A glucosamine derivative affects metastatic activity in two prostate cancer cell lines by stimulating Maspin expression

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Il piccolo principe sedette su una pietra e alzò gli occhi verso il cielo:

"Mi domando" disse "se le stelle sono illuminate perché ognuno possa un giorno trovare la sua"

A Papà, Mamma, Enzo e Peppe
le mie stelle

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1 ABSTRACT

Prostate Cancer (CaP) is the most common male tumor and is the third leading cause of cancer death, with an incidence of 1.28 million cases worldwide, according to the data collected by the Global Cancer Observatory in 2018.

PC3 and LNCaP cell lines represent suitable models to study CaP development, due to their different metastatic origin and their distinct sensitiveness to androgen signaling. PC3 is a hormone-insensitive cell line isolated from a vertebral metastatic prostatic tumor. It lacks of androgen receptor (AR) and its abnormal growth could be attributed to enhanced expression levels of TGF-α, EGF, and EGF-R. LNCaP cell line was isolated from a human metastatic prostate adenocarcinoma found in a lymphnode. It expresses a T877A mutated AR form, which results in an enhanced binding affinity for several steroid compounds. Both cell lines present very low basal levels of Maspin expression. Maspin, an unusual member of the Serine Proteases superfamily, has been characterized as a class II tumor suppressor gene in many cancer types, among them CaP, due to its ability to inhibit cell invasiveness and proliferation and to increase apoptosis, thus inhibiting metastasis. In normal prostate epithelial cells, Maspin is highly expressed, whereas in prostate cancer cells its expression is almost completely suppressed. Previously in our laboratory, a glucosamine derivative, NCPA, has been proved to be effective in stimulating Maspin expression and to induce its nuclear localization in an osteosarcoma cell line, 143B.

The aim of my PhD project was to evaluate the ability of NCPA to affect metastatic activity in two prostate cancer cell lines, the hormone-insensitive PC3 and the hormone-sensitive LNCaP cells, respectively representative of late-stage and early-stage CaP.

2 INTRODUCTION

2.1 Human Prostate: anatomy and development

The human prostate, a gland of the male reproductive system, is commonly described as a small walnut-sized organ. It is located anterior to the rectum at the base of the bladder, surrounding the prostatic urethra. The prostate is formed of both muscular and glandular tissues [Amin and Khalid, 2011]. It is an accessory gland enhancing male fertility by producing and secreting fluids containing many enzymes (acid phosphatase, β-glucuronidase, amylase, fibrinolysin, proteases), prostaglandins, spermine and spermidine, immunoglobulins and zinc, that constitute the ejaculate [Drake, 2008]. The prostate is highly susceptible to benign prostatic hypertrophy (BPH) and prostatitis onset and undergoes to oncogenic transformation more than other tissues in men. Investigating prostate anatomy and organogenesis is of great importance in understanding the site and origin of prostatic diseases. McNeal first described four anatomic zones of the prostate on the basis of biological and histological concepts. Each zone originates from prostatic urethra and displays specific architectural features: the peripheral zone, the central zone, the transitional zone and the peri-urethral zone (Figure 1) [Mcneal, 1981].

Peripheral zone covers almost 75% of the glandular tissue of the adult prostate. It forms a disc of tissue whose ducts radiate laterally from the urethra and distal to the verumontanum, a structure located on the floor of the posterior urethra, which limits the membranous and the prostatic segment [Geavlete et al., 2016]. Almost all carcinomas arise here.

Central zone constitutes nearly 25% of the glandular tissue of the prostate. Its ducts open close to the ejaculatory ducts into the prostatic urethra. Their orifices arise separately from those of the peripheral zone, even if its distal part

is surrounded by the peripheral zone. This region is relatively resistant to carcinoma and other diseases.

Transitional zone represents an evolution of the embryonic development of the urogenital sinus (UGS), above the base of the verumontanum into the pre-prostatic urethral segment. In this zone, no major ducts are present in the proximal segment, but there is an elongation of the lateral rows of the peripheral zone. Only tiny channels of near-microscopic size and vastly simplified structure, constituting less than 1% of the mass of glandular prostate, are present in adult subjects. Although insignificant in size and probably in function importance, the transitional zone has great significance for adult pathology. It grows with age and is the common site of BPH origin.

Peri-urethral zone is a non-glandular region, representing about one third of the volume of the tissue within the prostatic capsule. It produces a convexity in its anterior surface, covered by fibromuscular stroma. It is made of a thick sheath of tissue, continuous with the detrusor muscle at the bladder neck. In this region are present small ducts and acini not completely developed [Mcneal, 1981].

During embryogenesis the primitive UGS, the structure giving rise to urinary and reproductive organs, resulting from an endodermal origin [Seifert et al., 2008], forms a caudal elongation of the hindgut. In humans, the UGS and the hindgut are initially connected in a single excretory tract at the embryonic cloaca. Progressively, by 8 weeks of gestation, the cloaca undergoes to division into separate urogenital and anorectal tracts [Hynes and Fraher, 2004]. Subsequently, the primitive UGS is subdivided into the bladder at its rostral end, the UGS in the middle and the penile urethra in the caudal part [Toivanen and Shen, 2017]. Through epithelial development, from the UGS, starting at

10 weeks of gestation, the prostate takes shape [Kellokumpu-Lehtinen et al., 1980]. During birth and pre-pubertal ages prostate organogenesis continues, under the influence of circulating androgens, and reaches its mature size during puberty. Prostate development could be divided, more in detail, into the following stages: (1) pre-bud UGS origin, directly or indirectly mediated by androgens, (2) emergence of solid prostatic epithelial buds from UGS epithelium (UGE), initiating tissue outgrowth, (3) bud elongation and branching, to form a system of ducts composed of solid epithelial cords, a process that involves paracrine signaling, in which androgen receptor (AR) function mediates epithelial growth, (4) canalization of the solid epithelial cords, leading to the formation of the mature ductal network, (5) differentiation of luminal and basal epithelial cells, (6) secretory cytodifferentiation, to give origin to functional glandular epithelium with completely differentiated cell types (Figure 1) [Cunha et al., 2018; Timms, 2008].

