]_i$	Intracellular calcium concentration
CaM	Ca^{2+}/calmodulin-dependent protein
cAMP	Cyclic adenosine-3-5-monophosphate
CaR	Calcium-sensing receptor
cDNA	Complementary deoxyribonucleic acid
Cl^-	Chloride ion
CO_2	Carbon dioxide
DAG	Diacylglycerol
DCIS	Ductal carcinoma in situ
DEPC	Diethylpyrocarbonate
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DHT	5α-dihydrotestosterone
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DFP	Diisopropyl phosphorofluoridate
DTT	Dithiothreitol
E2	17ß-estradiol
EGFR	Epidermal growth factor receptor
ER	Oestrogen receptor
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT2	Glucose transporter 2
GnRH	Gonadotropin releasing hormone
GTP	Guanosine-5'-triphosphate
HER-2	Human epidermal growth factor receptor 2
IDC	Infiltrating ductal carcinoma
IDL	Infiltrating lobular carcinoma
IGF-1	Insulin-like growth factor-1
IP3	Inositol-1,4,5-triphosphate
K^+	Potassium ion
Kf	Association constant
LCIS	Lobular carcinoma in situ
LH	Luteinising hormone
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
Mef2c	Myocyte-specific enhancer factor 2C
Mg^{2+}	Magnesium ion
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
mRNA	Messenger ribonucleic acid
Na^+	Sodium ion
NCX	Na+/Ca^{2+}-exchanger
NF-κB	Nuclear factor-κB
NF1-A1	Nuclear factor 1-A1
NO	Nitric oxide
nt	Nucleotide
pH	Potential hydrogen
pI	Isoelectric point
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMCA	Plasma membrane Ca^{2+}-ATPase
PR	Progesterone receptor
pRb	Retinoblastoma protein
RGN	Regucalcin
RGPR-p117	Regucalcin gene promoter region-related protein 117
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction

SERCA	Sarcoplasmatic/endoplasmatic reticulum Ca^{2+}-ATPase
SH	Sulfhydryl group
SHBG	Sex hormone-binding globulin
SMP30	Senescence marker protein-30
Sp1	Specificity protein 1
SPCA	Secretory pathway Ca^{2+}-ATPase
TBS-T	Tris-buffered saline with Tween 20
TG	Transgenic
TNF-α	Tumour necrosis factor-α
TRPM8	Transient receptor potential cation channel subfamily M member 8
TRPP8	Transient receptor potential cation channel subfamily P member 8
TRPV6	Transient receptor potential cation channel subfamily V member 6
tRNA	Transfer ribonucleic acid
U	Enzyme unit
UV	Ultraviolet
Wt	Wild-type
Zn^{2+}	Zinc ion

I. Background and Aim

1. Breast – Anatomy and Pathophysiology

The breasts and the mammary glands are often mistakenly regarded as one and the same. Breasts are present in both sexes, and are considered as one of the secondary sexual characteristics (*1*). The mammary glands are considered as highly modified apocrine sweat glands and milk-producing glands, that develop within the female breast only during pregnancy and lactation (*1, 2*).

1.1. Overview of anatomical and physiological features

The breasts are positioned between the second and sixth ribs and overlies the pectoral muscles, and portions of the serratus anterior and external abdominal oblique muscles (Figure 1). The axillary process of the breast extends upwards and laterally towards the axilla, where it comes into close relationship with the axillary vessels. This region of the breast is clinically significant because of the high incidence of breast cancer cells within the lymphatic drainage of the axillary process (*3*).

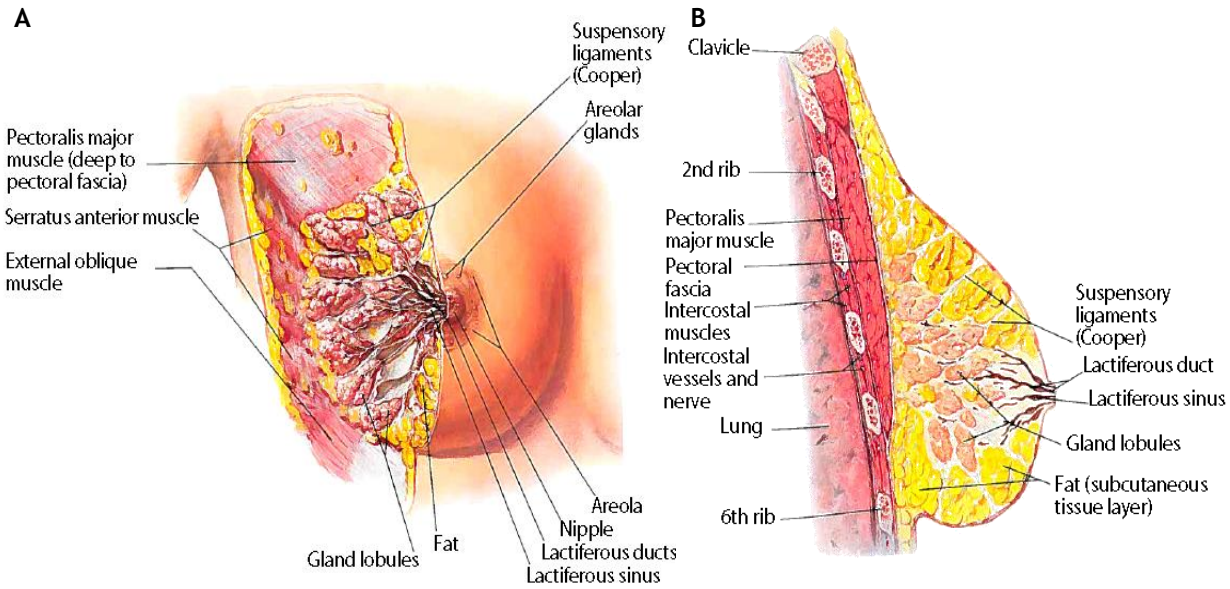


Figure 1. Human breast anatomy. (A) An anterolateral view partially sectioned. (B) A sagittal section (Adapted from (*4*)).

Each mammary gland is composed of 15 to 20 lobes (*5*) (Figure 1), which lobes are separated by dense connective tissue and by varying amounts of adipose tissue which determines the size and shape of the breast. In turn, the lobes are subdivided into a variable number of lobules that contain the mammary glandular alveoli, the structures that produce milk in a lactating female (*3, 6*). The lobes are arranged radially at different depths around

the nipple with a single large duct, the lactiferous duct, draining each lobe via a separate opening on the surface of the nipple. (*3*).

Each lobe is a system of ever-branching ducts which penetrate deep into the fibroadipose tissue of the breast. This extensive branching duct system is surrounded by relatively dense fibrous interlobular tissue, at the periphery of which there is adipose tissue. Each duct is lined by two columnar or cuboidal epithelium, with a continuous surface layer of epithelial cells with oval nuclei and an outer discontinuous layer of myoepithelial cells which have clear cytoplasm (Figure 2). The ducts are surrounded by loose fibrocallegenous support tissue containing a rich capillary network. Elastic fibbers are present within this fibrous sheath in all but the smallest, most peripheral branches (*2, 7*).

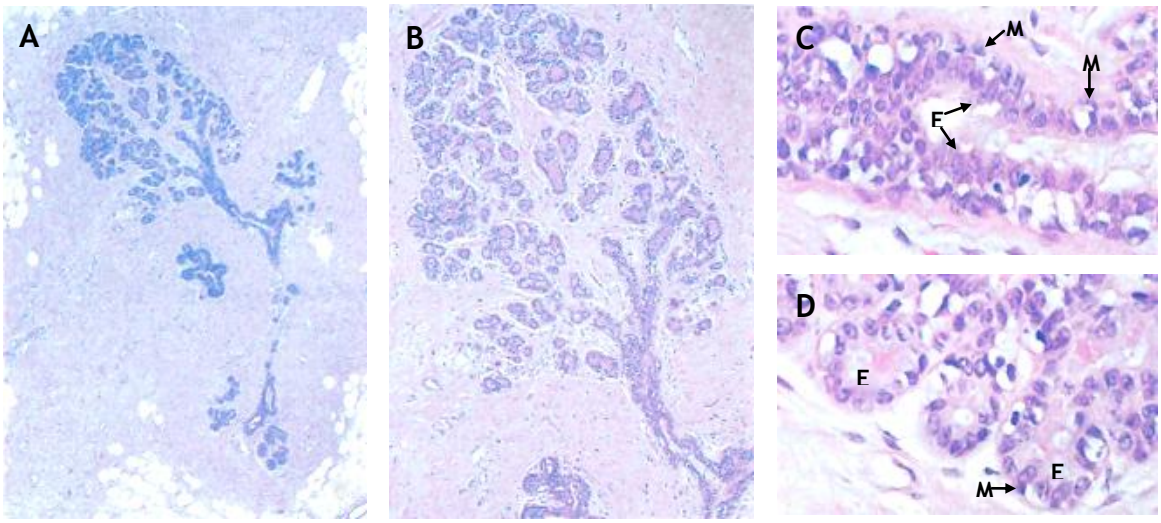


Figure 2. Histology of the human mammary gland. (A) Hematoxylin and eosin staining x30. (B) Hematoxylin and eosin staining x60. (C, D) Hematoxylin and eosin staining x400. E-epithelium, M-myoepithelium (Adapted from (*2*)).

1.2. Development and hormonal regulation

The breasts of both sexes follow a similar course of development until puberty, after which the female breasts develop under the influence of growth factors and pituitary, ovarian and other hormones (*2*). The majority of breast development occurs during puberty and pregnancy. Puberty is controlled by hormonal signals elicited by the hypothalamic-ovarian-pituitary axis. The first release of gonadotropin releasing hormone (GnRH) from the hypothalamus signals the onset of puberty. GnRH stimulates the release of luteinising hormone (LH) and follicle stimulating hormone (FSH) from the pituitary, which in its turn act upon the ovaries to promote the maturation of the ovarian follicles, and subsequent release of oestrogen and progesterone (*8*). Oestrogens, progesterone and prolactin predominantly control the developmental changes of mammary gland. In particular, oestrogen induces ductal growth, progesterone trigger alveolar lobules secretory activity and prolactin induces the alveoli to produce milk (*9*). After menopause, the breasts, like the other female reproductive tissues, undergo progressive atrophy and involution (*2*).

1.3. Carcinogenesis

Carcinogenesis is a very rare process in which a normal cell is converted to a cancer cell. These cells differ from normal ones in many important characteristics, including loss of differentiation, uncontrolled growth, immortalization, loss of contact inhibition, increased invasive capacity, evading the host immune surveillance processes and the apoptotic signal restraints, and induction of neo-angiogenesis (*10, 11*). Carcinogenesis is considered as a multistep process, in which a normal epithelial cell develops into a premalignant cell, which after clonal expansion becomes a premalignant lesion, a carcinoma in situ. After some time, such lesion may become invasive, then disseminates and, after evading the immune system, forms metastases. At each step, an important genetic event is assumed to occur that gives the cell new properties with a resulting clonal selective advantage for that cell (*12-14*) (Figure 3).

The genetic alterations associated with cancer development include inherited mutations or polymorphisms of cancer susceptibility genes, environmental agents that influence the acquisition of somatic genetic changes, and other systemic and local factors including lifestyle, diet, hormones and growth factors (*10, 14*). These genetic events range from small point mutations, via chromosomal deletions, translocations and amplifications to large-scale changes as whole chromosome losses or duplications. The result of these alterations could be the modification of gene expression or functional alteration of gene products that regulate the physiological balance between proliferation, apoptosis and differentiation, or the expression of hormones receptors, cell adhesion molecules and angiogenic factors, and of various other proteins important for invasion and the establishment of metastases (*15*). Mutational activation of oncogenes coupled with inactivation of tumour suppressor genes are probably early events in this multi-step process. Subsequently, more independent mutations occur in at least four or five other genes, the chronological order of these events possibly being less important (*16*).

Breast cancer is currently the most frequent cancer and the most frequent cause of cancer-induced deaths in women in Western countries. In the United States, one woman in eight women, and in Europe one in ten, will be affected by this disease during her lifetime (*17, 18*). It is estimated than over 1 million people are newly diagnosed with the disease in the world, annually (*18*). Men are also susceptible to breast cancer. It is 100 times less frequent than in women, but it is usually fatal (*3*). Although there have been recent declines in breast cancer mortality rates in some countries essentially due to systematic early detection through screening, effective diagnostic pathways and optimal treatment, this disease remains an important public health problem (*19, 20*).

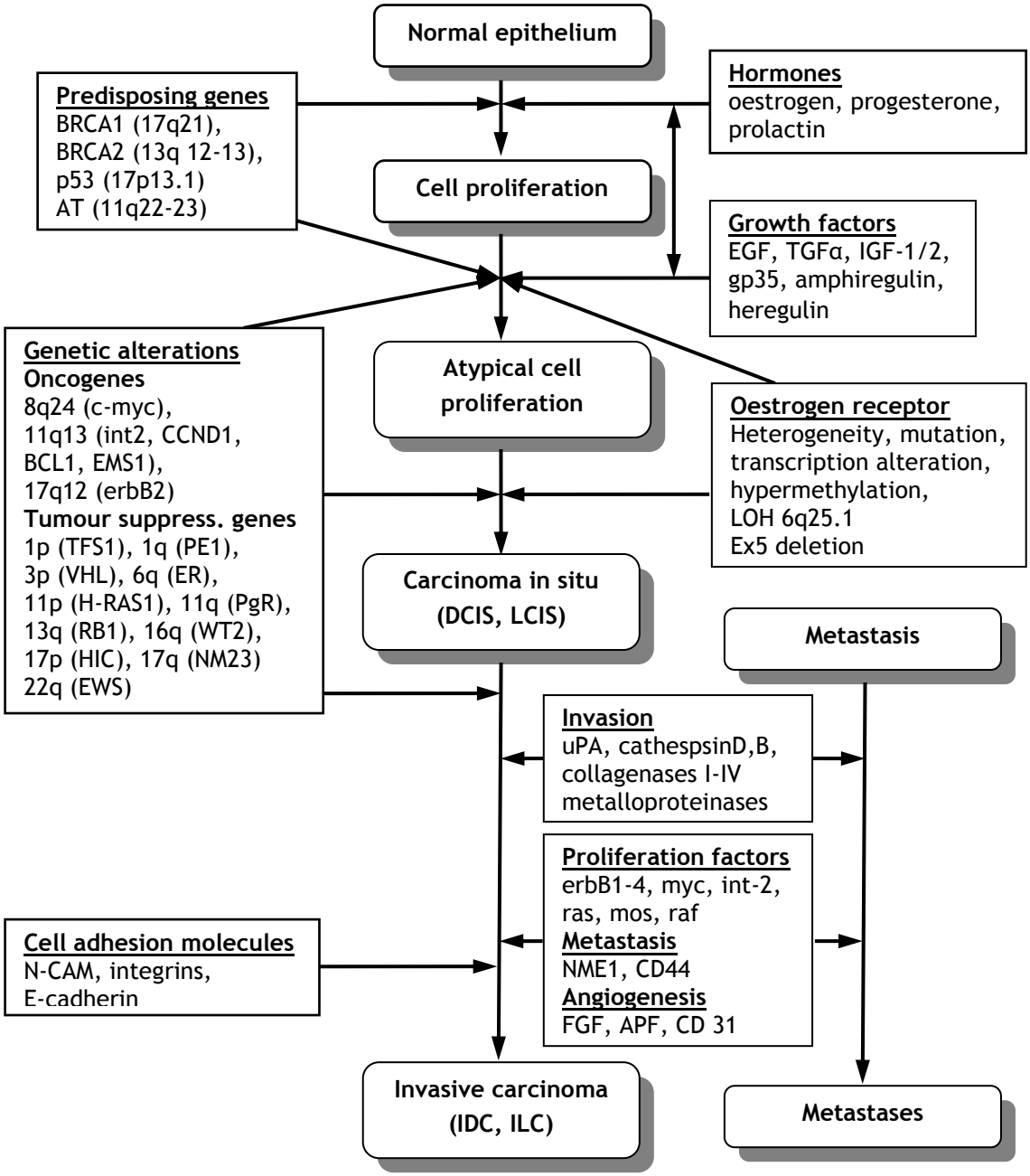


Figure 3. Model of the multistep carcinogenesis in breast cancer. A normal epithelial cell develops into a premalignant cell, which after clonal expansion becomes a premalignant lesion, a carcinoma in situ. After some time, such lesion may become invasive, then disseminates and, after evading the immune system, forms metastases. At each step, an important genetic event is assumed to occur that gives the cell new properties with a resulting clonal selective advantage for that cell (Adapted from (*14*)).

Most of breast tumours arise in the terminal duct lobular unit and, according to presentation, morphology and molecular profiling, they may be classified in ductal or lobular carcinoma *in situ* (DCIS or LCIS) and in infiltrating ductal or lobular carcinoma (IDC or IDL). DCIS and LCIS are non-invasive tumours, whereas IDC and IDL break through the wall of the duct and invade the fatty tissue of the breast, which can spread to other parts of the body (*14, 21*).

Breast cancer progression involves a sequential progression through clinical and pathologic stages, starting with hyperplasia, progressing into *in situ* and invasive carcinomas, and culminating in metastatic disease (*22*). Hyperplasia, characterized by proliferation of unevenly distributed epithelial cells is often a first sign of pathology, although the cells are benign. The next step is the development of carcinoma *in situ*, either ductal or lobular, which is defined as a proliferation of cells with cytological characteristics of malignancy, but without stromal invasion across the basement membrane. As cells detach from the basement membrane and invade the stroma, the tumour becomes invasive. Through dissemination via blood and lymph vessels, invasive cells can give rise to metastases, either to locoregional lymph nodes or to distant organs (*16, 22*).

Several factors are known to affect the risk of breast cancer however three of them deserve special attention. First, it is possible that this tumour may be influenced by a number of lifestyle and environmental factors, including dietary factors, whose importance in the development of human cancer is gaining increasing support (*23*). Second, and considering that breast carcinoma arises in an hormone regulated tissue, it is conceivable that common hormone alterations, namely androgens, could play a role on the onset and progression of this tumour (*24*). Third, it seems clear that, in addition to being hormone related, breast cancer may also share some genetic abnormalities that could contribute to the acquisition of the malignant phenotype (*25*).

2. Role of androgens in breast cancer

Until recently, androgen biology has largely focused on male reproduction whereas the role of these steroid hormones in the female reproductive tract has been largely unexplored, perhaps with the exception of androgens as precursors of oestrogen biosynthesis in the ovary (*26*). This is rather surprising as the androgen receptor (AR) is found in virtually every tissue in women (as well as men), including breast, which indicate that androgens and their metabolites play an important role in normal tissue homeostasis and possibly in pathologies like breast cancer (*27*).

2.1. Androgen biosynthesis in women

In women, androgens are secreted by the ovaries and adrenal glands in response to their respective tropic hormones. Like other steroid hormones, these C19-steroids are derived from cholesterol (*28*). The principal circulating androgens in women are dehydroepiandrosterone sulphate (DHEAS), dehydroepiandrosterone (DHEA), androstenedione, testosterone and dihydrotestosterone (DHT) (*29*). DHEAS is produced exclusively by the adrenal gland, whereas DHEA is produced in the adrenal gland, the ovary and elsewhere through peripheral conversion of DHEAS by steroid sulfatase, namely in skin, adipose tissue and liver (*30, 31*). Androstenedione is produced by the ovary and the adrenal gland (*32*). Testosterone is derived from the peripheral conversion of androstenedione and from the synthesis by the adrenal

gland and the ovary. DHT, the potent metabolite of testosterone, derives from the peripheral conversion of testosterone by the enzyme 5α-reductase (Figure 4) (*31*). Only testosterone and dihydrotestosterone are considered as "true" androgens with the ability to bind and activate the AR, while the remaining three act as prohormones (*33*). Testosterone is unique in its ability to act as both a prohormone for both DHT and oestradiol and directly as a hormone (*34*).

Female androgen levels exhibit cyclical changes: they circulate in a concentration similar to estradiol during the preovulatory peak and in a higher concentration during the rest of the menstrual cycle. After menopause the secretion of progesterone and oestrogens falls dramatically, but androgens continue being secreted in an even higher proportion than during the fertile period of life (*35*).

The mammary gland is also capable of synthesizing testosterone. All the steroidogenic enzymes necessary for the formation of androgens from steroid precursors have been reported in normal mammary tissues, breast cancer specimens and cell lines (*36*) (Figure 4).

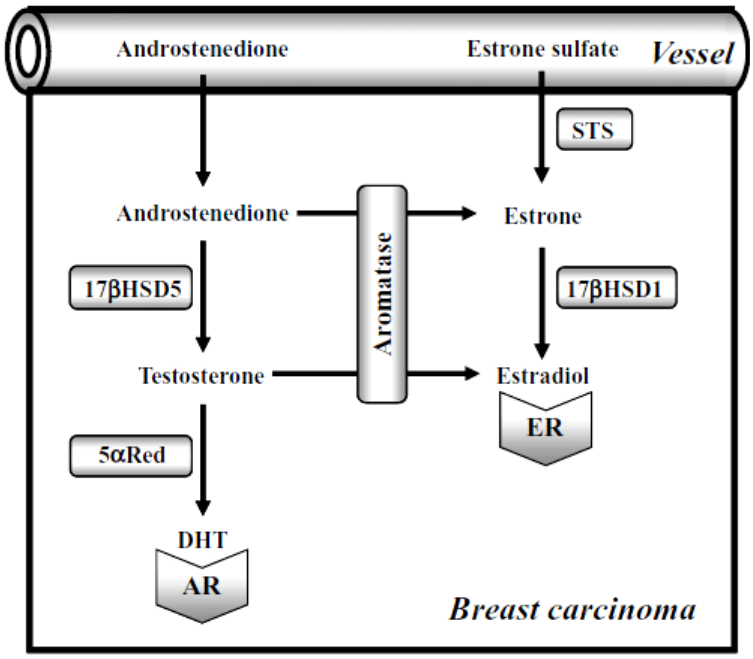


Figure 4. Scheme representing *in situ* production of androgens in breast carcinoma tissue. Biologically active DHT is locally produced from circulating androstenedione by 17β-hydroxysteroid dehydrogenase type 5 (17β-HSD5) and 5α-reductase (5α-Red) and acts on the breast carcinoma cells through androgen receptor (AR). In contrast, estradiol is synthesized by aromatase, steroid sulfatase (STS), and 17β-HSD1, then acts on the breast carcinoma cells through oestrogen receptor (ER) (*37*).

2.2. Androgens and breast carcinogenesis

Sex hormones play a critical role in breast cancer development and have been associated with an increased epithelial cell proliferation and in turn facilitating malignant transformation. In particular, two sex hormones have been very well characterized triggering these processes both *in vitro* and *in vivo*: oestrogen and progesterone. Studies have shown that the direct action of these steroid hormones on different breast tissues is dependent upon

their specific receptors (*38*). The proposed mechanism of hormonal carcinogenesis drives cell proliferation, and thus, increases the opportunity for genetic mutations to accumulate (*39*).

Also androgens play an important role in the normal development and function of many organs, as well as in the pathogenesis of endocrine-related cancers, namely breast cancer (*39*). Several studies have reported that AR is found in the majority of breast cancers and its expression is significantly higher among breast cancer samples than among normal breast (*35*). This high incidence suggests a potential role of AR in breast cancer development (*40, 41*). Moreover, some of breast cancer cases negative for oestrogen receptor (ER) and progesterone receptor (PR) still are positive for AR (*42*). However, the etiological role of androgens in breast cancer has been unclear (*43*).

Androgens mediate their biological effects, namely proliferation, differentiation, and homeostasis, through genomic and non-genomic mechanisms (*44*). The nature of the steroid-induced signal (*i.e.* genomic versus non-genomic) depend on the type of target cell, the receptor location within cells, as well as the ligand itself (*45*).

The genomic model for androgens action have been hypothesized to increase breast cancer risk either directly, by their binding and activation of AR, or indirectly, by their conversion to oestrogen and subsequent binding and activation of ER (*46*). Oestrogens may result from the conversion of testosterone by the enzyme aromatase (*37*). On the other hand, although DHT is a non-aromatizable androgen, it may be converted into 5α-androstane-3ß-17ß-diol (3ß-diol), a metabolite with intrinsic oestrogen-like effects (*47*). It is assumed that androgens and oestrogens freely cross the plasma membrane, enter the cytoplasm, and bind to and activate the intracellular receptor. The ligand-bound receptor acts as a transcription factor binding as heterodimer to specific deoxyribonucleic acid (DNA) response elements in target gene promoters, causing activation or repression of transcription and subsequently protein synthesis (*48-50*) (Figure 5A).

Studies in a variety of *in vitro* and *in vivo* models have shown that androgens and oestrogens can affect cellular processes in a non-genomic fashion. For instance, hormone bound/activated nuclear receptors are able to interact with other transcription factors on target gene promoters without direct binding to DNA (*51*). Rapid effects of androgens have been described in various tissues such as brain, blood, heart, bone, ovary, prostate and testis (*52*).

AR has been shown to activate second messenger pathways independent of their classical transcriptional activity (Figure 5B,C). Consistent with this mode of action, AR has been found to interact with and activate the tyrosine kinase c-Src. The activation of the c-Src mediates the activation of mitogen-activated protein kinase (MAPK), which is involved in multiple cellular processes, including migration, proliferation, and differentiation (*53*). Androgens can also induce cyclic adenosine-3-5-monophosphate (cAMP) production and, consequently, activation of protein kinase A (PKA) through the sex hormone-binding globulin (SHBG) receptor. SHBG is a liver derived glycoprotein that binds to sex hormones, specifically

testosterone, DHT, and oestradiol (*54*). A cell surface receptor for SHBG has been identified in a number of tissues namely in prostate and breast (*55, 56*).

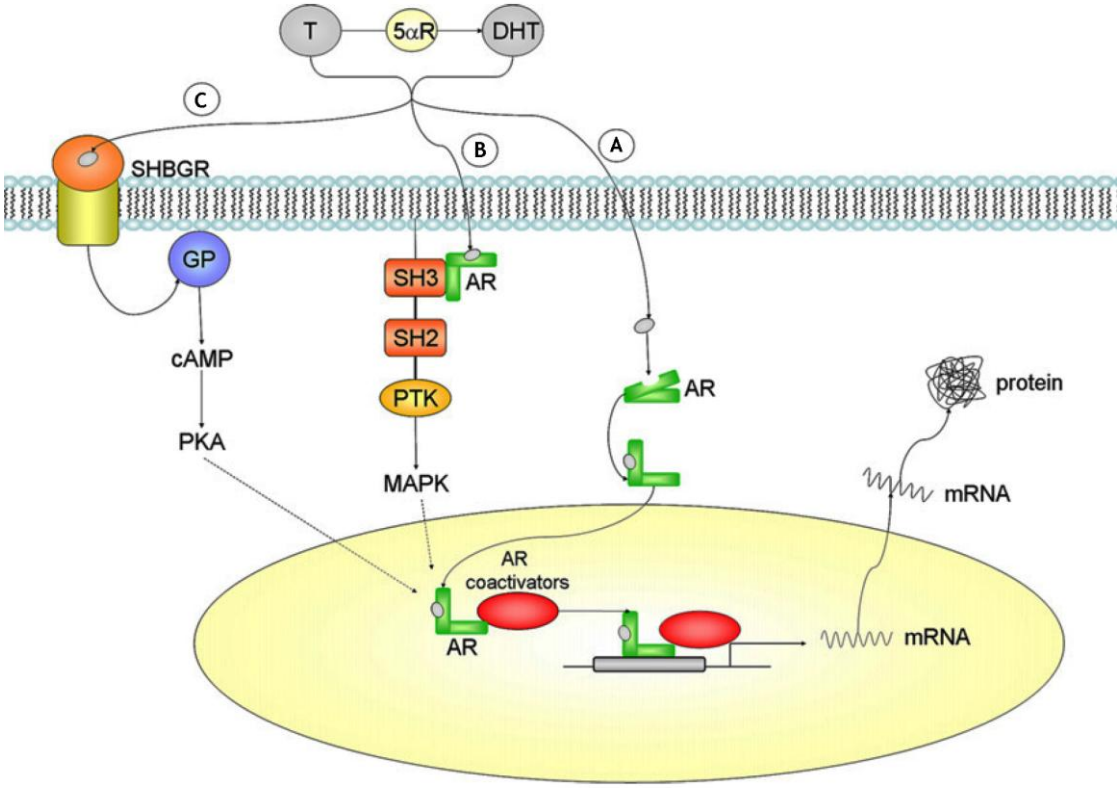


Figure 5. Androgen actions via intracellular androgen receptor-mediated pathways. Testosterone (T) can be converted to dihydrotestosterone (DHT) by the 5α-reductase enzyme. (A) In the classical pathway, androgen freely passes through the membrane bi-layer and binds cytoplasmic androgen receptor (AR). Bound AR translocates to the nucleus, binds to a DNA response element on a promoter of an androgen responsive gene and stimulates transcription. (B) Bound AR interacts with the tyrosine kinase c-Src to activate the MAPK pathway. (C) Androgen bound to steroid hormone-binding globulin (SHBG) can activate SHBG receptor (SHBGR) and lead to an increase in PKA activity (Adapted from (*44*)).

2.3. Androgens as calcium homeostasis regulators

Intracellular calcium (Ca^{2+}) is a second messenger involved in the regulation of many essential biological processes in the cell such as fertilization, proliferation, differentiation, secretion, contraction, transcription, phosphorylation and apoptosis (*57*). Ca^{2+} homeostasis is a steady state between Ca^{2+} influx, efflux, and storage, which is essentially regulated by the activity of Ca^{2+} transporters, Ca^{2+} channels and Ca^{2+} binding proteins (included cytosolic Ca^{2+} buffers) (*58*) (Figure 6). Under normal conditions, the intracellular calcium concentration $[Ca^{2+}]_i$ is kept very low at approximately 100 nM, whereas extracellular Ca^{2+} concentration is up to 10,000-fold higher at around 1–2 mM (*59*). A Ca^{2+} signal is induced when $[Ca^{2+}]_i$ is elevated beyond its resting concentration. Mechanisms for increasing $[Ca^{2+}]_i$ include the entry of extracellular Ca^{2+} via Ca^{2+} channels in the plasma membrane (voltage-operated channels, receptor-operated channels and members of the transient receptor potential ion channel family) or the release of stored Ca^{2+} from intracellular organelles via Ca^{2+} channels in internal membranes. Elevated $[Ca^{2+}]_i$ generates an intracellular Ca^{2+} signal that is modified and decoded by Ca^{2+} binding proteins to regulate cellular processes. Once Ca^{2+} has served its

signaling function, $[Ca^{2+}]_i$ is lowered to resting levels to maintain intracellular Ca^{2+} homeostasis. Ca^{2+} is either sequestered into intracellular organelles by pumps such as the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) or is extruded to the extracellular environment by transporters such as the Na^+/Ca^{2+}-exchanger (NCX) or the plasma membrane Ca^{2+}-ATPase (PMCA) (*57*). Mitochondria are important organelles for the sequestration of intracellular Ca^{2+} via the mitochondrial Ca^{2+}-uniporter that is located in the inner mitochondrial membrane and is driven by the negative electrical potential across this membrane (*60*). When signaling has ceased, Ca^{2+} can then be released back slowly from mitochondria to the cytosol via the mitochondrial NCX, for uptake by the endoplasmic reticulum or removal from the cell (*57, 60*). Another important organelle vital for cellular Ca^{2+} homeostasis is the Golgi apparatus, which sequesters intracellular Ca^{2+} and modulates Ca^{2+} signals via action of secretory pathway Ca^{2+}-ATPases (SPCAs) (*61*) .

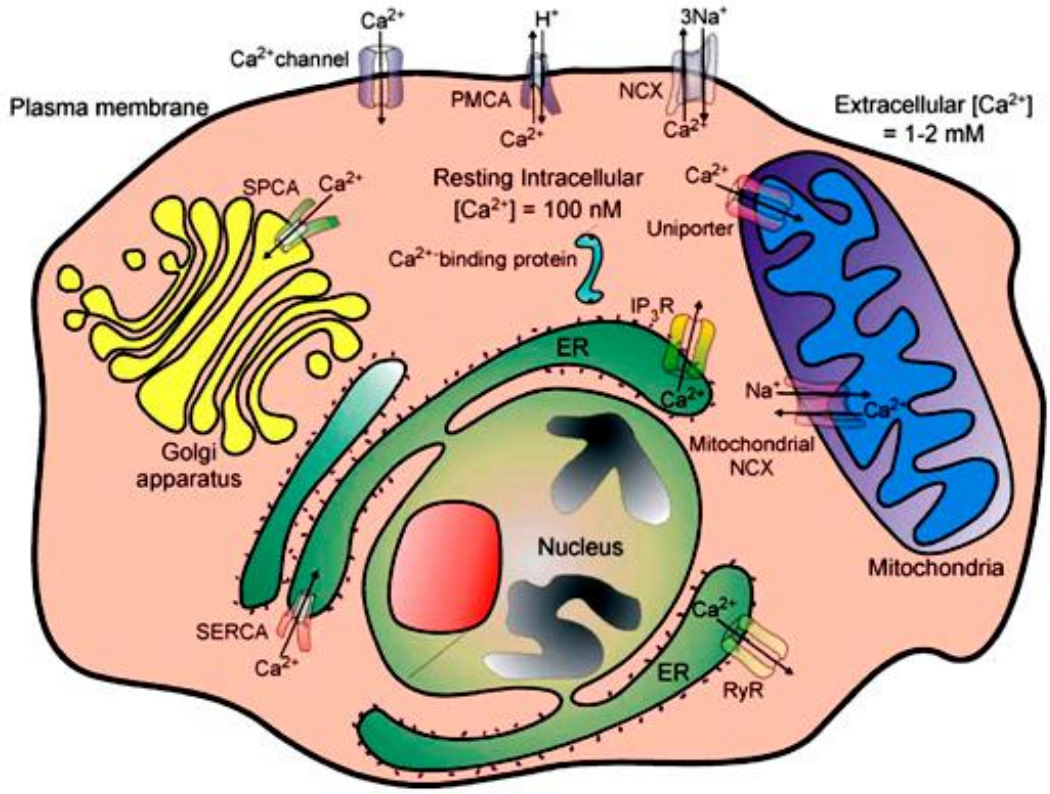


Figure 6. Calcium (Ca^{2+}) regulation in cells. The Ca^{2+} transporters, channels and binding proteins, which are likely to contribute to Ca^{2+} homeostasis and signaling in cells of the mammary gland (Adapted from (*62*)).