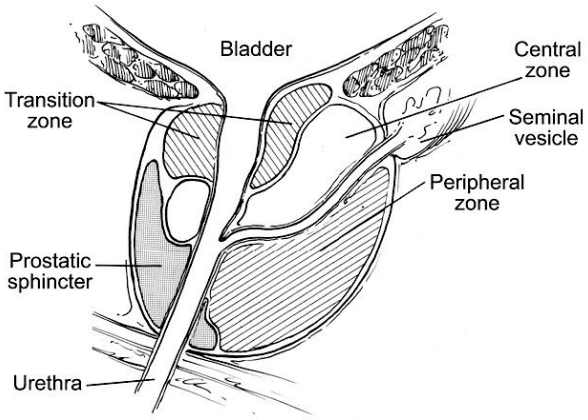

Figure 1. Overview of human prostate [Cunha et al., 2018]

Luminal and basal cells mainly constitute the mature prostatic epithelium (Figure 2). These cells derive from common progenitor/stem cells, highly concentrated in the embryonic UGS (from which prostatic epithelium origins) and presumably within the basal compartment of adult prostatic epithelium [Wang et al., 2001]. Luminal cells express cytokeratins [Verhagen et al., 1988] and secretory proteins such as prostate specific antigen (PSA) [Liu et al., 1997], largely used as biomarker for the diagnosis and screening of prostate cancer. Below the luminal layer, at the basement membrane level, basal cells express also cytokeratins [Verhagen et al., 1988], as well as p63, a biomarker required for prostate development [Signoretti et al., 2000]. Luminal cells, in addition, express high levels of AR compared to basal cells, which result negative to AR immunoreactivity tests [El-Alfy et al., 1999]. Intermediate cells co-expressing luminal and basal markers are often present in the basal layer. Ultimately, few neuroendocrine basally localized cells express secreted neuropeptides and other hormones, such as somatostatin and bombesin, to date good target for drug development in the treatment of prostate cancer [Abrahamsson, 1999; D'Angelillo et al., 2014; Kim et al., 2017b].

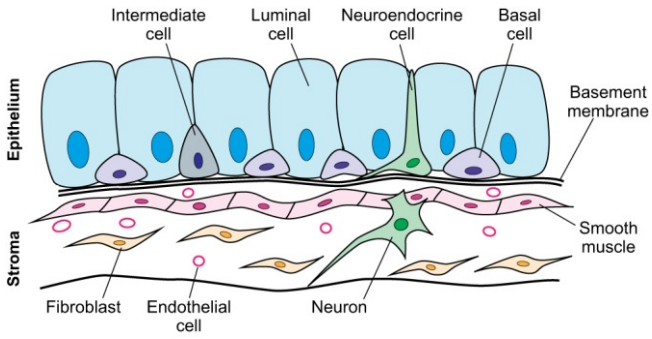

Figure 2. Differentiated cell types in the prostate [Toivanen and Shen, 2017]

2.2 Prostate cancer

Prostate Cancer (CaP) is the most common male malignancy and is the third leading cause of cancer death, with an incidence of 1.28 million cases worldwide [Bray et al., 2018]. Epidemiological studies report how cancer risk, aggressiveness and prognosis vary by race, ethnicity and geography [Rebbeck, 2017]. CaP incidence is higher in developed countries (North America, Western and Northern Europe and Australia), in contrast its mortality is higher in African men, based on 2018 data from the Global Cancer Observatory. The variation in incidence and mortality rates among men from different countries of the world reflect the differences in lifestyle, screening and treatment behavior, individual risk factors and genetic pattern.

In the past, CaP has been considered a rare disease, due to the shorter life perspective and the few detection and screening methods. Over time, histological studies of a number of autopsies provided important informations about the development of CaP: the peripheral zone of the prostate is site of origin of the disease and the distribution of the tumor depends on the gland density; contrary to some hypotheses, the carcinoma does not origins from atrophic glandular epithelium, but it selectively rises in gland with an active epithelial activity; CaP increases with age and its growing rate has a logarithmic trend; the malignancy course of the carcinoma has a direct relationship with tumor size, for its ability to locally invade gland capsule, peri-urethral tissue and verumontanum [McNeal, 1969].

CaPs have been mainly characterized as acinar adenocarcinoma, but exists a minority of cancers classified as acinar and non-acinar variants. Subtypes of adenocarcinoma and other carcinomas rarely occur in prostate gland, such as ductal adenocarcinoma, mucinous carcinoma and signet ring cell carcinoma [Grignon, 2004; Humphrey, 2012]. The disease originates from a high-grade

prostatic intraepithelial neoplasia (HGPIN), considered a pre-cancerous lesion. Prostatic intraepithelial neoplasia (PIN) is histologically characterized by three increasing levels of severity [McNeal and Bostwick, 1986]. As PIN grade increases, a progressive disruption of the basal cells layer occurs, as demonstrated by the absence of basal cell-specific keratins in immunoreactivity tests [Brawer et al., 1985]. Histological studies report three ways by which PIN growth, through prostatic ducts, occurs: the first involves the replacement by neoplastic cells of the normal luminal secretory epithelium with the preservation of the basal cell layer and the basement membrane; the second involves permeation of neoplastic cells along ducts between the basal cell layer and luminal secretory layer; the third involves invasion through the duct wall, with disruption of the basal cell layer and basement membrane [Bostwick and Brawer, 1987]. The loss of the basal cell layer confirms the histological diagnosis of CaP.

2.2.1 Gleason grading system

During 1960-1975 the Veterans Administration Cooperative Urological Research Group (VACURG) conducted a series of controlled and randomized clinical trials in order to develop a histological grading system for CaP. The classical Gleason system (Figure 3) identifies 5 histological grades analyzing by light microscope the glandular tissue: the grade is essentially based on phenotype of tumor cells compared to normal prostatic cells and a lower grade indicates a better prognosis. In the original grading system, i) Gleason pattern 1 is characterized by a well-circumscribed, nodular lesion composed of approximately uniform, well-differentiated glands of regular size; ii) Gleason pattern 2 shows variations in sizes of the neoplastic glands, slightly increased stroma between the glands, and even mild irregularity at the periphery of the nodule; iii) Gleason pattern 3, described in many series as the most common pattern, is composed of individual, discrete and distinct neoplastic glands, typically small, but often of variable sizes and infiltrating into the stroma, between the benign glands. This original pattern includes cribriform structures, with irregular contours, or within small, smooth, rounded glandular spaces, or "gland in gland formation", i.e., glomerulation; iiii) Gleason pattern 4 exhibits fused glands, which are no longer individual or distinct, resulting in irregular fused glandular or cribriform patterns; iiiii) Gleason pattern 5 includes comedo type necrosis, solid or cordlike growth or infiltration of individual tumor cells, without any trace of gland formation (Figure 3) [Gleason, 1992].

Figure 3. Prostatic adenocarcinoma (histologic patterns). Standardized drawing for grading system [Gleason, 1992]

Since its definition, the Gleason score underwent to constant updates, due to the modern surgery protocols and the revised cancer management, in combination with the increased use of PSA serum level measurement as screening method. The International Society of Urologic Pathology (ISUP) significantly modified the original Gleason grading system during two conferences held respectively in 2005 and 2014. The follow-up studies provided a database for the Gleason grading system, based on the extent of glandular differentiation and the tumor growth pattern in the prostatic stroma, in correlation with clinical data such as staging and prognosis. To date, the original 1 and 2 scores are included in the Gleason 3 pattern and considered as non-aggressive, thus only Gleason scores from 3 to 5 are accepted. The Gleason scores 4 and 5 are considered the more aggressive, with a metastatic potential and worse clinical outcomes. Since in most cases prostate adenocarcinoma presents cells with different grade of differentiation, two grades could be assigned. A primary grade is given to cells covering the largest area of the tumor and the secondary grade is given to the next largest area. The sum of the primary (e.g., Gleason 3) and secondary (e.g., Gleason 4) grades gives the Gleason score (e.g. Gleason score =3+4=7). Tumors with Gleason 6 (3+3) are considered low-grade, while scores ≥7, with 4 and 5 grades are considered high grade [Chen and Zhou, 2016]. Currently, the Gleason grading system is one of the best prognostic value to predict the clinical outcome of the CaP, combined with the PSA serum level measurement, a common screening method to detect prostate abnormalities.

The World Health Organization (WHO) incorporated the revised Gleason grading system for the classification of CaP.

2.2.2 PSA screening and molecular markers in Prostate Cancer diagnosis

Prostate-specific antigen (PSA), or kallikrein-related peptidase 3 (KLK3), is a protein belonging to kallikreins trypsin-like or chimotrypsin-like serine proteinases. The gene is one of the fifteen kallikrein subfamily members located in a cluster of chromosome 19 [Sutherland et al., 1988]. Kallikreins are known to be involved in several pathways that regulate skin desquamation, tooth enamel formation, lung and kidney function, seminal liquefaction, synaptic neural plasticity, brain function and immune system response [Kalinska et al., 2016]. PSA protein is secreted by prostatic epithelial cells as a preproenzyme and requires post-translational modification to become catalytically active. The preproenzyme (preproPSA), with a length of 261 aminoacids, is processed to a 244-aminoacids non-catalytic zymogen (proPSA), which is secreted into the seminal lumen. Human kallikreins 2 (hK2) and human kallikrein 4 (hK4) remove the pro-leader peptide, by a proteolytic cleavage of 7 aminoacids, to form the active PSA of 237 aminoacids [Väisänen et al., 1999]. The mature and catalytically active PSA forms stable complexes with α_1-antichymotrypsin (ACT), α_2-macroglobulin (AMG), α_1-protease inhibitor and protease C inhibitor [Christensson et al., 1990; Christensson and Lilja, 1994; Rannikko et al., 1991].

PSA physiological function is related to the liquefaction of semen coagulum, by the cleavage of Semenogelis [Lee et al., 1989]. It is highly expressed specifically in the prostate gland and secreted into the seminal fluid at concentrations ranging from 0.2 to 5 mg/ml [Sensabaugh, 1978]. Normally, PSA is detected in the blood at very low levels, between 0 and 4 ng/ml [Martins et al., 2002]. Several clinical studies correlated the increased levels of serum PSA with the presence of prostatic adenocarcinoma. During 90's, PSA has

been widely used in clinical practice as the most relevant biomarker in the diagnosis of CaP [El-Shirbiny, 1994]. However, recently it has been accepted that PSA serum level measurement by itself is not a sufficient and specific method to detect the presence of CaP, because of its onset in patients with the complete absence of increasing PSA. On the other hand, some pathological conditions, such as prostate infections, inflammation, benign prostatic hypertrophy or hyperplasia (BPH) could lead to an increase in PSA amount in the blood [Hennessy et al., 2008].

It has been proposed to combine the detection of new molecular biomarkers to PSA serum level measurement, in order to improve its accuracy in the early diagnosis of CaP. New promising biomarkers include: 1) prostate cancer antigen 3 (*PCA3*); 2) transmembrane protease serine 2:v-erytrhoblastosis virus E26 transforming specific (*ets*) oncogene homolog gene fusion (*TMPRSS2:ERG*); 3) specific micro RNAs (miRNAs); 4) circulating tumor cells (CTCs); 5) AR variants; 6) phosphatase and tensin homolog (*PTEN*). Moreover, two tissue biomarkers tests are commercially available such as Prolaris®, measuring the aggressiveness of CaP by evaluating a panel of 31 cell cycle progression genes and Oncotype DX®, a quantitative RT-PCR assay evaluating five housekeeping controls and 12 CaP related genes belonging to four biological pathways with a role in prostate tumorigenesis, as androgen signaling, cell proliferation, cellular organization and stromal response [Filella et al., 2018].

PCA3, an androgen responsive gene, as putative ncRNA could be involved in transcriptional regulation, RNA splicing, DNA imprinting and demethylation, as well as in carcinogenesis, and it was found to be highly overexpressed in CaP [Bussemakers et al., 1999; Costa, 2005].

TMPRSS2:ERG gene fusion plays an important role in prostate tumorigenesis, contributing to cellular invasiveness *in vitro* and it has been identified as the most recurrent chromosomal aberration in CaP [Perner et al., 2007; Prensner and Chinnaiyan, 2009].

miRNAs act as post-transcriptional gene regulators in several cellular processes, such as cell differentiation, proliferation, apoptosis and carcinogenesis. An aberrant expression profile of miRNAs was detected in many cancer types, including CaP. In particular, miR-21, miR-141 and miR-375 were often found upregulated in urinary exosomes isolated from patients with CaP, compared to healthy subjects [Foj et al., 2017].

CTCs by definition acquired the ability to migrate from the primary tumor site into the blood stream in a process called epithelial-mesenchymal transition (EMT). In CaP early stage, CTCs may be used to predict the risk of generating metastasis or to evaluate therapy response in advanced malignancy [Doyen et al., 2012].

AR-Vs are abnormally truncated isoforms of AR protein lacking the C-terminal domain, with intact N-terminal and DNA-binding domains, thus remaining constitutively active, even in the absence of ligands. AR-Vs overexpression, detected in clinical specimens such as blood samples, occurs frequently in castration resistant prostate cancer (CRPC) patients and they have been proposed as potential markers and therapeutic targets [Antonarakis et al., 2016].