Several studies have demonstrated that androgens are able to modulate the $[Ca^{2+}]_i$ in different cell systems, including cardiomyocytes, macrophages, osteoblasts, neuroblastomas cells, and Sertoli cells (*63-72*). Androgens have been shown to have profound effects on the cells of the cardiovascular system where they can induce relaxation of the aorta and coronary arteries (*64*), but they can also facilitate vasoconstriction (*65, 68*). In cardiomyocytes, androgens have been found to induce a rapid $[Ca^{2+}]_i$ increase due to release of Ca^{2+} from internal stores through the activation of a plasma membrane AR associated with the G-

protein, and consequent activation of phospholipase C (PLC)/inositol-1,4,5-triphosphate (IP3) signaling pathway (*67*) (Figure 7B). Moreover, the activation of AR associated with the G-protein can also activate voltage-gated Ca^{2+} channels (e.g. L-type Ca^{2+} channels), and then contribute to the $[Ca^{2+}]_i$ increase (*63*) (Figure 7A). In macrophages, treatment with androgens induced an increase in $[Ca^{2+}]_i$ through the activation of AR associated with the G-protein, and subsequent activation of PLC/IP3 pathway signaling and non-voltage-gated (*71*). A rapid AR-independent effects of testosterone on intracellular $[Ca^{2+}]_i$ has also been shown in neuroblastomas cells. The initial transient rise in $[Ca^{2+}]_i$ was dependent upon production of IP3, but propagation of the Ca^{2+} rise required both Ca^{2+} influx from extracellular sources as well as Ca^{2+} release from intracellular stores (*70*). A similar response is found in rat osteoblasts, where androgens induced both the influx of extracellular Ca^{2+} via Ca^{2+}-channels and Ca^{2+} release from internal stores through G-protein coupled receptors activating PLC (*69*). The ability of androgens to induce a rapid influx of Ca^{2+} has also been reported in primary cultures of rat Sertoli cells. The $[Ca^{2+}]_i$ increases in Sertoli cells can be inhibited by the AR antagonist flutamide, which suggest that androgens bind to a classical-AR and activate a transmembrane influx of extracellular Ca^{2+} (*66*).

Androgens also modulate the $[Ca^{2+}]_i$ in pathophysiolocal states, namely in prostate cancer. In fact, they have been also shown to increase $[Ca^{2+}]_i$ in human prostate cancer cells LNCaP and PC3. Such as in cardiomyocytes, the androgens induce a $[Ca^{2+}]_i$ increase due to release of Ca^{2+} from internal stores through the activation of a plasma membrane AR associated with the G-protein, and subsequent activation of PLC/IP3 signaling pathway and voltage-gated Ca^{2+}-channels (*72, 73*). Such as described above, activation of Ca^{2+}-channels and PLC/IP3 signaling pathway contribute to an $[Ca^{2+}]_i$ increase. This increase can lead, on the one hand, to the activation of protein kinase C (PKC), and via calmodulin activate PKA and MAPK pathways (Figure 7A), and to the other hand, to the activation of the RAS/MEK/ERK pathway (Figure 7B), which may influence gene transcription (*44*). As well as possibly regulating lactation in the breast, intracellular Ca^{2+} signaling may have physiological roles in controlling normal mammary gland epithelial cell proliferation, differentiation and apoptosis. To keep Ca^{2+} homeostasis, the mammary gland co-ordinately express and regulate Ca^{2+}-transporters, Ca^{2+}-channels and Ca^{2+}-binding proteins. Deregulation of $[Ca^{2+}]_i$ via altered Ca^{2+}-transporters, Ca^{2+}-channels and Ca^{2+}-binding proteins expression and/or function may have implications for mammary gland pathophysiology (*58*). Several studies have reported that androgens may induce alterations in these proteins expression. Concerning Ca^{2+}-channels, androgens have been shown to increase L-type Ca^{2+}-channel expression in coronary smooth muscle cells and myocardium (*74*). They also control the transient receptor potential cation channel subfamily M member 8 (TRPM8) in prostate cells (*75*). Concerning Ca^{2+}-binding proteins, androgens have been shown to decrease the regucalcin expression in several cell types and tissues (*76-78*).

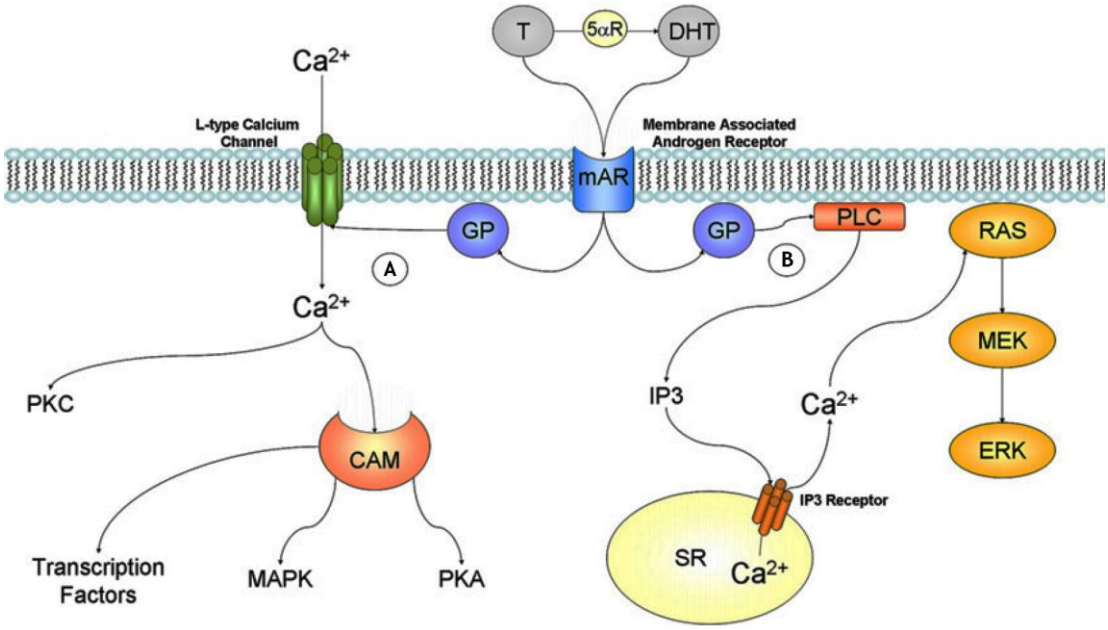


Figure 7. Non-genomic androgen actions via changes in intracellular ion concentrations and membrane fluidity. (1) Androgen interacts with a membrane associated androgen receptor (mAR) leading to the activation of L-type Ca^{2+} channels through an inhibitory G-protein (GP). This increase in intracellular Ca^{2+} can lead to activation of PKC, and via calmodulin (CAM) activate PKA and MAPK pathways, ultimately influencing gene transcription through phosphorylation. (2) Androgen interacts with a mAR leading to modulation of G-protein activity and subsequent activation of phospholipase C (PLC). Resulting increases in IP3 lead to the release of intracellular Ca^{2+} stores from the sarcoplasmic reticulum (SR), and consequently the activation of the RAS/MEK/ERK pathway (Adapted from (*44*)).

3. Regucalcin: structural and functional characterization

Regucalcin was discovered in 1978 as a Ca^{2+}-binding protein differing from other Ca^{2+}-related proteins, by the fact that it does not contain the EF-hand motif as Ca^{2+}-binding domain (*79-82*). The name regucalcin was proposed due to its capacity to regulate the activity of several Ca^{2+}-dependent enzymes in various cell types (*83-87*). It is also known as senescence marker protein-30 (SMP30), since the protein with a molecular mass of 30 kDa shows a significantly decreased expression in aged rats (*88-90*).

3.1. Physicochemical properties, cell localization and tissue distribution

Regucalcin protein is encoded by a gene linked to the X-chromosome. The human regucalcin gene is localized on the Xp 11.3 – Xp 11.23 regions (*91*), while the rat regucalcin gene has been assigned to the proximal end of the chromosome, region Xq 11.1-12 (*92*). The rat regucalcin gene seems to be about 18 kb long and consists of seven exons, six introns and several consensus regulatory elements upstream of the 5'-flanking region (*93*). The rat regucalcin complementary deoxyribonucleic acid (cDNA) segment, 1,600 base pairs (bp) in length, has an open reading frame of 897 bp, which encodes a protein with 299 amino acids (aa), an estimated molecular weight of 33,388 Da and a pI of 5.101 (*89*). Primer extension analysis revealed two major transcription initiation sites located 101 and 102 bp upstream

from the ATG translation initiation codon. In the proximal promoter region, a TATA-like sequence, a CAAT box and specificity protein 1 (Sp1) sites seems to be located at nucleotide (nt) 229, 272, and 2169, respectively. In addition to these conventional transcription factor binding sites, there were found two clustered Sp1 boxes with activator protein-2 (AP-2) at nt 2900 and 21376 in the distal promoter region (*94*) (Figure 8).

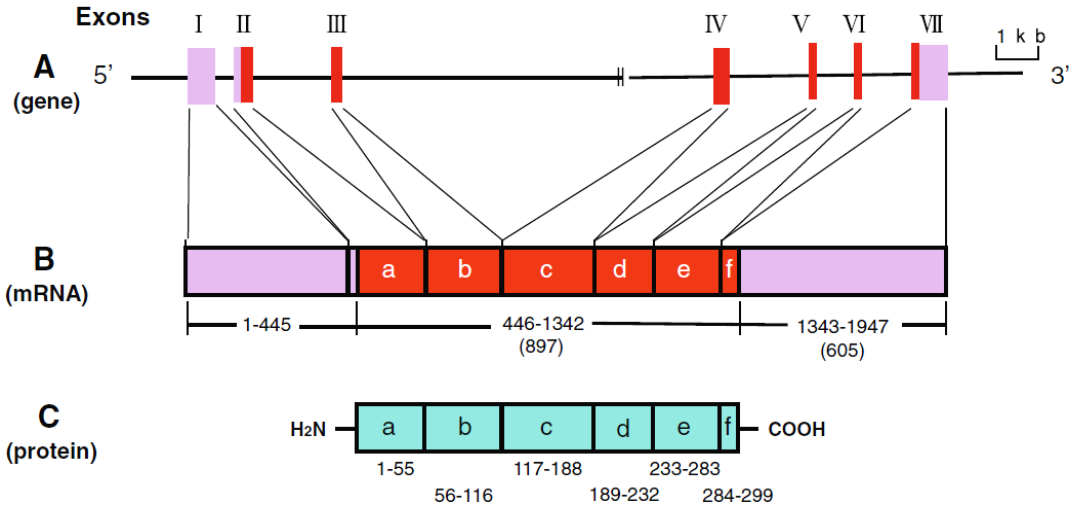


Figure 8. Alignment of the exons for the rat regucalcin gene with the cDNA. (A) Genomic organization of rat regucalcin gene. The positions of exons, which are shown as boxes (I-VII), are indicated in the agreement with the protein coding regions. Intones are depicted by connecting lines. (B) Diagram of the regucalcin cDNA from rat liver. (C) Organization of amino acids residues of regucalcin (*78*).

At messenger ribonucleic acid (mRNA) level, recently was shown that regucalcin gene can be transcribed three generating different transcripts: the full-length regucalcin transcript (wild-type, wt) and two transcript variants, regucalcin exon 4-deleted (Δ4) and regucalcin exon 4- and exon 5-deleted variant (Δ4,5) (Figure 9). The Δ4 and Δ4,5 transcripts which could be detected in neoplastic and non-neoplastic tissues (Figure 9A), are likely generated by alternative splicing events and if translated may encode proteins with 227 and 183 aa, respectively (*77*).

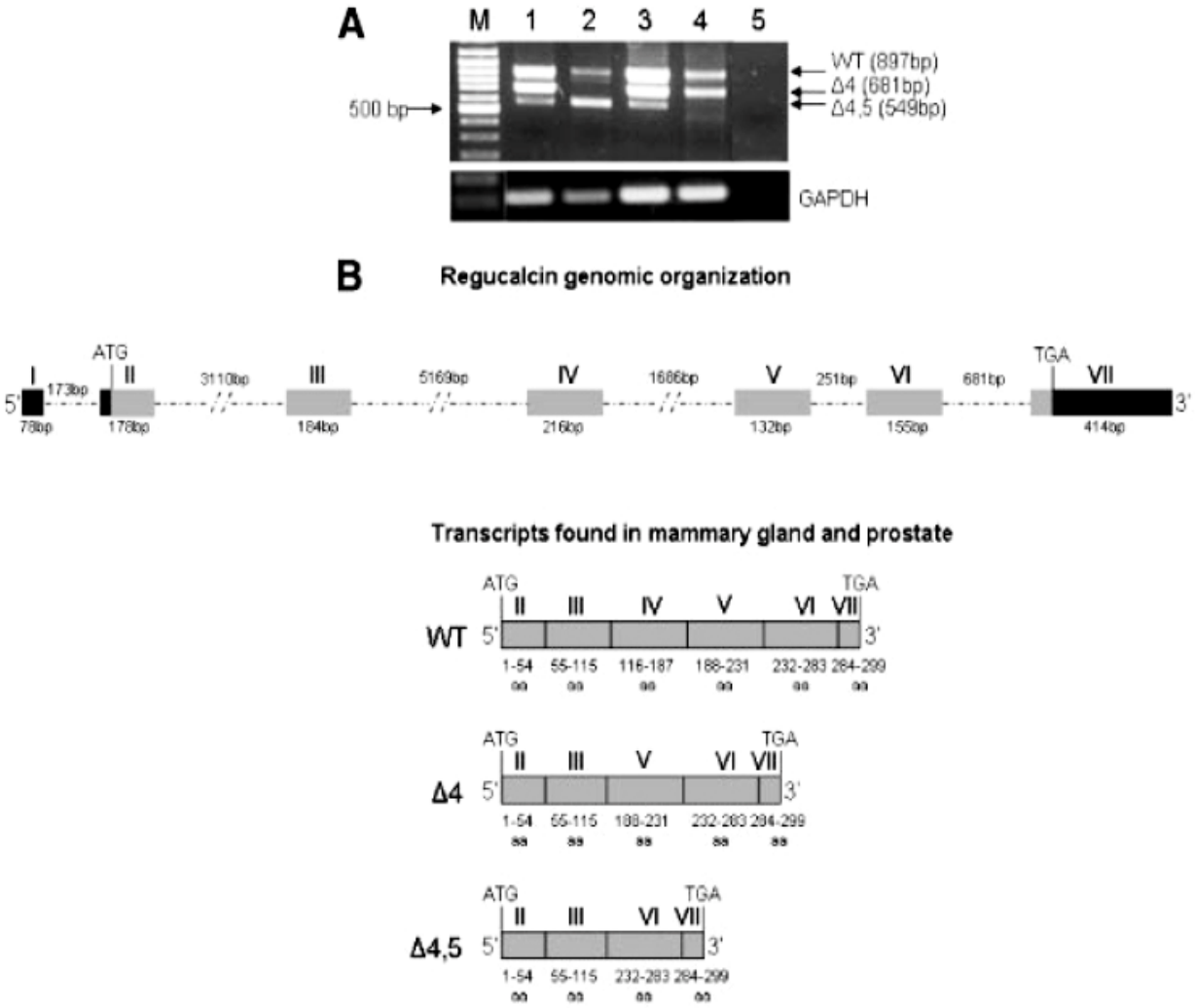


Figure 9. Regucalcin mRNA transcripts in human breast and prostate tissue and cell lines. (A) RT-PCR analysis using specific primers spanning the entire coding region of regucalcin. Lane M: DNA Molecular Weight Marker; Lane 1: Non-neoplasic breast tissue; Lane 2: MCF-7 cells; Lane 3: Non-neoplasic prostate tissue; Lane 4: LNCaP cells; Lane 5: Negative control with total RNA not reverse transcribed. (B) Diagram of the organization of human regucalcin gene and mRNA variants amplified in RT-PCR reactions. Gray and black boxes indicate coding and non-coding exons, respectively. Dotted lines correspond to introns. Exons are marked with roman numerals. Arabic numerals indicate the number of base pairs per exon or intron, or the number of aa encoded by each exon (*77*).