PTEN is a tumor suppressor gene that is mutated in several cancer types, including CaP, or inactivated by epigenetic mechanisms, such as promoter methylation or aberrant expression of miR-21 [Folini et al., 2010; Whang et al., 1998].

2.2.3 The molecular biology of Prostate Cancer

The investigation of molecular mechanisms involved in CaP initiation and progression is of great importance for developing early screening methods and selective therapeutic drugs. CaP development is driven by chromosomal alterations consisting of genetic predisposition to mutation of genes, somatic mutations that amplify oncogenes and/or lead to the loss-of-function of tumor suppressor genes. Chromosomal alterations include deletions and duplications. Deletions usually lead to regional loss-of-function, while duplications often result in regional gain-of-function of genes.

Chromosome 8, 13, 7, 10, 16, 6 and 17 are the most frequently altered in CaP [Schulz and Hoffmann, 2009]. A study conducted in 62 prostate cancer patients (53% European American and 47% African American), with mean age of 60.8 years, revealed that chromosome 8 is the most common region of genomic instability, displaying 66% loss-of-function and 19% gain-of-function at 8p locus and 24% loss-of-function and 56% gain-of-function at 8q locus of all tumors analyzed, with any racial differences [Cheng et al., 2012]. Anyway, besides genetic inheritance, at early stage CaP remains often euploid and numerical and structural abnormalities occur in advanced stages.

Key regulatory genes have been mapped on chromosomal region that undergoes to copy number alterations, such as *NKX3.1*, a negative regulator of epithelial cell growth in prostate tissue, at 8p21, *MYC*, a proto-oncogene involved in cell cycle progression, apoptosis and cellular transformation at 8q24, and *PTEN*, a tumor suppressor gene mutated at high frequency in several tumors, at 10q23 [Taylor et al., 2010].

NKX3.1 loss-of-function represents a frequent and critical event in prostate cancer initiation, due to its important role in cell differentiation and growth [Abate-Shen et al., 2008].

MYC oncogene was found to be highly amplified in a subset of advanced CaP and nuclear *MYC* protein is up-regulated in many PIN lesions, from which CaP origins [Gurel et al., 2008; Jenkins et al., 1997].

PTEN undergoes to copy number loss during the early stage of prostate carcinogenesis and its inactivation has been shown to cooperate with loss-of-function of *NKX3.1*, up-regulation of *MYC* proto-oncogene, or the *TMPRSS2:ERG* fusion [Carver et al., 2009; Kim et al., 2002, 2009]. *PTEN* loss-of-function is also related to an up-regulation of the Akt/mTOR signaling pathway, mediated by activation of p110β isoform of phosphoinositide 3-kinase (PI3K) [Hill et al., 2010; Thomas et al., 2004]. The consequence of Akt induction are also mediated by the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling via the stimulation of IκB Kinase (IKK) complex [Dan et al., 2008]. Moreover, analyses of autochthonous transgenic adenocarcinoma of mouse prostate (TRAMP) model and cell lines revealed that nuclear IKKα, one of the two catalitic subunits of IKK complex, enhances CaP metastatic phenotype through down-regulation of *maspin,* a class II tumor suppressor gene [Luo et al., 2007]. A recapitulation of molecular mechanisms is depicted in Figure 4.

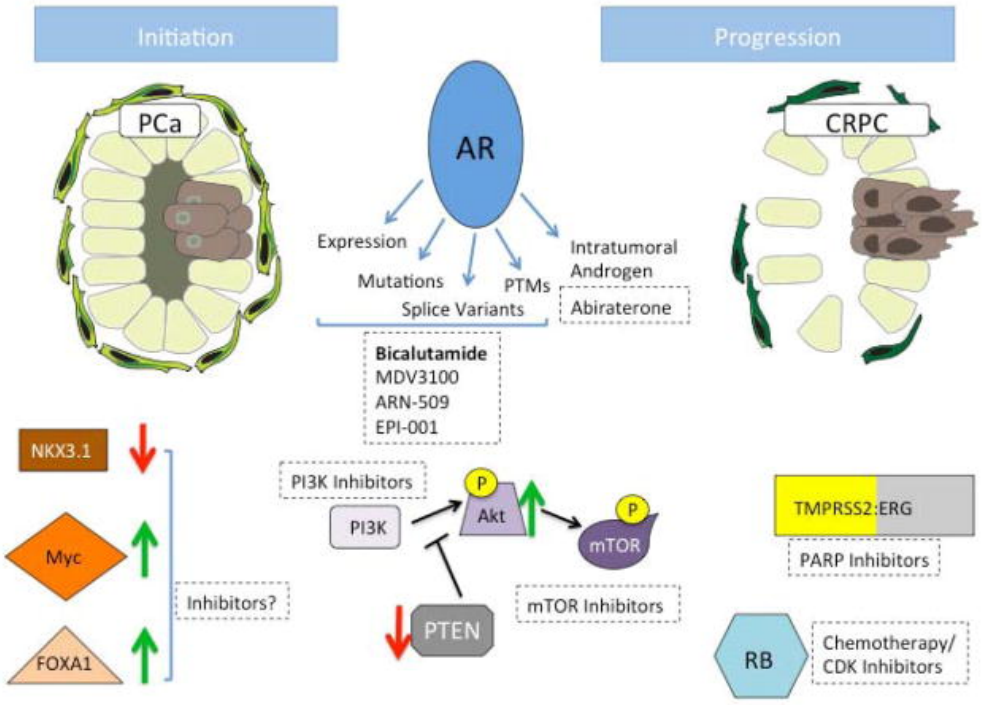

Figure 4. Molecules implicated in CaP initiation and progression [Schrecengost and Knudsen, 2013]

An overexpression of *ERG* oncogene was found in more than 55% prostate cancer cases and it is driven by the fusion with *TMPRSS2* gene, both located on chromosome 21 [Tomlins et al., 2012; Wang et al., 2017]. The most frequent alteration of *TMPRSS2* and *ERG* is gene fusion, a consequence of missense mutations found in both genes [Tomlins et al., 2005; Wang et al., 2017]. *TMPRSS2*, a prostate-specific and androgen-response gene, encodes a protein belonging to the serine protease family, which functions in prostate carcinogenesis and relies on gene fusion with *ets* transcription factors, such as *ERG* and *ets* variant 1 (*ETV1*) [Yu et al., 2010]. *ERG* is an oncogene that

encodes a member of the erythroblast transformation-specific family of transcription factors, and is a key regulator of cell proliferation, differentiation, angiogenesis, inflammation and apoptosis [Brenner et al., 2013]. Wang et al. demonstrated that *ERG* gene silencing significantly affected prostate cancer progression, by inhibiting tumor cell proliferation and by arresting cell cycle progression in G_0/G_1 stage [Wang et al., 2017].

CaPs are mainly acinar adenocarcinoma, expressing AR. *AR* gene is located on chromosome X, it is more than 90 kb long and codes for a protein that has 3 major functional domains: the N-terminal domain, DNA-binding domain, and androgen-binding domain. *AR* gene contains a polymorphic trinucleotide repeat region $(CAG)_{(n)}$ in its N-terminal transactivation domain (NTD) that encodes a polyglutamine (polyQ) tract. Upon binding the hormone ligand, the receptor dissociates from accessory proteins, translocates into the nucleus, dimerizes, and then stimulates transcription of androgen responsive genes. The most abundant androgen is testosterone, which is synthesized by the testis and converted into the more active metabolite dihydrotestosterone (DHT) in prostate tissue through the activity of 5α-reductase [Thigpen et al., 1993]. Mutations in *AR* gene are associated with complete androgen insensitivity [Taplin et al., 1995]. A study conducted in the TRAMP model revealed that, among 15 unique somatic mutations, all mutations were single base substitutions, 10 were missense and 5 were silent [Han et al., 2001]. The main mechanism by which mutations lead to abnormalities in AR function is alterations in cofactor binding or reduction in ligand specificity. Somatic missense mutations were found in the polyQ tract. This mutation leads to a reduction in ligand induced N- and C-terminal interaction, but increases receptor activity more than the wild type AR [Buchanan et al., 2004]. It is possible to postulate that enhanced interactions with coactivators may

potentiate AR signaling in presence of low levels of circulating androgens or weaker agonists, bringing to a therapy failure. The first AR mutation frequently found in late stage CaPs was a threonine to alanine substitution at aminoacid 877, which leads to a reduction in ligand specificity by altering pocket dimension [Veldscholte et al., 1992].

Circulating androgens are essential for normal prostate homeostasis as well as CaP initiation through their interaction with AR. Androgen ablation therapy, by chemical or surgical castration, proved to be an effective strategy for the regression of prostate tumor, but it has a short-term and the recurrence of aggressive and essentially untreatable CaP is very common, an event termed "castration resistance". Recent studies demonstrated that *PTEN* undergoes copy number loss as an early event in prostate carcinogenesis and this abnormality is related to the progression through aggressive, castration-resistant malignancy [Taylor et al., 2010]. Furthermore, an aberrant expression of specific miRNAs was found in castration-resistant prostate cancer, such as miR-125b, which is responsible of Bak1 down-regulation, a pro-apoptotic protein from the Bcl-2 protein family, and miR-221 and miR-222, both targeting p27/kip1, a cell-cycle regulator that blocks the cell cycle in G_0/G_1 stage [Shi et al., 2007; Sun et al., 2009].

Point mutations, also called somatic single nucleotide variations (SNVs), were found in several genes including *FOXA1, TP53, CDKN1B, KRAS* and *EGF-R* [David, 2017].

A variable over-expression of Cyclin D1 among prostate carcinoma cells was observed [Pereira et al., 2014]. Cyclin D1-positive cancer cells show greater motility, increased invasion capability and a hormone-independent phenotype in cell cultures, supporting the hypothesis that cyclin D1 plays an important role in aggressive prostate carcinogenesis [He et al., 2007].

Inflammation is highly related to prostate carcinogenesis. Infections deriving from exposure to environmental factors, such as bacterial or viral agent, dietary carcinogens, hormonal perturbation such as altered androgen and estrogen levels or physical trauma lead to chronic inflammation which is causally linked to CaP onset [Sfanos and de Marzo, 2012]. Other aging-associated mechanisms involved in prostate carcinogenesis are oxidative stress and DNA damage, telomere shortening and cellular senescence.

2.2.3.1 PC3 and LNCaP cell lines: How androgen sensitivity affects metastatic activity

Human derived PC3 and LNCaP cells are suitable models for prostate cancer studies. PC3 cells were isolated from a vertebral metastatic prostatic tumor in 1979 [Kaighn et al., 1979]. PC3 cell line is hormone-insensitive, lacks of AR and PSA expression at mRNA and protein level and abnormal growth could be attributed to enhanced expression levels of Transforming Growth Factor- α (TGF-α), Epidermal Growth Factor (EGF), and Epidermal Growth Factor Receptor (EGF-R) [Ching et al., 1993]. LNCaP cells were isolated from a human metastatic prostate adenocarcinoma found in a lymphnode [Horoszewicz et al., 1980]. LNCaP cells express AR, but they contain a T877A mutation in the AR coding sequence that enhances binding affinity for several steroid compounds [Veldscholte et al., 1990].

In absence of ligand, AR is located in the cytoplasm, associated with heat shock proteins (HSPs), cytoskeletal proteins and other chaperones. Androgen pathway activation is mediated by androgens as testosterone or its activated form, DHT. Upon ligand binding, a conformational change in AR occurs and helices 3, 4 and 12 within the ligand-binding domain (LBD) form the activation function 2 (AF2) binding surface. The amphipathic structure of helix 12 is positioned across the ligand-binding pocket, reducing ligand dissociation. As result of this conformational change AR dissociates from HSPs, interacts with co-regulators such as filamin-A, importin-α and AR-associated protein 70, ARA70, by binding to the AR nuclear localization signal (NLS). This event enhances nuclear targeting of AR and the consequent nuclear dimerization (Figure 5).