Regucalcin and its gene are identified over 15 species, including human, rat, and mouse (*82, 86, 89, 90, 95-97*) and sequences comparison between different vertebrate species have shown that the coding regions of the regucalcin gene have a high degree of similarity (*96*).

At the protein level, alignment of the amino acid sequence of human regucalcin shows 98% of similarity with primates, 93-96% with other mammalian species and 79-85% with non-mammalian vertebrates (*98*). These findings demonstrate the strong evolutionary conservation of regucalcin in higher animals suggesting that it may play a fundamentally important biological role in vertebrates (*82, 99*).

The crystal structure of the human regucalcin has been determined by X-ray diffraction and shows that regucalcin has a six-bladed ß-propeller fold and a single metal binding site (Figure 10). This is interesting when compared to the structural homologues described previously, which have at least two metal ions per molecule: one catalytic and the other structural. Moreover, it was demonstrated that zinc ion (Zn^{2+}) and Ca^{2+} bind to this same metal-binding site, in an identical manner (*100*). This is also interesting, as normally the coordination of Zn^{2+} is quite distinct from that of Ca^{2+} in enzymes. The binding of Ca^{2+} to regucalcin induces conformational changes in this Ca^{2+}-binding protein. In fact, this result has been demonstrated by means of the UV absorption spectrum, circular dichroism spectrum and fluorescence spectroscopy, suggesting that Ca^{2+}-binding loosens the conformation of regucalcin (*79, 90*).

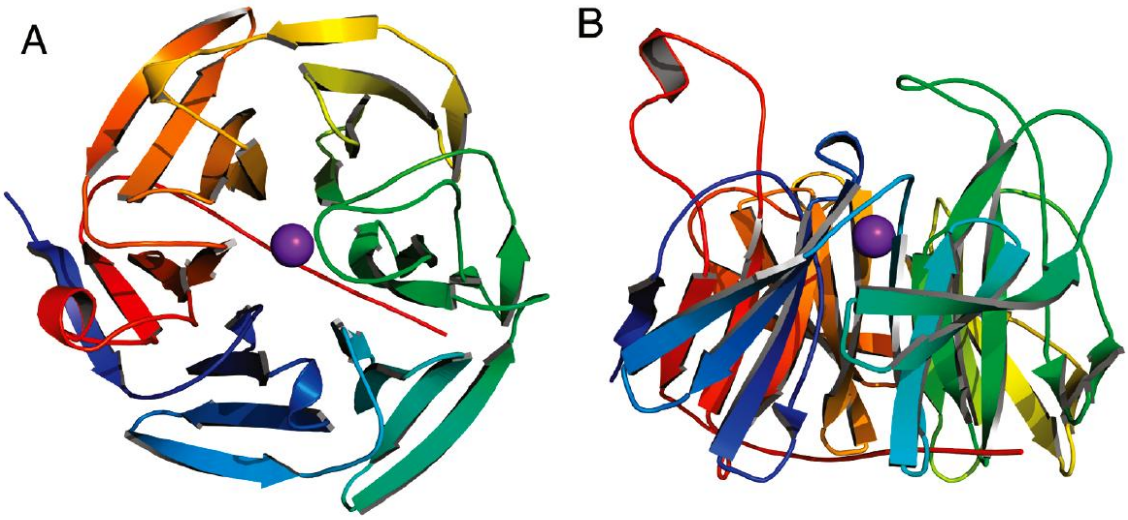


Figure 10. Crystal structure of human regucalcin with Ca^{2+} bound. (A) The ribbon structure of regucalcin displays the six-bladed ß-propeller fold with each blade displayed in a rainbow colour. The active site Ca^{2+} is shown in the middle of the ß-propeller as a purple sphere. (B) A 90° x-axis rotation of the view in panel A (*100*).

Nothern blot and immunohistochemical analysis showed that regucalcin is greatly expressed in the nuclei and cytoplasm of liver and kidney cortex in rat tissues, more precisely in centrilobular to midlobular areas of hepatocytes and in renal proximal tubular epithelia (*88, 101, 102*). The immunoreactivity for regucalcin is also detected in other tissues, including, brain, heart, bone, lung, epidermis, stomach, duodenum, adrenal gland, ovary, testis, mammary gland and prostate, although only slightly (*77, 91, 103-106*). In human tissues, regucalcin is widely distributed but is also strongly reactive in the liver (*91*). In addition to the expression in the liver and kidney, regucalcin is moderately expressed in the pancreas, heart and in the citosol and nuclei of breast and prostate epithelial cells (*77, 89, 99*). More recently regucalcin was identified in tissues of male reproductive tract, namely, epidydimis, seminal vesicles and testis (*76*).

3.2. Regulation of gene expression

The tissue-specific gene expression may be regulated at the process of transcription that is mediated through the interaction of trans-acting factors with cis-acting DNA sequences (*107-110*). This interaction is important for the tissue-specific expression of regucalcin mRNA (*111*).

There are many trans-acting regulatory factors in the 5'-flanking region of regucalcin gene, namely, activator protein-1 (AP-1), nuclear factor 1-A1 (NF1-A1), regucalcin gene promoter region-related protein 117 (RGPR-p117), ß-catenin, nuclear factor-κB (NF-κB) and ER (*78, 111-120*) (Figure 11). The transcription factors Ap-1, NF1-A1 and RGPR-p117 are translocated from the cytoplasm to the nucleus in a process that is mediated through PKC, Ca^{2+}/calmodulin-dependent protein (CaM) kinase, MAPK kinase, and phosphatidylinositol 3-kinase (PI3K) (*115, 117*), and, such as the others factors previously described, these are related to enhance the promoter activity of the regucalcin gene (*114*).

Regucalcin mRNA expression is also regulated by several factors, namely, Ca^{2+} (*101, 111, 121*), calcitonin (*122*), insulin (*121*), and oestrogen (*106, 120, 123*) suggesting that the expression of regucalcin mRNA is enhanced through various hormonal stimuli. In addition to these hormones, regucalcin is also regulated by androgens in prostate cancer (*77*). In a recent report, it was shown that androgens also regulate regucalcin expression in testicular tissue cultured *in vitro*, which seems to be associated with cell survival (*76*).

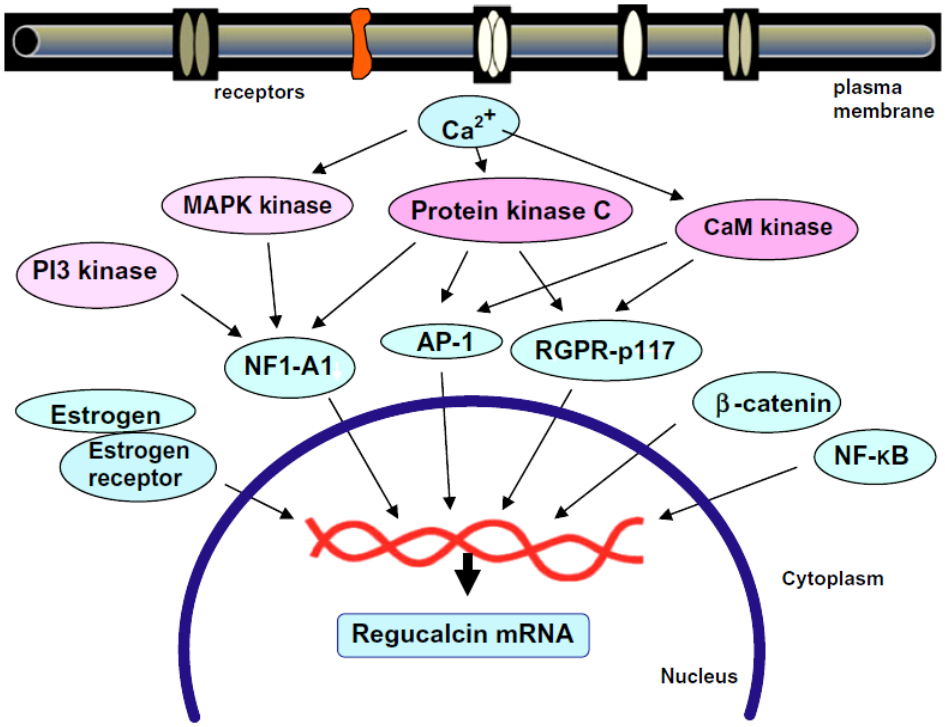


Figure 11. Regulation of regucalcin gene transcription activity. The transcription activity of regucalcin activity is regulated through various cell signaling factors. These transcription factors enhance the promoter activity of regucalcin gene in the nucleus (*78*).

3.3. Cellular functions

Regucalcin has a multifunctional role in maintaining intracellular Ca^{2+} homeostasis by activating Ca^{2+} pump enzymes; suppressing Ca^{2+} signaling from the cytoplasm to the nucleus in the proliferative cells; inhibiting protein kinases, protein phosphatases, protein synthesis, DNA and ribonucleic acid (RNA) synthesis in the cytoplasm and nucleus of cells; and suppressing cell proliferation and apoptotic cell death induced by various signaling factors (*124-129*). From these findings, regucalcin has been proposed to play a pivotal role in maintaining cell homeostasis and function as the regulatory protein of intracellular signaling system (*129*).

3.3.1. Intracellular calcium homeostasis

Regucalcin plays a role as a regulatory protein involved in the maintenance of intracellular Ca^{2+} homeostasis by enhancing Ca^{2+}-pumping activity in the plasma membrane, endoplasmic reticulum, mitochondria and nuclei of many cell types, namely, liver, renal cortex cells, heart, and brain (*104, 130-132*) (Figure 12).

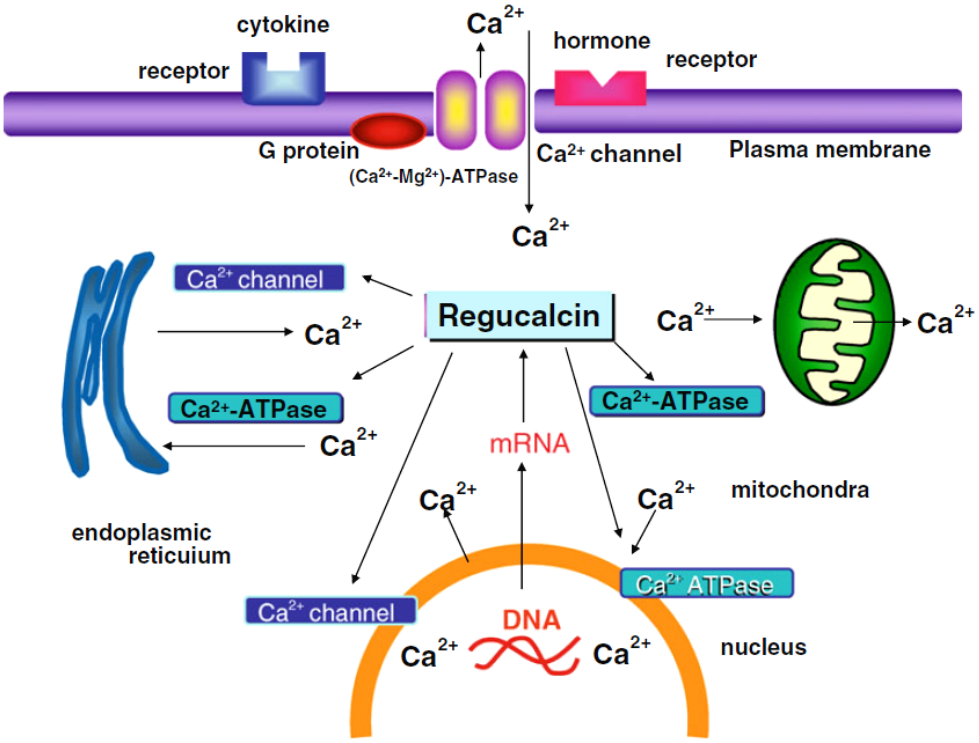


Figure 12. Regulatory role of regucalcin in Ca^{2+} homeostasis of liver cells. Regucalcin has a pivotal role in keeping intracellular Ca^{2+} homeostasis that is attenuated with various stimulating in cells. In fact, regucalcin increases plasma membrane (Ca^{2+}-Mg^{2+})-ATPase, mitochondrial Ca^{2+}-ATPase and microsomal Ca^{2+}- ATPase activities in cells. Regucalcin also stimulates Ca^{2+} release from the microsomes. Regucalcin has an inhibitory effect on nuclear Ca^{2+}-ATPase and a stimulatory effect on Ca^{2+} release from the nucleus. Through this mechanism, regucalcin keeps the rise of cytosolic Ca^{2+} concentration and nuclear matrix Ca^{2+} levels in cells (*133*).

The high-affinity (Ca^{2+}-Mg^{2+})-ATPase is located on the plasma membranes of liver, kidney cortex and neuronal cells (*134-137*). This enzyme acts as a Ca^{2+}-pump to exclude the

metal ion from the cytoplasm of these cells, due its capacity to directly activates (Ca^{2+}-Mg^{2+})-ATPase independently of Ca^{2+}-stimulated phosphorylation of the enzyme (*138-140*), and to stimulate adenosine triphosphate (ATP)-dependent Ca^{2+} transport across the plasma membrane vesicles (*141*). Regucalcin has been shown to bind the lipid components of plasma membrane, and it acts on the sulfhydryl (SH) groups that are an active site of (Ca^{2+}-Mg^{2+})-ATPase (*140*). These results suggest an involvement of endogenous regucalcin in the regulation of Ca^{2+} pump activity, binding Ca^{2+} in the cytoplasm of the cells previously cited, and transporting subsequently the metal ion into the organelle dependent on ATP (*139*).

Regucalcin can also stimulate the uptake of Ca^{2+} by mitochondria in rat liver, heart and brain. Studies involving regucalcin transgenic (TG) rats demonstrated an increase of regucalcin expression in the mitochondria and an increase of Ca^{2+}-ATPase activity as compared with normal rats (*132, 142-144*).

Regucalcin has been also demonstrated to activate Ca^{2+}-pump enzymes (Ca^{2+}-ATPase) and to stimulate ATP-dependent Ca^{2+} uptake by microsomes in rat liver, kidney cortex, heart and brain (*105, 131, 145-147*). Also, this protein regulates Ca^{2+} storage in the endoplasmic reticulum of these cells, stimulating Ca^{2+} release from the microsomes to restore the microsomal Ca^{2+} accumulation to regulate Ca^{2+}-related microsomal functions (*146*). Such as in (Ca^{2+}-Mg^{2+})-ATPase, it is suggested that regucalcin binds to the lipids at the close site of Ca^{2+}-ATPase in microsomes, and that it acts on the SH group which may be an active site of the enzyme and stimulates Ca^{2+}-dependent phosphorylation of Ca^{2+}-ATPase (*105, 146*).

Regucalcin has also a role in the regulation of Ca^{2+} concentration in liver and kidney nuclei. The existence of an ATPase, which is stimulated by Ca^{2+} in the presence of magnesium ion (Mg^{2+}), is found in liver nuclei, and it generates a net increase in nuclear matrix free Ca^{2+} concentration. This system may play an important role in the regulation of intranuclear Ca^{2+}-dependent processes (*148*). Presumably, regucalcin has a role in the regulation of liver nuclear function through the effect on Ca^{2+} transporting system in the nuclei (*130, 149*).

Moreover, overexpression of regucalcin causes a remarkable decrease on the gene expression of L-type Ca^{2+} channel and calcium-sensing receptor (CaR), which regulates intracellular Ca^{2+} signaling in the cloned normal rat kidney proximal tubular epithelial cells, suggesting that regucalcin regulates Ca^{2+}-channel expression (*150*).

3.3.2. Calcium-dependent enzymes and signaling pathways regulation

Protein phosphorylation–dephosphorylation is a universal mechanism by which numerous cellular events are regulated (*151*). The phosphorylation is catalyzed by protein kinases and consists of the attachment of phosphoryl groups to specific amino acid residues of a protein. The dephosphorylation consists of the removal of phosphoryl groups, catalyzed by protein phosphatases (*152*).