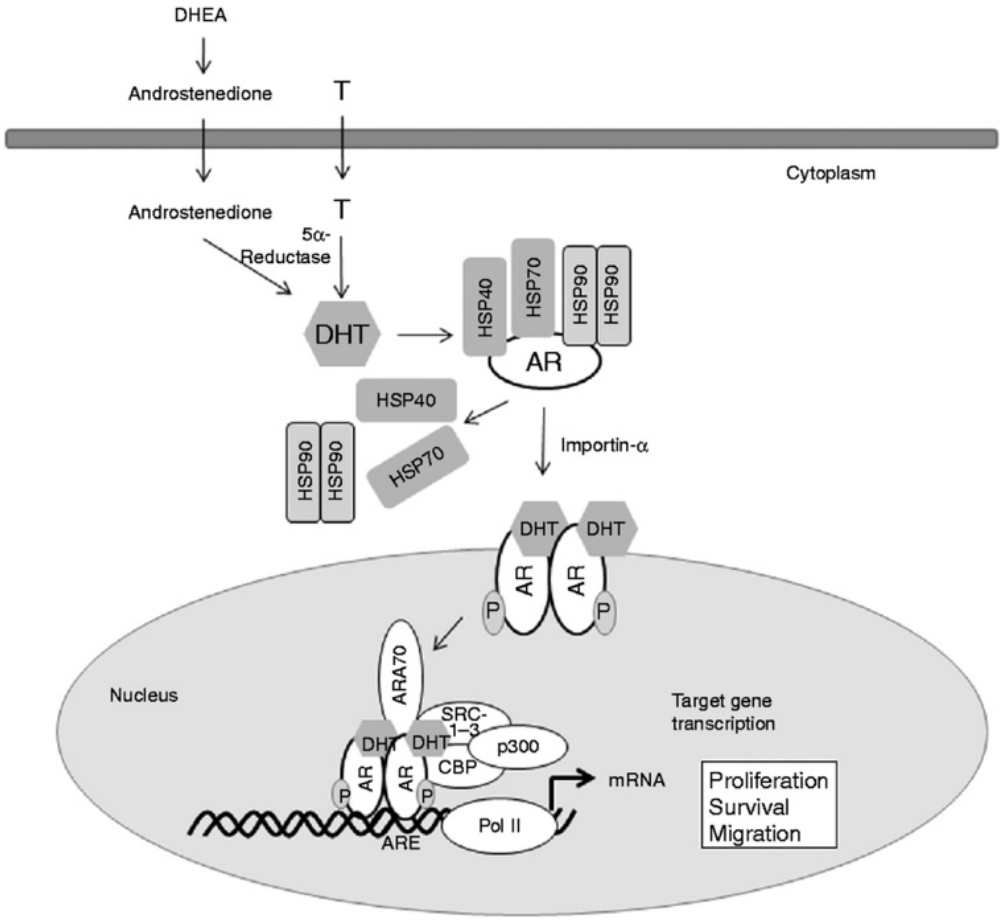

Figure 5. Classic AR activity via androgen [Sampson et al., 2013]

Several other co-activators bind to AR in the nucleus and the AR-DNA binding domain, AR-DBD, facilitates nucleic acid binding at androgen responsive elements (ARE), which promote recruitment of co-activators with histone acetyltransferase (HAT) activity (CBP/p300, P/CAF), via SRC/p160 co-activator family members, resulting in chromatine remodelling. Chromatin remodelling in turn allows binding of TATA binding protein (TBP) followed

by general transcription factors (GTF) and RNApolII to begin transcription. Non-ligand bound AR can shuttle back to the cytoplasm and recovered in preparation for further ligand binding or can be processed for proteosomal degradation mediated by ubiquitination under E3 ubiquitin ligase control. The nature of AR-bound ligands determines the stability of AR-DNA complexes and the amount of transcription [Bennett et al., 2010]. AR mediates cell proliferation, differentiation and apoptosis, as well as the secretory activity of proteins such as PSA.

Somatic mutations in metastatic CaPs and CPRCs have been found at the level of LBD of the AR [Yeh et al., 1998]. Mutated AR is susceptible to be bound and activated by several steroids and other molecules, which increase AR nuclear activity as indicated by an accumulation of AR dimers in the nucleus.

The main mechanism by which PC3 and LNCaP display different metastatic properties regard the absence of AR expression in PC3 and its mutation in LNCaP cells. In particular, PC3 cells lack AR expression, and their metastatic features are regulated by elevated levels of EGF and EGF-R. PC3 cell proliferation is also stimulated by TGF-α. EGF and TGF-α both bind to EGF-R [Jarrard et al., 1994; Sherbet, 2011]. T877A AR mutation in LNCaP cells reduces binding specificity to its natural androgen ligand, so other molecules such as progesterone, estrogens, growth factors and many antiandrogens can also activate the protein. Among growth factors, enhanced Insulin Growth Factor-1 (IGF-1) and EGF binding to AR increases receptor signaling in cell proliferation, leading to an induction of metastatic activity [Culig et al., 1994].

2.3 Maspin (MAmmary Serin Protease INhibitor): An unusual member of Serpins superfamily

Maspin (MAmmary Serin Protease INhibitor or SerpinB5) is a 42 kDa protein, non-classical member of the ovalbumin clade of SERine Protease INhibitors (Serpins), with a lenght of 375 aminoacids (RefSeq ID: NM_002639). In humans the gene is located on chromosome 18 in a cluster containing genes for other Serpins such as Squamous Cell Carcinoma Antigen (SCCA) 1 and 2 and Plasminogen Activator Inhibitor type 2 (PAI-2) [Law et al., 2006].

Although Maspin shares a common structure with classical Serpins, the reactive site (or center) loop (RSL or RCL) possesses a non-standard hinge region, on the N-terminus of the RSL, which prevents it from undergoing a critical structural change from the stressed (S) to relaxed (R) state. This uncommon feature prevents Maspin from being able to trap and inhibit serine proteases [Narayan and Twining, 2010]. Maspin consists of nine α-helices (helix A-I) and three β-sheets (sheets A-C) and adopts the native Serpin fold with the RLS fully expelled from the A β-sheet, exhibiting a high grade of flexibility. The RSL of Maspin is unique in length, structure and position. Although the RSL is exposed and cleaved by some proteases, it functions in the uncleaved form. The RSL makes minimal contacts with the core of the protein and is very flexible. A buried salt bridge causes an unusual bulge in the region of the D and E α-helices, an area demonstrated to be important for cofactor recognition in many other Serpins. Additionally, intact Maspin can undergo a major conformational change in the G α-helix and adjacent sequences, switching between an “open” and “closed” (Figure 6) conformation, thereby altering the electrostatic properties of a presumptive cofactor binding surface of the molecule, which may affect its function [Law et al., 2005].

Maspin has been characterized as a class II tumor suppressor gene in many cancer types, among them CaP, in which even though its expression is down-regulated, it is not mutated. Its anti-tumor ability is due to the inhibition of cancer cell invasion, attachment to extracellular matrices, increased sensitivity to apoptosis and inhibition of angiogenesis [Bailey et al., 2006].

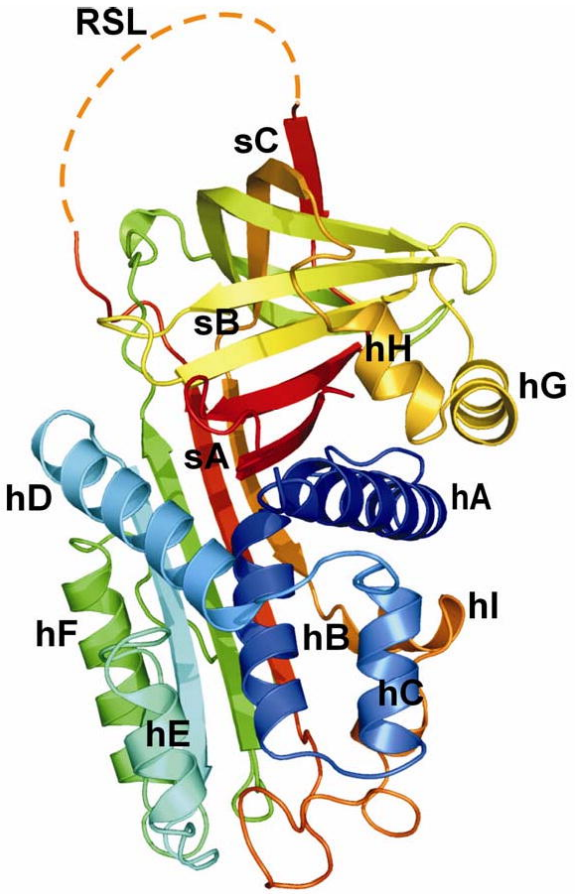

Figure 6. Cartoon representation of human maspin in the closed form (PDB ID: 1xu8)

2.3.1 Maspin molecular and biological functions in normal development

In order to understand the role of a tumor suppressor protein in cancer it is often useful to clarify its mechanism in physiological conditions. Maspin expression has been found in multiple tissues including epithelium of the breast, prostate, epidermis and lung and in the stromal cells of the cornea (Table 1) [Ngamkitidechakul et al., 2001; Reis-Filho et al., 2002; Yatabe et al., 2004].

Organ/ tissue	Subcellular expression in normal tissue	Subcellular expression in pathologic conditions
Placenta	Cytoplasm: Syncytio- and cytotrophoblasts, and endothelial cells; Nucleus: Chorionic plate	Preeclampsia: Upregulation
Mammary gland	Cytoplasm: Myoepithelial cells (intense in pregnancy and lactation); Nucleus: Myoepithelial cells	Invasive breast cancer: Maspin positivity is more frequent in ductal than lobular carcinomas; Cytoplasm only: Negative prognostic indicator, ER and PgR negativity; Nucleus: Better prognosis, ER and PgR positivity; Negativity: Loss or cytoplasm to nuclear translocation in metastatic tissue
Ovary	Negative	Benign tumors: Negative or infrequent nuclear; Ovarian carcinomas: Cytoplasm only: Cisplatin sensitivity; Mixed expression (cytoplasm and nucleus): Indicator of low malignant potential

Organ/ tissue	Subcellular expression in normal tissue	Subcellular expression in pathologic conditions
Uterine cervix	Squamous epithelium: Cytoplasmic and nuclear staining	CIN3: Cytoplasm: Down regulation; Nucleus: Upregulation; Squamous cell carcinoma: Cytoplasm: Tumor suppressor role; Adenocarcinoma: Cytoplasm: Aggressive behavior
Uterine body	Negative or positive (mostly nuclear) staining in normal endometrial glands; Low intensity in atrophic endometrium	Endometrial hyperplasia: Nucleus: Indicator of atypia; Endometrioid endometrial adenocarcinoma: Cytoplasm: Aggressive behavior; Nucleus: Better prognosis
Prostate	Basal cells: Positive; Secretory cells: Negative	HGPIN: Basal cells: Positive (same intensity as normal); Secretory cells: Positive; Adenocarcinoma: Low-grade carcinoma: Reduced expression compared with HGPIN; High-grade carcinoma: Low or no expression
Urinary bladder	Positive in epithelial cells	Urothelial carcinoma: Nucleus: Better prognosis

Table 1. Maspin expression in placenta, mammary gland and urogenital organs [Banias et al., 2019]

At cellular level, Maspin has been found in the cytoplasm, in the nucleus, at cell surface or secreted by extracellular vesicles [Pemberton et al., 1997; Reis-Filho et al., 2001; Zou et al., 1994]. Its different subcellular localization is related to its heterogeneous functions (Figure 7).

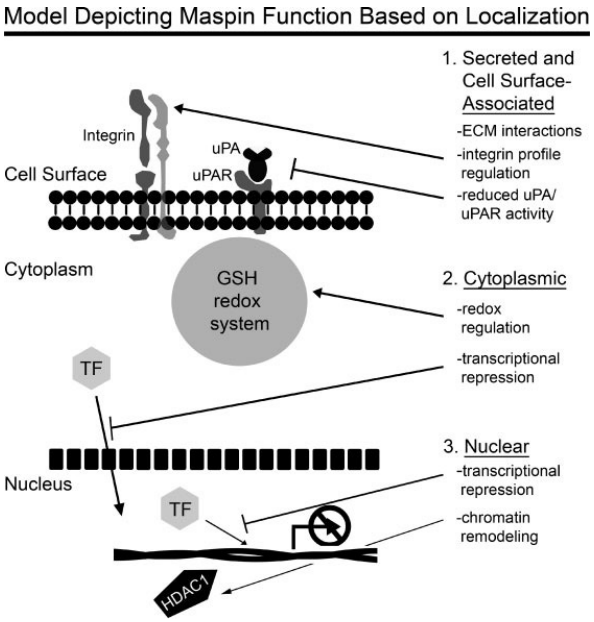

Figure 7. Possible function of Maspin depending on its cellular localization [Bailey et al., 2006]

Maspin interacts with several intracellular proteins, such as heat shock proteins (Hsp90 and Hsp70), glutathione S-transferase (GST), interferon regulatory factor 6 (IRF6), early growth response protein 1 (Egr-1), GCrich-binding factor 2 (GCF2) and histone deacetylase 1 (HDAC1).

Hsp90 and Hsp70 are important stress-related proteins that primarily act in hormonal regulation by binding and maintaining the native conformation of AR in the cytoplasm in absence of ligands. Furthermore, Hsp90 and Hsp70 are involved in apoptotic process, thus it has been demonstrated that Maspin interaction plays an important role in the regulation of AR signaling and cell sensitivity to apoptosis upon binding with heat shock proteins [Jones et al., 2004; Müller et al., 2005].