There are many protein kinases, namely, the multifunctional CaM kinases, which play an important role in the response of the cells to a Ca^{2+} signal (*153, 154*). Regucalcin has an inhibitory effect on enzyme activation by Ca^{2+}/calmodulin in liver, kidney and brain cells

(*155-158*). In fact, it can inhibit the activation of several enzymes, namely, pyruvate kinase (*85*); 5'-nucleotidase (*86*); ATPase (*159*); cyclic adenosine monophosphate (AMP) phosphodiesterase (*160, 161*); nitric oxide (NO) synthase (*162-164*) and PKC (*157, 158, 165, 166*) . The mechanism of the reversible effect on the activation and inhibition of regucalcin on various enzymes, which are regulated through Ca^{2+}, has not been well known. However, the mechanism of action of regucalcin may be partly based on its binding to Ca^{2+} and/or calmodulin. Moreover, it is possible that regucalcin may directly bind to enzymes and thus, inhibits their functions (*125*). In addition to the inhibitory effect on protein kinases, regucalcin has been shown to inhibit nuclear protein phosphatase activity (*167*). Presumably, regucalcin may be a unique protein, which has inhibitory effects on protein tyrosine phosphatase and protein serine/threonine phosphatase (*168-170*).

From these findings, regucalcin has been proposed to play a pivotal role in regulating the process of signal transduction from the cytoplasm to the nucleus, and regulating nuclear functions (*133*).

3.3.2.1. Regulation of protein synthesis and degradation

Regucalcin has been shown to have a regulatory effect on protein synthesis and protein degradation, suggesting that it plays a role in the regulation of protein turnover in cells (*133*).

Protein synthesis is depressed in a variety of eukaryotic cell types exposed to conditions depleting Ca^{2+} (*171*). It has been proposed that hormones, which are known to mobilize sequestered Ca^{2+} within liver cells, inhibit amino acid incorporation by influencing a Ca^{2+} requirement associated with protein synthesis (*172*). In addition to its capacity of binding Ca^{2+}, regucalcin has been also shown to inhibit hepatic aminoacyl-tRNA synthase activity (*173*). Thus, the inhibitory effect of regucalcin on hepatic protein synthesis may be partly based on its capacity of binding Ca^{2+} and on a remarkable decrease of aminoacyl-tRNA synthetase activity caused by regucalcin (*133*).

This protein can activate neutral cysteinyl-protease including Ca^{2+}-activated neutral protease (calpain) in liver and kidney cells, in a mechanism independent of Ca^{2+} (*174-176*). The ability of calpain to alter the limited proteolysis, the activity or function of numerous cytoskeletal proteins, protein kinases, receptors, and transcription factors suggests an involvement of the protease in various Ca^{2+}-regulated cellular functions (*177, 178*). Such as regucalcin activates cysteinyl proteases including calpain, it may be implicated in many cellular functions which are regulated by cysteinyl proteases in liver and renal cortex cells, namely in protein degradation (*125*).

3.3.3. Regulation of nuclear functions

Exogenous regucalcin has been shown to be transported into the nucleus isolated from normal rat liver and kidney (*179, 180*). It seems that the nuclear translocation of regucalcin is independently of Ca^{2+} and is not related to nuclear localization signal that is responsible for

selection for intranuclear active transport (*133*). Presumably, regucalcin is passively transported to the nucleus through nuclear pore in liver cells, since a molecular weight of regucalcin is about 33 kDa (*90*).

Regucalcin has been shown to bind proteins and DNA in liver nucleus (*181*), thus it may have a regulatory effect on signaling pathways that modulate transcriptional activity in liver cells (*133*). In fact, regucalcin has an inhibitory effect on DNA fragmentation that may be partly based on binding of Ca^{2+} since DNA endonuclease activity is Ca^{2+}-dependent (*182, 183*). This protein can also inhibit GTPase activity on liver nuclear extracts. This inhibitory effect is revealed independent of Ca^{2+}, since it seems to be due to the binding of regucalcin to the enzyme, inhibiting directly GTPase activity in liver nucleus (*184*).

In addition to these enzymes and such as described above, regucalcin can inhibit various protein kinases and protein phosphatases, in nucleus of rat liver (*167, 185*). It has been also shown to have an inhibitory effect on DNA synthesis activity in the nuclei of normal rat liver in a mechanism independent of Ca^{2+} (*186-188*).

Moreover, regucalcin has been shown to have an inhibitory effect on RNA synthesis in the nuclei isolated from control rat liver and regenerating rat liver. This suppressive effect of regucalcin in nuclear RNA synthesis activity is partly resulted from its inhibitory action on RNA polymerase II and III (*189, 190*). Inactivation of RNA polymerase III transcription has been shown to be Ca^{2+} dependent; changes in Ca^{2+} concentration possibly mediated through Ca^{2+}-dependent protein kinase, activation of calpains, and consequent proteolytic degradation of RNA of transcription factors has been suggested to be involved in the regulation of RNA polymerase III transcription in the presence of Ca^{2+} (*191*). Such as described above, regucalcin may be involved in the inhibition of Ca^{2+}-dependent protein kinase and calpains activity, which can influence RNA synthesis (*133*).

3.3.4. Cell proliferation and apoptosis

Regucalcin has been shown to have a suppressive effect on cell proliferation (Figure 13). In fact, the nuclear translocation of regucalcin is increased in regenerating rat liver and its mRNA expression is enhanced in the proliferative cells after partial hepatectomy in rats (*192*). Moreover, cell proliferation is suppressed in rat hepatoma and rat kidney proximal tubular epithelial cells overexpressing regucalcin, suggesting that regucalcin plays a role in the proliferation of cells (*150, 193*).

Regucalcin may play an important role as a suppressor for the enhancement of cell proliferation due to inhibiting various nuclear protein kinases, namely MAPK kinase, CaM kinase, protein tyrosine kinase and PI3-kinase, and protein phosphatases activities, namely protein tyrosine kinase, which are involved in signal transduction to the nucleus, and it causes an inhibition in nuclear DNA synthesis in proliferative liver cells (*194-196*). Moreover, regucalcin has a suppressive effect on cytosolic protein synthesis (*197, 198*) and nuclear RNA synthesis (*189, 190*) in rat liver, suggesting that the effect of regucalcin in suppressing cell proliferation is also partly mediated through its suppressive effect on protein and RNA

synthesis in the cells. On the other hand, regucalcin enhances p21 mRNA expression, which participates in cell cycle arrest, and suppresses IGF-I mRNA expression, a growth factor in cell proliferation, in the hepatoma cells (*129, 199*). In addition, regucalcin is found to bind nuclear proteins or DNA, modulating nuclear transcriptional activity (*181*). Its overexpression suppresses the expression of oncogenes c-myc, Ha-ras, or c-src and enhances the expression of tumour suppressor genes p53, which stimulates p21 mRNA expression, and retinoblastoma protein (pRb) in the cloned hepatoma cells (*200, 201*).

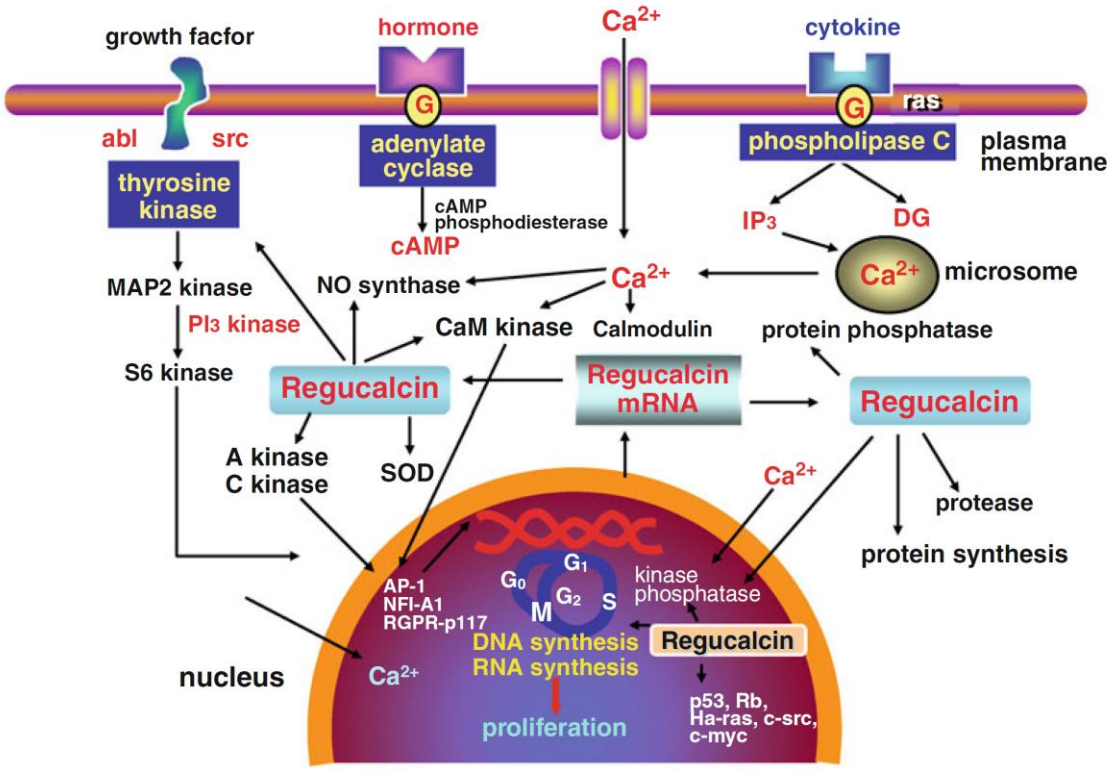


Figure 13. Suppressive role of regucalcin on the enhancement of cell proliferation. Regucalcin mRNA expression is stimulated through the pathway of signaling mechanism concerning CaMK, PKC, PKA, and thyrosine kinase due to normal stimulation. Regucalcin inhibits the activities of various protein kinases and protein phosphatases in the cytoplasm and nucleus of cells, and it also can inhibit Ca^{2+}/calmodulin-dependent enzyme activity. Cytoplasmic regucalcin translocates into nucleus. Regucalcin inhibits nuclear DNA and RNA synthesis. Regucalcin has an inhibitory effect on the expression of c-myc, Ha-ras, and c-src mRNAs, which are tumour stimulator genes. Regucalcin also stimulates the expression of p53 and Rb mRNAs that are tumour suppressor genes. Moreover, regucalcin can inhibit protein synthesis and it can stimulate protein degradation. Regucalcin induces G1 and G2/M phase cell cycle arrest in cells. The suppressive effect of regucalcin on cell proliferation is mediated through regulating many signaling systems (*133*).

Contrastingly, there are also reports describing regucalcin roles suppressing cell death and apoptosis (Figure 14). Regucalcin has been shown to inhibit inducible and endothelial Ca^{2+}/calmodulin-dependent NO synthase (*163*). This enzyme produces NO, an important signaling factor in many cells which plays an important role in apoptosis of hepatoma cells (*202, 203*). When present at high concentrations, NO has been shown to inhibit cell proliferation and to induce cell apoptosis (*203*). Presumably, regucalcin has an inhibitory effect on NO synthase activity due to binding to calmodulin and/or the enzyme independently of Ca^{2+} in proliferative cells, suggesting that regucalcin has a suppressive role in apoptosis (*163*).

Regucalcin has also a suppressive effect on cell death, due to inhibition of tumour necrosis factor α (TNF-α) and lipopolysaccharide (LPS) in rat liver and kidney cells (*204-206*). TNF-α induces apoptosis in mammary adenocarcinoma cells by an increase in intracellular free Ca^{2+} concentration and DNA fragmentation whereas LPS acts to modulate the expression of a large number of genes that favour apoptosis of fibroblastic cells that are dependent upon activation of caspase-8 (*206, 207*).

As previously described, regucalcin has been shown to have a suppressive effect on Ca^{2+}-activated DNA fragmentation in isolated rat liver nucleus, suggesting that the protein has an inhibitory effect on apoptosis in liver cells (*183*). In addition, regucalcin also inhibits apoptosis due to its ability to keep intracellular Ca^{2+} homeostasis. In fact, Ca^{2+} is an apoptosis inducing factor since its entry into cells is known to induce cell death (*208, 209*). Such as previously described, regucalcin may have a suppressive effect on Ca^{2+} entry due to activating Ca^{2+} pump enzymes in the plasma membranes, mitochondria, endoplasmic reticulum and nucleus of rat liver cells, since it could rescue cells from an apoptotic death induced by a high intracellular Ca^{2+} level (*125, 133*).

On the other hand, regucalcin overexpression has an inhibitory effect on other apoptotic inducing factors such as insulin, insulin-like growth factor-1 (IGF-1) and sulforaphane, inhibiting signaling pathways which involves NO synthase, caspase-3, and Ca^{2+}-dependent endonuclease and activating Bcl-2 in cells (*133, 210, 211*).

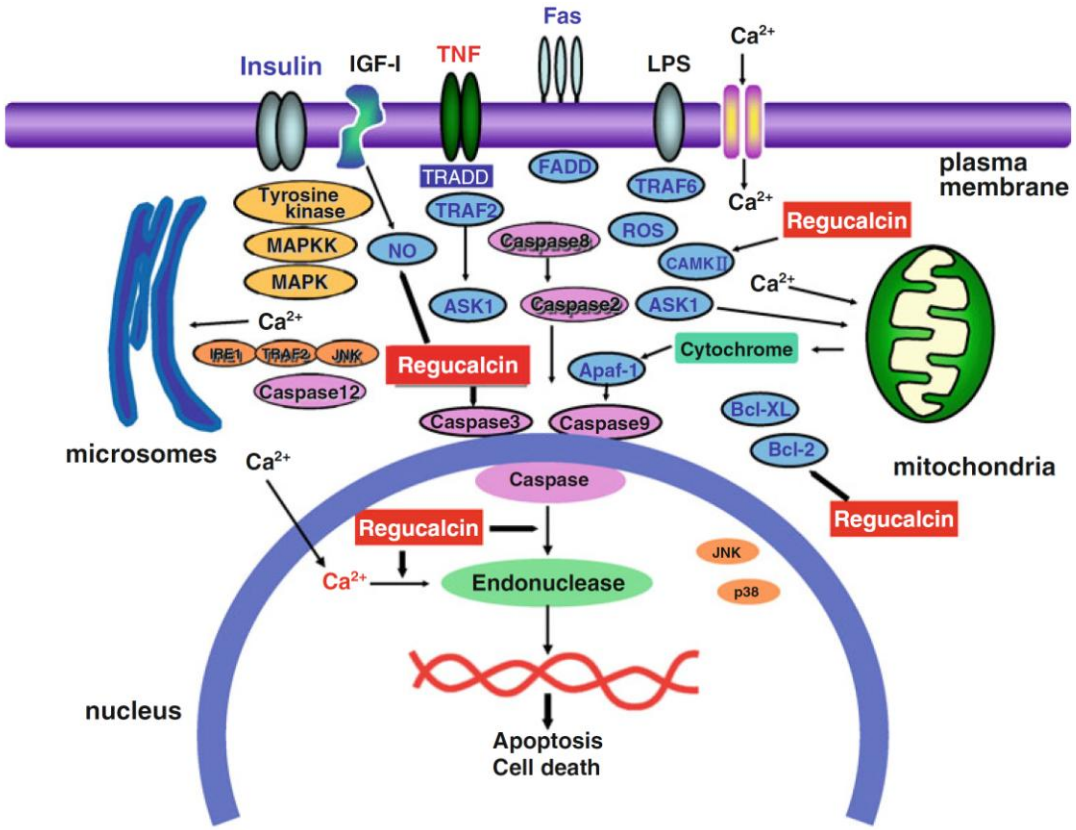


Figure 14. Suppressive role of regucalcin in cell death and apoptosis induced by various factors. Regucalcin suppresses cell death induced by various factors. The suppressive effect of regucalcin on cell death and apoptosis is mediated due to inhibiting the activities of NO synthase, caspase-3, and Ca^{2+}-dependent endonuclease and activating Bcl-2 in the cells (*133*).

3.4. Regucalcin in breast pathophysiology

Considering the information provided in previous sections it is quite clear that regucalcin play a multifunctional role in cell physiology, being highly predictable that deregulation of its functions would be associated with pathological conditions. In fact, several studies have been linking regucalcin with several diseases, namely osteoporosis (*103*), diabetes (*212*), muscular dystrophy (*213*), Alzheimer disease (*214*), liver injury (*215*), male infertility (*98*) and different types of cancer (*77, 106, 216*).

Studies in hepatoma cell lines showed that regucalcin suppresses cell proliferation (*129, 205*), inhibits expression of oncogenes c-myc, H-ras, and c-src, and increases the expression of tumour suppressor genes p53 and pRb (*181, 201*), suggesting that regucalcin may have a protective role against carcinogenesis and, consequently, loss of regucalcin expression may contribute to tumour development (*77*). Recently, it was demonstrated that regucalcin mRNA and protein expression are diminished in samples of human breast cancer cases (*77*). Moreover, regucalcin immunoreactivity was associated with the tumour differentiation grade (*77*).

4. L-type calcium channels

Voltage-dependent L-type Ca^{2+} channels mediate Ca^{2+} influx into both excitable and non-excitable cells, including cardiac and skeletal myocytes, vascular smooth cells, neurons, endocrine cells, osteoblasts and osteoclasts (*217-219*). They acutely contribute to neurotransmitter and hormone release from neurons and endocrine cells, contraction of cardiac, smooth, and skeletal muscle, and Ca^{2+}-dependent signal transduction. The roles of voltage-dependent Ca^{2+} channels have been extensively studied in these excitable cells, but their roles in the non-excitable cells have not been studied as much (*220*).

4.1. Structural features

L-type Ca^{2+} channels are heteromultimeric complexes consisting of a pore-forming α_1 subunit and auxiliary α_2, ß, δ, and γ subunits (*221*) (Figure 15).

The α_1 subunit (170-240 kDa) serves as the main functional component of the channel complex, containing the channel pore, voltage sensors, the gating machinery and the receptors for various classes of drugs and toxins. They consist of four homologous domains (I-IV), each composed of six transmembrane segments (S1-S6). To date, ten different α_1 subunit genes have been identified and separated into four classes: $Ca_v1.1$ (α_{1S}), $Ca_v1.2$ (α_{1C}), $Ca_v1.3$ (α_{1D}), and $Ca_v1.4$ (α_{1F}), which provide unique functional properties to Ca^{2+} channels present in different cell types (*222*).

L-type Ca^{2+} channel α_{1C} subunit is encoded by the $Ca_v1.2$ gene that is located on chromosome 6 in mouse and 12 in human. Human $Ca_v1.2$ contains seven predicted alternative promoters with variations in the mRNA including 5′ and 3′ truncations (*223*). L-type Ca^{2+} channel α_{1C} subunit expression is regulated by α- and ß-adrenergic stimulation (*224*),

androgens (*225*), and Ca^{2+} entry through the channel (*226*). Binding sites for transcription factors such as NKX2.5, myocyte-specific enhancer factor 2C (Mef2c), a cAMP response element, and hormone binding sites were identified, along with a minimal promoter sequence (*227*).

Ca^{2+} channel auxiliary subunits further add to the functional diversity of these channels. The ß subunits (55-60 kDa) are encoded by four separate genes and are intracellular auxiliary subunits co-expressed with α_1 subunit, which promote trafficking of the channel complex to the plasma membrane and modulate gating properties of the channel (*228, 229*). The α_2 subunits are closely associated with the α_1 subunit by surface interaction and are intracellularly linked through a disulfide bridge to a small protein, the δ subunit (175 kDa). The α_2 subunit is entirely extracellular and the δ subunit has a single transmembrane region with a very short intracellular part. The α_2 and δ subunits are encoded by the same gene, which is separated by proteolytic cleavage (*230*). They have been shown to modify both channel gating properties and surface membrane expression of the L-type Ca^{2+} channel complex (*228*). To date, 8 genes encoding a variety of γ subunit (25-38 kDa) isoforms have been identified. The γ subunits are only expressed in some tissues, and unlike the other auxiliary subunits (ß and $\alpha_2\delta$), they do not have a significant role in the membrane trafficking of the Ca^{2+} channel (*231*).

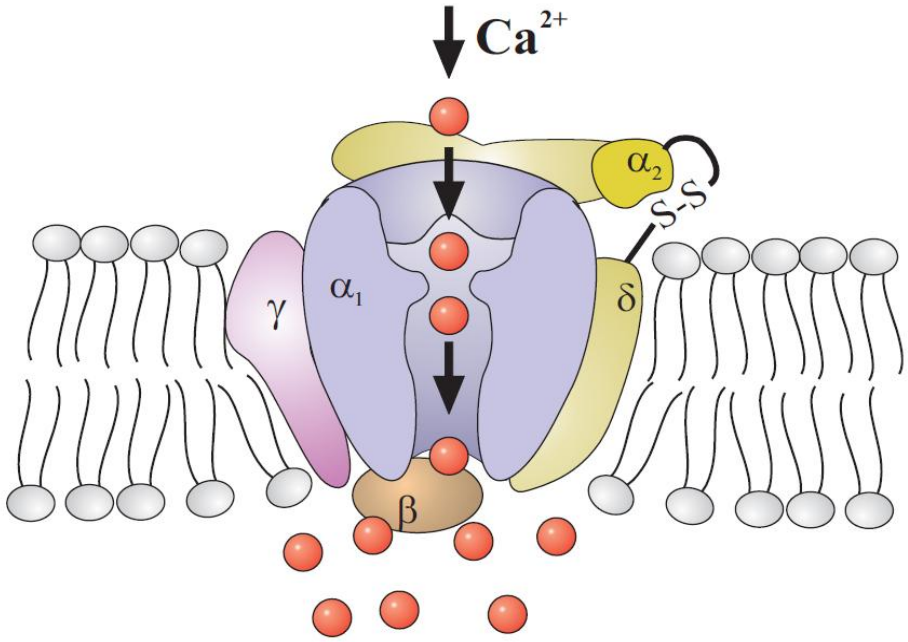


Figure 15. A proposed schematic structure of voltage-activated Ca^{2+} channel. The principal a_1 subunit is a transmembrane protein containing a conducting pore, through which Ca^{2+} ions can pass upon opening. a_1 subunit is further regulated by auxiliary subunits: intracellular *ß* subunit, transmembrane *y* subunit and a complex of extracellular a_2 subunit and transmembrane *δ* subunit, connected by a disulphide bridge (*217*).

In non-excitable cells, L-type Ca^{2+} channels are regulated by PKA- and PKC-mediated signalling pathways. Binding of several agonists and activation of their specific receptors may result in the phosphorylation of the L-type Ca^{2+} channels and consequent opening and entry of Ca^{2+} from the extracellular space (*232, 233*).

Multiple G protein–coupled receptors, including ß-adrenergic receptors (ßAR), act through cAMP/PKA pathways to regulate many cellular proteins, including the L-type Ca^{2+} channel (Figure 16). These receptors are coupled to heterotrimeric G proteins, which either stimulate (G_s) or inhibit (G_i) adenylyl cyclase (AC). An increase in AC activity leads to increased cellular cAMP, which binds to the regulatory subunits of PKA, liberating the catalytic subunits to phosphorylate their substrates on L-type Ca^{2+} channel specific serine and threonine residues. Phosphorylation of L-type Ca^{2+} channel induces its opening and consequent entry of Ca^{2+} from the extracellular space. Muscarinic M_2 receptors can oppose the ßAR up-regulation by acting through G_i to inhibit AC (*232*).

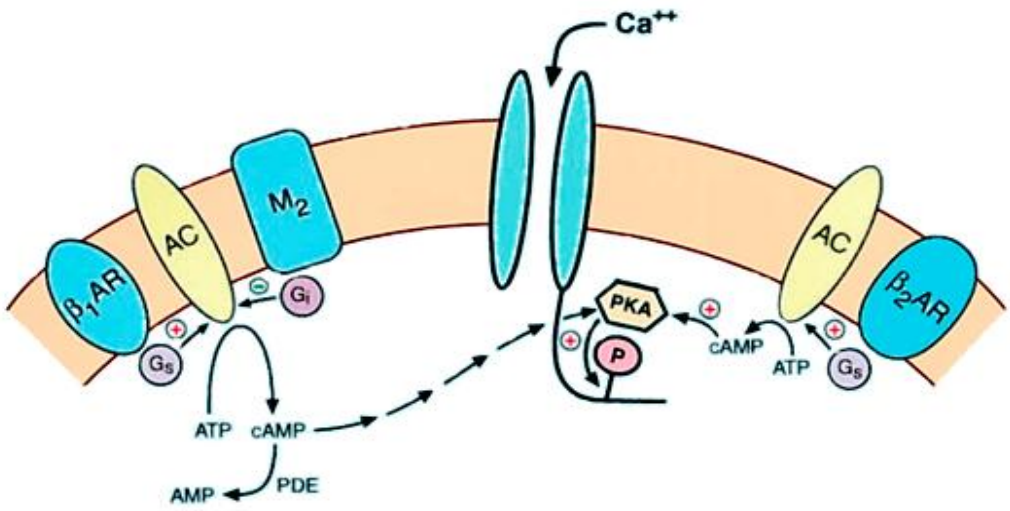


Figure 16. Schematic of the cAMP/PKA cascade regulating L-type channels. Stimulation of the ß-adrenergic receptors (ßAR) 1 and 2, leads to G**s**-mediated activation of AC and increased production of cAMP, which stimulates PKA. PKA can then phosphorylate the channel at multiple potential sites indicated schematically by the single P in the diagram. Whereas $ß_1$AR regulation causes more global increases in cAMP, $ß_2$AR stimulation can result in highly localized cAMP level changes and regulation. Muscarinic M_2 receptors can oppose the ßAR up-regulation by acting through G_i to inhibit AC (Adapted from (*232*).

Multiple G_q protein–coupled receptors, including endothelin (ET), α1-adrenergic, and angiotensin II receptors, trigger the signaling cascade leading to activation of PKC (Figure 17). Binding of an agonist to a G_q protein-coupled receptor activates PLC, which breaks down phosphoinositides in the plasma membrane to form IP3 and diacylglycerol (DAG). The IP3 triggers Ca^{2+} release from intracellular stores through IP3 receptor channels, producing the initial transient peak increase in $[Ca^{2+}]_i$. Then, DAG, phosphatidylserine, and in some cases Ca^{2+} collectively activate PKC, which in turn, phosphorylate L-type Ca^{2+} channel on specific serine and threonine residues. This phosphorylation of L-type Ca^{2+} channel induces its opening and consequent entry of Ca^{2+} from the extracellular space (*232*).

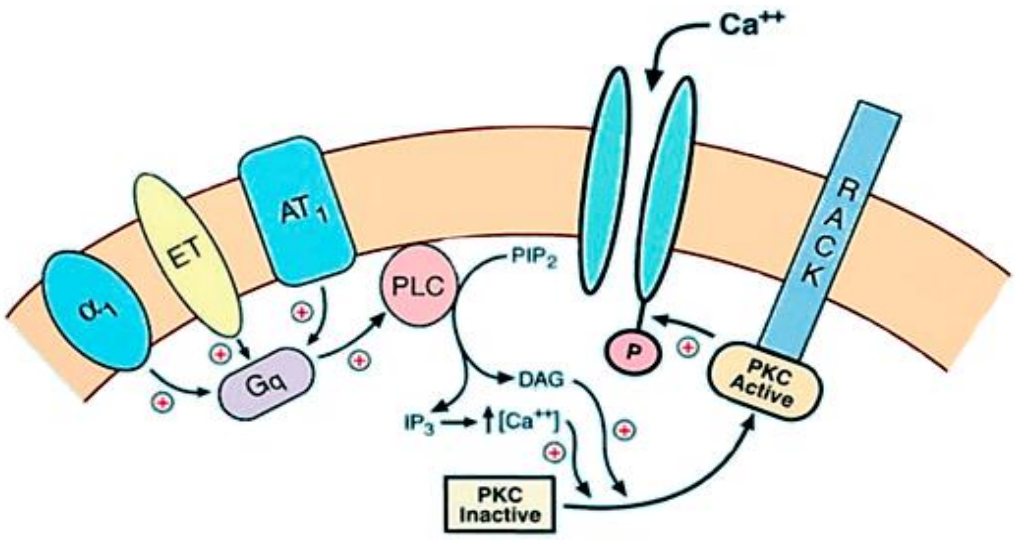


Figure 17. PLC/PKC signaling cascade regulating L-type Ca^{2+} channels. Activation of a1-adrenergic, ET, or AT_1 receptors stimulates G_q with resulting activation of PLC, which leads to the production of diacylglycerol and activation of PKC. PKC is proposed to target to the membrane by binding a RACK protein in the vicinity of the L-type Ca^{2+} channel, which it then phosphorylates. A Ser/Thr phosphatase counterbalances this phosphorylation. IP3 indicates inositol trisphosphate; PIP_2, phosphatidylinositol 4,5 bisphosphate (Adapted from (*232*).

4.2. Regulation of L-type calcium channels by androgens

L-type Ca^{2+} channels are regulated by a variety of neurotransmitters, cytokines and hormones, including the androgens (*232*). Studies have demonstrated that androgens modulate L-type Ca^{2+} channel expression in several cells, such as coronary smooth muscle cells, myocardium, human prostate cancer cells, and Sertoli cells. In all these cells, androgens have been shown to increase L-type Ca^{2+} channel expression (*74, 234-237*). In addition, androgens can also modulate L-type Ca^{2+} channel activity. In fact, androgens have been shown to inhibit L-type Ca^{2+} channel activity in rat aorta vascular smooth muscle cells (*238, 239*). L-type Ca^{2+} channel expression may be regulated through classic genomic mechanism, but also by a non-genomic androgen action associated with a G-protein (*232, 233*). L-type Ca^{2+} channel activity may also be regulated through non-genomic signaling pathways. Binding of androgens to its membrane associated receptor can lead to phosphorylation of L-type Ca^{2+} channel and consequent entry of Ca^{2+} from extracellular space (*232*) (Figure 7).

4.3. L-type calcium channels in breast pathophysiology

Such as previously described, Ca^{2+} is a key regulator of many essential biological processes, including cell proliferation (*57*). Deregulation of $[Ca^{2+}]_i$ via altered Ca^{2+}-transporters, Ca^{2+}-channels and Ca^{2+}-binding proteins expression and/or function may contribute to cancer development (*58*). Studies have demonstrated that voltage-sensitive ion channels play a role in the onset, proliferation and malignant progression of various types of cancer, such as prostate, breast, lung, thyroid, colon, and glioma. This has initially been

demonstrated for K^+ channels and is meanwhile also suggested for other Ca^{2+}, sodium ion (Na^+) and chloride ion (Cl^-) channels (*240*).

Alterations in several ion channels have been associated to breast cancer. In fact, a significant increase in K^+ channel ($K_v1.3$, $K_v10.1$-2 and $K_{2p}9.1$), transient receptor potential cation channel subfamily V member 6 (TRPV6), transient receptor potential cation channel subfamily P member 6 (TRPP6), TRPM8 and Cl^- channels expression have been shown in breast cancer cell compared to normal cells (*241-244*).

To date, alterations in L-type Ca^{2+} channel expression or functions have been implicated only in colonic cancer (*245*). Wang et al. have demonstrated that mRNA and protein L-type Ca^{2+} channel (α_{1c} subunit) expression is increased in colonic carcinoma cells compared to normal cells (*245*). However, although function of L-type Ca^{2+} channel has been demonstrated in distinct types of non-excitable epithelial cells, including Sertoli cells and other endocrine cells (*218, 237*), their involvement in breast cells physiology remains to be deciphered.

5. Aim of the thesis

The role of androgens in the female reproductive tract has been largely unexplored. However, AR is found in virtually every tissue in women, including breast, indicating that androgens and their metabolites play an important role in normal tissue homeostasis. In human breast cancer, AR expression is significantly increased and the majority of cases, including those negative for ER and PR, still are positive for AR, highlighting the role of androgens in breast cancer. Moreover, it has been also established that deregulation of Ca^{2+} homeostasis is associated with mammary gland pathophysiology, and that androgens play a role in Ca^{2+} homeostasis in other cells types. Therefore we hypothesised that androgens may regulate the expression of Ca^{2+}-binding proteins and Ca^{2+} channels in human breast cancer cells. In this way, the present study aims to:

- Analyse the expression of Ca^{2+}-binding protein regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) in human breast cancer cells (MCF-7);

- Study the effect of androgens on the expression of Ca^{2+}-binding protein regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) in human breast cancer cells (MCF-7);

- Disclose the mechanisms underlying the androgenic effects on the regulation of regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) expression.

II. Materials and Methods

1. Cell lines

The human breast cancer epithelial cell line (MCF-7) was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). MCF-7, is the acronym of Michigan Cancer Foundation-7, and these cells are the most commonly used breast cancer cell model for studies of tumor biology and hormone mechanism of action, due to the expression of oestrogen, progesterone, androgen and glucocorticoid receptors (*246, 247*).

2. Cell culture and treatment

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) at pH of 7.4, in an incubator at 37°C equilibrated with 5% CO_2. For this assay, cells were seeded in 12-well and 6-well plates for further RNA and protein extraction, respectively. When growth confluence of 60% was achieved, cells were grown in phenol red-free DMEM supplemented (Gibco) with 5% charcoal-stripped FBS (Gibco) for 24h. Then, cells were exposed to four different concentrations of DHT (0, 1, 10 and 100 nM) (Sigma, Saint Louis, USA) during different periods (0, 6, 12, and 24h). Control treatments with flutamide (1μM; Sigma), cyclohexamide (1μg/mL; Sigma), and ICI 182,780 (100nM; Tocris Cooksob, Bristol, UK) were carried out with 1nM of DHT for 24h. Inhibitors were added to cell cultures 30min before hormone stimulation. All assays were carried out in hexaplicate.

3. Total RNA extraction

Total RNA was extracted from MCF-7 cells using TRIzol reagent (Ambion, USA) according to the manufacturer's instructions. An appropriate volume of TRIzol was added to cells and incubated for 5min at room temperature (RT) to dissociate nucleoprotein complexes. 200 μL of chloroform (Sigma) per mL of TRIzol reagent was added, and the sample was homogenized vigorously for 15s and incubated for 2-3min at RT. After the incubation period, samples were centrifuged (12000g for 15min at 4ºC) and the mixture was separated into three distinct phases: a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase, since upper aqueous phase was transferred to a new tube and 500μL of 100% isopropanol per mL of TRIzol reagent was added. Samples were incubated for 10min at RT and then centrifuged at 12000g for 10min at 4ºC. The supernatant was removed and the RNA pellet washed with 1mL of 75% ethanol in diethylpyrocarbonate (DEPC)-treated water (Sigma) at 20°C, per mL of TRIzol reagent. After centrifugation at 7500g for 5min at 4ºC, the supernatant was removed and the

wash step was repeated. Finally, the resultant RNA pellet was air dried for 5-10min and resuspended in an appropriate volume of DEPC-treated water.

In order to assess the quantity and quality of total RNA, its optical density was determined by spectrophotometry (NanoPhotometer, Implen, Munich, Germany) measuring absorbance at 260 nm and 280 nm. RNA extracts were also inspected by agarose gel electrophoresis using GreenSafe (NZYtech, Lisbon, Portugal) as staining method.

4. cDNA synthesis

Firstly, 1µg of total RNA was denatured at 70°C for 5min in a reaction containing 5µg/µL random hexamer primers (Invitrogen) and DEPC-treated water up to 10µL and chilled in ice before added to a 10µL reverse-transcription solution containing 5X Reaction Buffer (provided with M-MLV Reverse Transcriptase) (Promega, Madison, USA), 0,8µL M-MLV Reverse Transcriptase (Promega), 1µL deoxynucleotide triphosphates mix (dNTPs; Amersham, GE Healthcare, Uppsala, Sweden), and DEPC-treated water up to 10µL. The cDNA synthesis reaction was carried out at 37°C for 1h and was stopped at 70°C for 15min. Synthesized cDNA was stored at -20°C until further use.

5. RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) was performed to analyze the regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) expression in human breast cancer cell line MCF-7. Reactions were carried out using 1µL of cDNA synthesized from human breast cancer cell line MCF-7 in a 25µL reaction containing 1X Taq DNA polymerase buffer (provided with Platinum Taq DNA polymerase) (Promega), 500µM dNTPs (Amersham), 3.0mM of $MgCl_2$ (Promega), 300nM of each primer pair specific to the target regucalcin and L-type Ca^{2+} channel genes, 1µL of Platinum Taq DNA polymerase (Promega) and sterile water. Prior amplification of regucalcin and L-type Ca^{2+} channel (α_{1C} subunit), the integrity of cDNA samples was assessed by amplification of the 18S housekeeping gene. Cycling conditions, primer sequences and corresponding amplicon sizes are indicated in Table 1.

Table 1. Primer sequences, amplicons size and cycling conditions used in RT-PCR amplification of human regucalcin, L-type Ca^{2+} channel (α_{1C} subunit) and 18S.

Gene and Accession numbers	Sequence (5' – 3')	Amplicon size (bp)	Cycling conditions
Human Regucalcin NM_004683.4	S: GCA AGT ACA GCG AGT GAC AS: TTC CCA TCA TTG AAG CGA TTG	177	40x { 95°C – 5min 95°C – 30s 60°C – 30s 72°C – 1min 72°C – 5min
Human L-type calcium channel (α_{1C} subunit) NG_008801	S: AAT GCC TAC CTC CGC AAC GGC TG AS: TGA TGC CGT GCT TGG GAC CAT CC	469	40x { 95°C – 5min 95°C – 30s 62°C – 30s 72°C – 1min 72°C – 5min
Human 18S NR_003286.2	S:AAG ACG AAC CAG AGC GAA AG AS: GGC GGG TCA TGG GAA TAA	152	25x { 95°C – 5min 95°C – 30s 58°C – 30s 72°C – 1min 72°C – 5min

6. Real-time PCR

Real-time PCR was performed to compare the mRNA levels of regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) in MCF-7 cells subjected to DHT treatments. Specific primers to regucalcin (human regucalcin) and L-type α_{1C} (human L-type Ca^{2+} channel (α_{1C} subunit)), located in different exons, were the same used in RT-PCR (Table 1). To normalize the expression of regucalcin and L-type α_{1C}, human GAPDH expression was used as internal control (Sense primer: CGC CAG CCG AGC CAC ATC; Anti-sense primer: CGC CCA ATA CGA CCA AAT CCG). The efficiency of real-time PCR was determined for all designated primers performing serial dilutions (1, 1:5, 1:25 and 1:125) of the cDNA from MCF-7 cells. Real-time PCR reactions were carried out using 1µL of cDNA synthesized in a 20µL reaction containing 10µL of Maxima^{TM} SYBR Green/Fluorescein qPCR Master Mix (Fermentas) and 300nM of primers for each gene. Reaction conditions comprised 5min of denaturation at 95ºC, followed by 40 cycles at 95ºC for 10 seconds, 60ºC to regucalcin and 62°C to L-type Ca^{2+} channel (α_{1C} subunit) for 30 seconds, and 72ºC for 10 seconds. Samples were run in triplicate in each PCR assay. Normalized expression values were calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta Ct}$ (*248*).

7. Western blot

Total protein was extracted from MCF-7 cells using an appropriate volume of radioimmunoprecipitation assay buffer (RIPA) (150mM NaCl, 1% Nonidet-P40 substitute, 0,5% Na-deoxycholate, 0,1% SDS, 50mM Tris, 1mM EDTA), 10% PMSF and 1% Protease cocktail. The mixture was incubated for 20 min in ice and then, centrifuged at 14000g for 20min at 4°C. The supernatant was recovered to new tubes and stored at -80°C. Finally, quantification of total protein extracts was assessed using the Bradford method (Bio-Rad, Hercules, USA) (*249*).

40µg, 50µg or 60 µg of total proteins were resolved in a 12% SDS-PAGE gel and electrotransferred to a PVDF membrane (Amersham), previously activated in methanol, water and electrotransferation solution (10mM CAPS in 10% methanol, pH=11), at 750mA and 4°C. Then, membranes were blocked in Tris-buffered saline (TBS-T) with Tween 20 (TBS-T; Applichem, Darmstadt, Germany) containing 5% milk (Regilait, France) for 1h30. The membrane was then incubated overnight at 4ºC with a primary antibody (Table 2). After washing in TBS-T, the membrane was incubated for 1h30 with a secondary antibody. Finally, the membrane was again washed in TBS-T and incubated with ECF substrate (Amersham) for 3min and visualized on the Molecular Imager FX Pro plus MultImager (Bio-Rad). Incubation periods, and specific primary and secondary antibodies used for regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) staining are indicated in Table 2.

Table 2. Electrotransfer details and specific primary and secondary antibodies used in Western blot analysis for regucalcin and L-type Ca^{2+} subunit (α_{1C} subunit) staining.

	Electrotransferation	Primary Antibody	Secondary Antibody
Regucalcin	1h30	Mouse monoclonal anti-human regucalcin 1:1000 Abcam (ab67336, Cambridge, UK)	Goat polyclonal antibody against mouse IgG 1:20000 Abcam (ab7069)
L-type α_{1c}	2h30	Rabbit anti-human calcium channel α_{1C} (L-type of voltage-Gated Ca^{2+} Channel) 1:200 Sigma (C 1603)	Goat anti-rabbit IgG alkaline phosphatase linked antibody 1:10000 Amersham (RPN5783)

8. Statistical analysis

The statistical significance of differences in regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) mRNA and protein expression among experimental groups was assessed by Student's paired *t*-test using GraphPad Prism software (version 5.0 for Windows). Significant differences were considered when $p<0.05$. All experimental data are shown as mean ± SEM.

III. Results

1. Expression of calcium-binding protein regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) in MCF-7 cells

In order to analyze the expression of regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) in human breast cancer MCF-7 cells, RT-PCR and Western blot were carried out using specific primers and antibodies, respectively.

RT-PCR results using specific primers to human regucalcin, allowed confirming its expression in human breast cancer cell line MCF-7 (Figure 18A). Western blot analysis also confirmed regucalcin expression in MCF-7 cells enabling the detection of an immunoreactive protein of the expected size with approximately 33 kDa (Figure 18B). In addition, it is shown that intensity of immunoreactive band is enhanced following increasing loads of MCF-7 protein extracts.

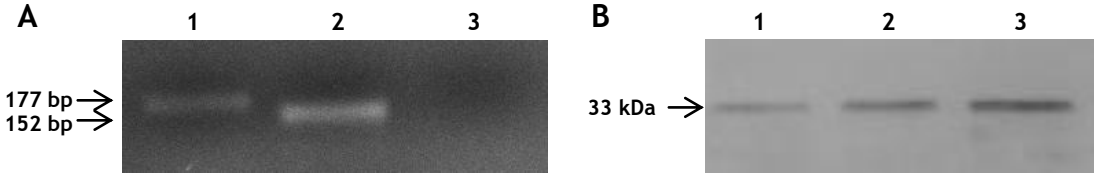
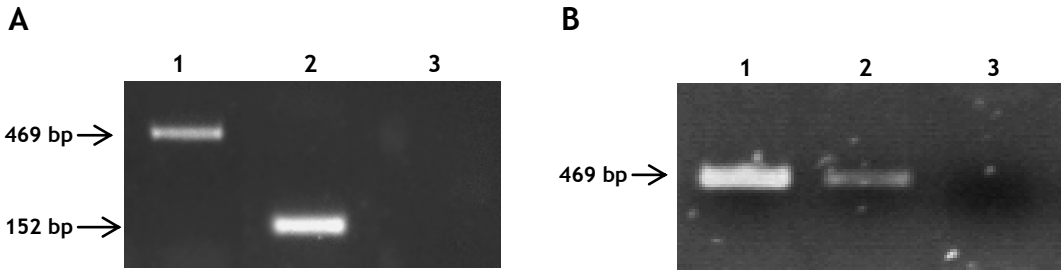


Figure 18. Expression of regucalcin in human breast cancer MCF-7 cells. (A) RT-PCR. 1: Regucalcin; 2: 18S used as positive control of cDNA synthesis; 3: Negative control, no cDNA added. Amplicons sizes are indicated on the left side. (B) Western blot analysis using a mouse anti-human regucalcin monoclonal antibody (1:1000). Different loads of MCF-7 total protein extracts were used. 1: 40µg; 2: 50µg ; 3: 60µg. Molecular weight is indicated on the left side.

RT-PCR results also allowed confirming L-type Ca^{2+} channel expression in MCF-7 cells using specific primers to the L-type Ca^{2+} channel (α_{1C} subunit) (Figure 19A). Western blot analysis enabled the detection of an immunoreactive protein of the expected size with approximately 190 kDa (Figure 19C). In addition, it is shown that intensity of immunoreactive band is enhanced following increasing loads of MCF-7 protein extracts. Total RNA and protein extracts of human and rat Sertoli cells (Figure 19B,D) were used as positive control, since L-type Ca^{2+} channel expression was already demonstrated in these cells (*237*).



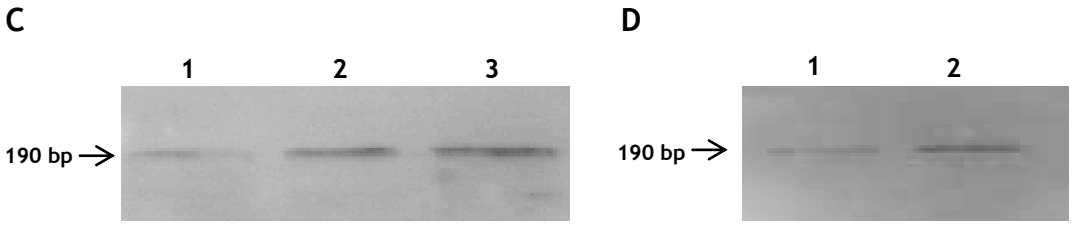


Figure 19. Expression of L-type Ca^{2+} channel (α_{1C} subunit) in human breast cancer MCF-7 cells . Sertoli cells were used in RT-PCR and WB as as a positive control for detection. (A) RT-PCR analysis in MCF7 cells. 1: L-type Ca^{2+} channel (α_{1C} subunit); 2: 18S used as positive control of cDNA synthesis; 3: Negative control, no cDNA added. (B) RT-PCR analysis in Sertoli cells: 1: L-type Ca^{2+} channel (α_{1C} subunit) in human cells; 2. L-type Ca^{2+} channel (α_{1C} subunit) in rat cells; 3: Negative control, no cDNA added. Amplicons sizes are indicated on the left side. (C) Western blot analysis using a rabbit anti-human L-type Ca^{2+} channel (α_{1C} subunit) antibody (1:200) in MCF-7 cells. Different loads of MCF-7 total protein extracts were used. 1: 40µg; 2: 50µg; 3: 60µg. Molecular weight is indicated on the left side. (D) Western blot analysis using a rabbit anti-human L-type Ca^{2+}-channel (α_{1C} subunit) antibody (1:200) in rat Sertoli cells. Different loads of rat Sertoli cells total protein extracts were used. 1: 20µg, 2:30µg.

2. DHT down-regulates expression of calcium-binding protein regucalcin in MCF-7 cells

The analysis of the effect of androgens on the expression of regucalcin in MCF-7 cells was performed by means of real-time PCR. For this purpose, a time-course experiment at 0, 6, 12 and 24h was carried out using 1nM of DHT. Real-time PCR analysis demonstrated that DHT induces a significant down-regulation of regucalcin mRNA expression (approximately two-fold) in MCF-7 cells in comparison to control group (Figure 20). A significant down-regulation was also observed with 10nM and 100nM of DHT (data not shown).

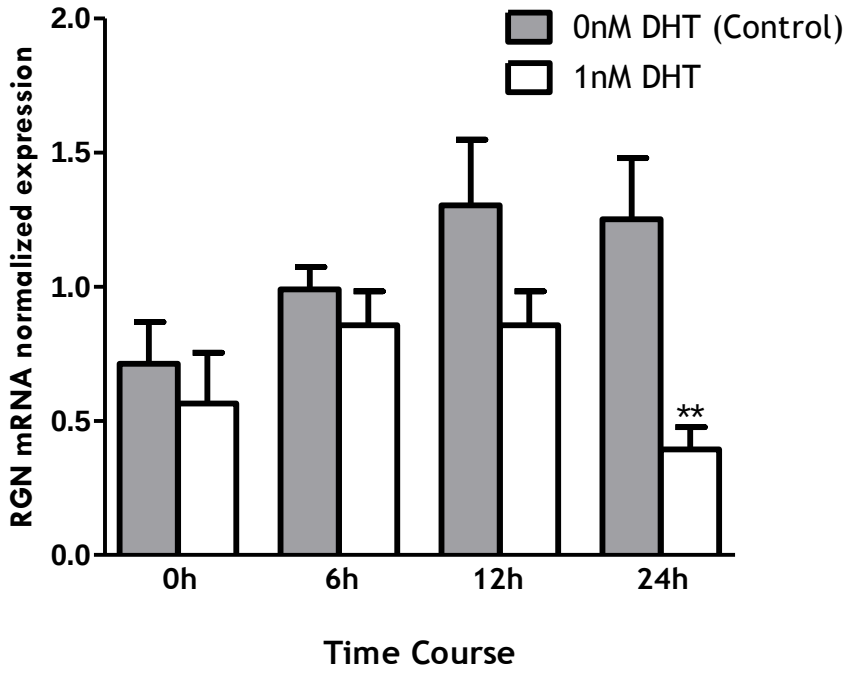


Figure 20. Effect of DHT on regucalcin mRNA expression in MCF-7 cells determined by real-time PCR. MCF-7 cells were either exposed to vehicle (0nM DHT –control group) or to 1nM of DHT for 0, 6, 12 and 24h. Regucalcin expression was normalized with that of GAPDH housekeeping gene. Results are indicated as mean ± SEM (n=6 in each experimental condition) **p<0.01 compared to control values.

3. DHT down-regulates expression of L-type Ca^{2+} channel (α_{1C} subunit) in MCF-7 cells.

Following the same strategy applied for regucalcin, the effect of androgens on the expression of L-type Ca^{2+} (α_{1C} subunit) in MCF-7 cells was also investigated. Real-time PCR analysis demonstrated that the mRNA expression of L-type Ca^{2+} channel (α_{1C} subunit) is down-regulated by DHT (approximately two-fold) in MCF-7 cells in comparison to controls (Figure 21). A significant down-regulation was also observed with 10nM and 100nM of DHT (data not shown).

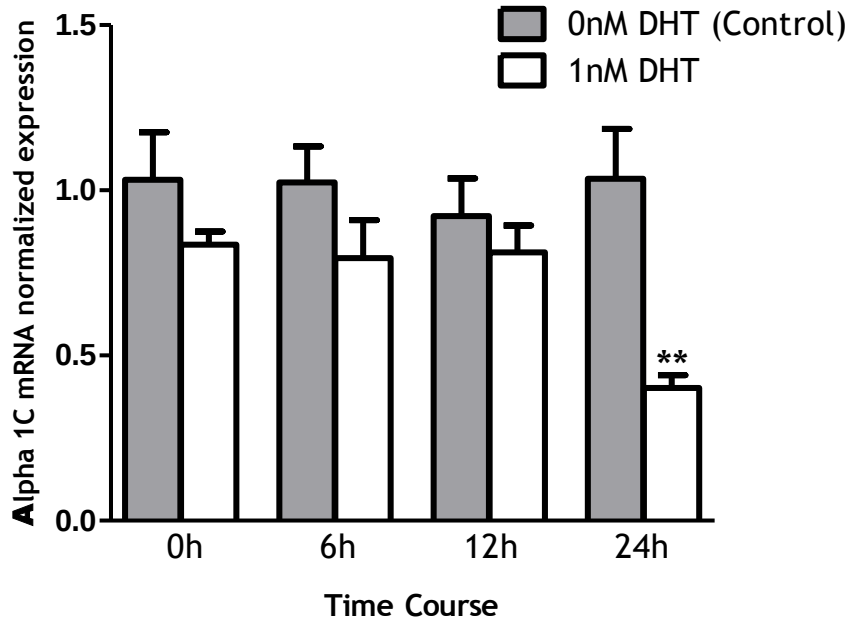


Figure 21. Effect of DHT on L-type Ca^{2+} channel (α_{1C} subunit) mRNA expression in MCF-7 cells by real-time PCR. MCF-7 cells were either exposed to vehicle (0nM DHT – control group) or to 1nM of DHT for 0, 6, 12 and 24h. L-type Ca^{2+} channel (α_{1C} subunit) expression was normalized with GAPDH housekeeping genes. Results are indicated as mean ± SEM (n=6 in each experimental condition) **p<0.01 compared to control values.

4. DHT effects regulating calcium-binding protein regucalcin and L-type calcium channel (α_{1C} subunit) mRNA expression are reverted by androgen and oestrogen inhibitors

To explore the mechanism underlying the androgenic down-regulation of regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) expression, MCF-7 cells were exposed to 1nM of DHT alone or in presence of AR inhibitor flutamide (Flut, 1μM), protein synthesis inhibitor cyclohexamide (Chx, 1 μg/mL) or ER inhibitor ICI 182,780 (ICI, 100nM) during 24h. This group was included since DHT has been shown to be converted to 3β-diol which is able to activate ERs (*47*).

These experiments further confirmed the down-regulation of regucalcin (Figure 22) and L-type Ca^{2+} channel (α_{1C} subunit) (Figure 23) in MCF-7 cells in response to DHT treatment. Moreover, the androgenic effect down-regulating regucalcin and L-type Ca^{2+} channel is reverted by both, androgen (Flut) and oestrogen receptor (ICI) inhibitors (Figures 22 and 23).

It is also noteworthy that Chx did not reverse the down-regulation of regucalcin expression induced by DHT. Moreover, MCF-7 cells stimulated with Chx alone display a down-regulated regucalcin mRNA expression (approximately two-fold), when compared to controls. No significant differences were observed using Flut or ICI alone (Figure 22).

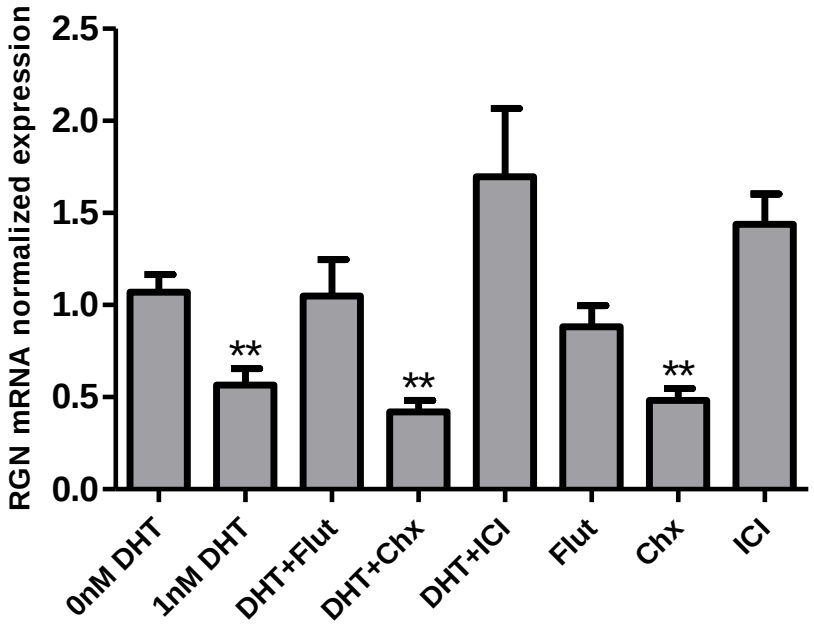


Figure 22. Effect of flutamide (Flut), cyclohexamide (Chx) and ICI 182,780 (ICI) on DHT regulation of regucalcin expression in MCF-7 cells. MCF-7 cells were exposed for 24h to DHT (1nM), DHT plus flutamide (1μM), DHT plus cyclohexamide (1μg/mL), DHT plus ICI 182,780 (100nm), flutamide, cyclohexamide, and ICI 182,780. Results are indicated as mean ± SEM (n=6 in each experimental condition) **p<0.01, compared to control values).

Considering L-type Ca^{2+} channel (α_{1C} subunit), in addition to Flu and ICI, also Chx reverted the DHT effect. Moreover MCF-7 cells stimulated with Flut, Chx or ICI alone display an up-regulated regucalcin mRNA expression (approximately two-fold by Flut and Chx, and three-fold by ICI), when compared to controls (Figure 23).

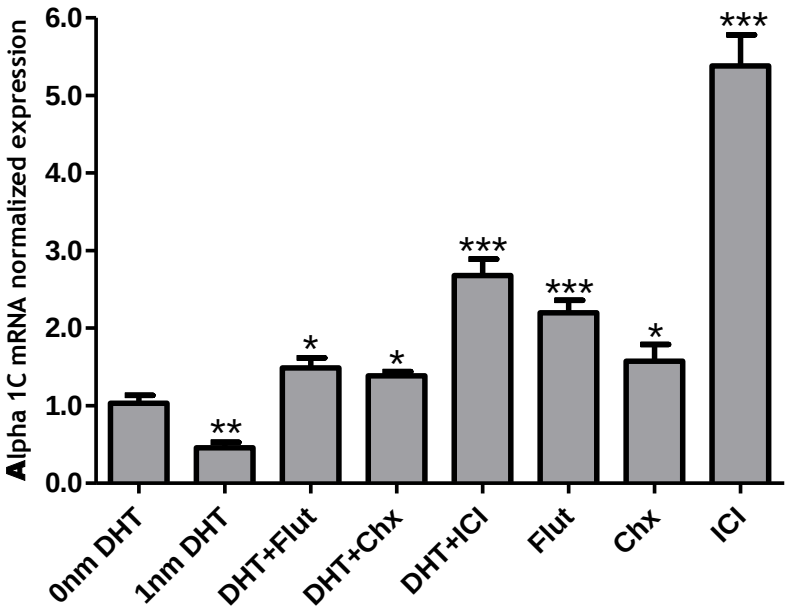


Figure 23. Effect of flutamide (Flut), cyclohexamide (Chx) and ICI 182,780 (ICI) on DHT regulation of L-type Ca^{2+} channel (α_{1C} subunit) expression in MCF-7 cells. MCF-7 cells were exposed for 24h to DHT (1nM), DHT plus flutamide (1μM), DHT plus cyclohexamide (1μg/mL), DHT plus ICI 182,780 (100nm), flutamide, cyclohexamide, and ICI 182,780. Results are indicated as mean ± SEM (n=6 in each experimental condition) *p<0.05, **p<0.01, ***p<0,001 compared to control value).

IV. Discussion

Ca^{2+} homeostasis is essential for several biological processes in the cell such as proliferation, differentiation and apoptosis. It is a steady state between Ca^{2+} influx, efflux, and storage, which is essentially regulated by the activity of Ca^{2+} transporters, Ca^{2+} channels and Ca^{2+} binding proteins. In this way, deregulation of $[Ca^{2+}]_i$ via altered expression and/or function of these proteins may have implications in mammary gland pathophysiology. Previous studies have demonstrated that androgens regulate the expression and/or activity of several Ca^{2+} regulator proteins, namely the Ca^{2+}-binding protein regucalcin and voltage-dependent L-type Ca^{2+} channel in distinct cell types. This raised the question whether androgens play the same role on these Ca^{2+} regulator proteins in human breast cancer cells.

In the present thesis, we further confirmed previous results of our research group demonstrating the presence of regucalcin mRNA and protein in human breast cancer MCF-7 cells cultured *in vitro* (*77, 106*). We also demonstrated the presence of mRNA and protein of L-type Ca^{2+} channel (α_{1C} subunit) in human breast cancer cells MCF-7. RT-PCR analysis allowed to detect a band of approximately 469 bp , which corresponds to the amplicon predicted size for the specific primer pair for L-type Ca^{2+} channel (α_{1C} subunit) (Figure 19A). An equivalent band was amplified in human and rat Sertoli cells using the same primer pair (Figure 19B), Western blot analysis showed an immunoreactive protein of approximately 190 kDa in MCF-7 cells (Figure 19C), which corresponds to the L-type Ca^{2+} channel (α_{1C} subunit) predicted size previously reported by Silva et al. (*237*). An equivalent immunoreactive protein was detected using the same antibody in human and rat Sertoli cells protein extracts (Figure 19D). The presence of L-type Ca^{2+} channel (α_{1C} subunit) has already been described in Sertoli cells (*237*), and their inclusion here as positive control reinforce the demonstration of L-type Ca^{2+} channel expression in human breast cancer MCF-7 cells. Others ion channels have been identified in MCF-7 cells, such as K^+ channel ($K_v1.3$, $K_v10.1$-2 and $K_{2p}9.1$), transient receptor potential cation channel subfamily V member 6 (TRPV6), transient receptor potential cation channel subfamily P member 8 (TRPP8), TRPM8 and Cl^- channels (*241-244*). However, and at least for our knowledge, this is the first report demonstrating the expression of L-type Ca^{2+} channel (α_{1C} subunit) in MCF-7 cells, which indicates that this channel may play an important role in Ca^{2+} homeostasis in human breast cancer cells. Regucalcin mRNA expression is regulated by several factors, namely, Ca^{2+} (*101, 111, 121*), calcitonin (*122*), insulin (*121*), and oestrogen (*106, 120, 123*) suggesting that the expression of regucalcin mRNA is enhanced through various hormonal stimuli. In addition, regucalcin is also regulated by androgens in prostate cancer cells and testicular tissue (*76, 77*). Several evidences also suggest that androgenic actions and calcium (Ca^{2+}) homeostasis alterations may contribute to the development of breast cancer. Moreover, androgens have been identified as Ca^{2+} regulators in many cell types (*63-72*). Altogether, this leads us to investigate the effect of the androgen

DHT on the regulation of regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) expression in MCF-7 cells.

For this purpose, MCF-7 cells were exposed to DHT (1nM) for different periods of time, either alone or in presence of inhibitors. Real-time PCR analysis demonstrated that DHT significantly down-regulated (approximately two-fold) both regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) mRNA expression at 24h of treatment in comparison with control group; at all other experimental times expression levels remained similar to those of the control (Figure 20, 21). A down-regulation of regucalcin mRNA expression induced by androgens was also demonstrated by our research group in prostate cancer LNCaP cells, treated with 10nM of DHT (*77*), which further supports the effects observed herein. Androgens modulate L-type Ca^{2+} channel expression in several cells, such as coronary smooth muscle cells, myocardium, human prostate cancer cells, and Sertoli cells (*74, 234-237*). However, in all these cells, androgens have been shown to increase L-type Ca^{2+} channel expression (*74, 234-237*), which highlight for the specificity of breast cancer cells in androgenic response.

It is assumed that androgens and oestrogens freely cross the plasma membrane, enter the cytoplasm, and bind to and activate the intracellular receptor. The ligand-bound receptor acts as a transcription factor binding as heterodimer to specific DNA response elements in target gene promoters, causing activation or repression of transcription and subsequently protein synthesis (*48-50*). DHT is a non-aromatizable androgen, however, it was recently shown that its metabolite 3β-diol, is able to bind ER having oestrogenic effects (*47*). Thus, DHT effects may be directly mediated by its binding and consequent activation of AR or indirectly by the binding of its metabolite 3β-diol through consequent activation of ER (*46*).

In order to explore the mechanisms underlying the androgenic down-regulation of regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) expression, MCF-7 cells were exposed to 1nM of DHT during 24h, alone or in presence of androgen receptor antagonist Flut, inhibitor of protein synthesis Chx or ER antagonist ICI. Real-time PCR analysis further confirmed the down-regulation of regucalcin (Figure 22) and L-type Ca^{2+} channel (α_{1C} subunit) (Figure 23) mRNA expression in MCF-7 cells in response to DHT treatment. This effect was reverted in presence of Flut, suggesting that DHT effect is directly mediated through AR. In addition, and at least for L-type Ca^{2+} channel expression, AR mediated effects seem to require *de novo* protein synthesis since Chx reverted the down-regulation effect induced by DHT. But, more than reverting the effect of DHT, Flut increased significantly L-type Ca^{2+} channel (α_{1C} subunit) expression relatively to control (Figure 23). Flut was developed to competitively bind to AR and interfere with androgen-AR association and action, however, emerging data indicate that this anti-androgen may not function as pure AR antagonist. In fact, Flut have been shown to display agonist activities at the level of neuroprotection (*250, 251*). This suggests that the increasing L-type Ca^{2+} channel (α_{1C} subunit) expression obtained in MCF-7 cells may also due to the agonist effect of Flut.

DHT down-regulation of regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) mRNA expression was also reverted by incubation with ICI, an ER inhibitor (Figure 22, 23). This

suggests the existence of DHT effects which could likely be indirectly mediated through ER by binding of its oestrogenic metabolite, 3β-diol. It is also noteworthy that in addition to revert the DHT effect, ICI also increase significantly regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) expression in MCF-7 cells. ICI binds to both ER subtypes with a comparable affinity to oestradiol and is considered as an efficacious antagonist of the ER-dependent proliferative actions of oestrogen in reproductive organs such as the breast and uterus (*252*). However, several studies have demonstrated that also this antagonist may have agonist effects, namely in specific cells of such as the sheep uterus and in hippocampal neurons (*253, 254*). These findings support and may explain the significant increase of regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) expression in MCF-7 cells in response to ICI alone or even in combination with DHT.

V. Conclusions and Future Perspectives

In this thesis we firstly demonstrated the expression L-type Ca^{2+} channel (α_{1C} subunit) in human breast cancer cells MCF-7. We also determined the down-regulatory effect of androgens on the expression of Ca^{2+}-binding protein regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) in MCF-7 cells, an effect that seems to be mediated by the AR, but also by likely by the ER. With the present work it was highlighted the importance of androgens modulating expression of Ca^{2+} regulator proteins in human breast cancer cells. Also, the molecular mechanisms underlying androgenic actions in human breast cancer cells started to be deciphered. Moreover, obtained data suggest that androgenic actions controlling breast cell death and proliferation may be associated with the control of Ca^{2+} homeostasis.

Additional studies should be realised in order to reinforce this conclusion. First, the DHT effect on regucalcin and L-type Ca^{2+} channel (α_{1C} subunit) expression should be studied at protein level, to confirm (or not) the data obtained at mRNA level. Second, it will be extremely useful to determine whether androgens may also regulate L-type Ca^{2+} channel activity by means of electrophysiological studies. Finally, it should also be of interest to use the same experimental approach to analyse the DHT on the expression of Ca^{2+} regulatory proteins.

VI. References

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