Maspin is an important regulator of cellular response to oxidative stress. An enzyme of the glutathione (GSH) redox system, glutathione S-transferase (GST) has been found to be a putative Maspin interacting protein [Yin et al., 2005]. The GSH redox system is essential in protecting cells against oxidative damage, thus Maspin has a role in reducing the amount of reactive oxygen species (ROS) generated during oxidative stress. ROS generation is also related to hypoxia-induced angiogenesis and GST regulation defines the role of Maspin as an anti-angiogenic molecule [Zhang et al., 2000].

Maspin plays an important role in the EMT biology, an important process associated with embryo implantation, embryogenesis and organ development, as well as tumor invasiveness and metastasis [Kalluri and Weinberg, 2009]. It has been reported a specific interaction between Maspin and IRF6, a member of IRF family associated to EMT, through the increase of N-cadherin, a cell adhesion protein [Bailey et al., 2005].

Maspin acts also at transcriptional level. It has been found an association between Maspin and *EGR1* and GCF2. Egr1 is a protein required for differentiation and morphogenesis and it is an immediate response molecule to tissue injury and cellular stress, whereas GCF2 is a transcriptional repressor induced by tissue damage and it is known to down-regulate the transcription of EGF-R, PDGF-A and TGF-α [Shankar et al., 2016]. The regulation of transcription is often associated to chromatin remodeling. Maspin interaction and inhibition of HDAC1 specifically upregulates genes promoting cell differentiation, cell cycle arrest or cell death and down-regulates genes involved in cell survival and EMT [Li et al., 2006].

2.3.2 The role of Maspin in tumor cells

Maspin was initially identified as a candidate tumor suppressor gene with a potential role in human breast cancer, due to its expression in normal mammary epithelial cells but not in most mammary carcinoma cell lines [Zou et al., 1994]. Maspin expression seems to be correlated with better prognosis in prostate, bladder, lung, gastric, colorectal, head and neck, thyroid and melanoma cancers. In prostate, breast and ovarian cancers Maspin relevance is related to its nuclear localization localization (Figure 8) [Berardi et al., 2013].

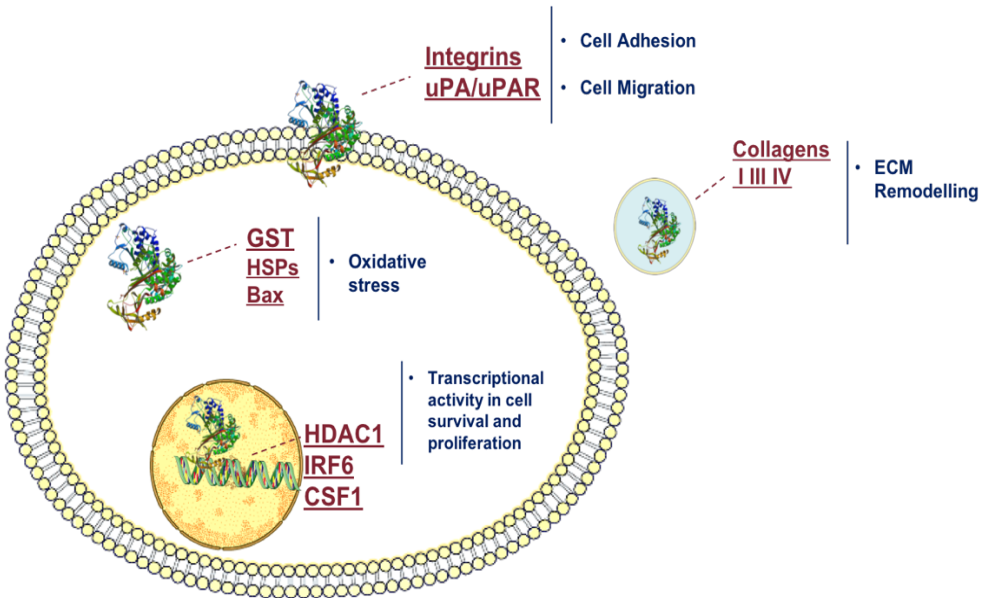

Figure 8. Schematic representation of the role of Maspin in tumor cells depending on its subcellular localization

It has been found that aberrant cytosine methylation and chromatin condensation of *maspin* promoter enhance Maspin silencing during cancer

development [Maass et al., 2002]. Maspin expression is differently regulated at transcriptional level: within the *maspin* promoter two cis elements, *ets* and hormonal responsive element (HRE), are present. In normal epithelial cells the *ets* element positively regulates Maspin expression, but it is inactive in tumor cells. AR can recognize and bind to the HRE which acts as a negative element, inhibiting the activity of *maspin* promoter [Zhang et al., 1997].

Maspin has been localized also in the Extracellular Matrix (ECM), where it interacts with and induces the expression of α5- and α3-containing integrins and reduces the levels of α2-, α4-, α6-, α(v)-, and some β1-containing integrins in metastatic human breast carcinoma cell line MDA-MB-435, stimulating cell adhesion and consequently inhibiting cell metastasis and invasion [Seftor et al., 1998]. Other potential binding proteins, whose regulation is involved in metastatic process, include type I, III and IV collagen and laminin [Blacque and Margaret Worrall, 2002; Ngamkitidechakul et al., 2001]. Extracellular Maspin interacts also with cell-surface-associated urokinase plasminogen activator, uPA, and its receptor uPAR, which converts plasminogen to plasmin, reducing the steady-state levels of both mRNA expression. The block of uPA and uPAR results in inhibition of pericellular proteolysis, correlated with significantly decreased cell invasion potential and motility [Biliran H. and Sheng, 2001]

Cytosolic relevance of Maspin has been linked to its ability to induce apoptosis. Maspin up-regulates the apoptotic inducer Bax, which in turn shuttles from the cytosol to the mitochondria. Bax stimulates the release of cytochrome c and Smac/DIABLO from the mitochondria into the cytosol. The cytochrome c release leads to APAf-1 mediated apoptosome assembly and a rapid activation of caspase-9, whereas Smac/DIABLO interacts with inhibitors of apoptotic proteases, blocking their inhibitory effects on activated caspase-9

and caspase-3 [Liu et al., 2004]. Nuclear Maspin anti-tumor activity relies on its mediation in the crosstalk between the tumor cells and the components of tumor microenvironment. It is associated with chromatin and is recruited to the promoter of the gene encoding the cytokine Colony-Stimulating Factor-1 (CSF-1), an important cytokine altered during metastatic process, significantly inhibiting its expression [Goulet et al., 2011]. Maspin interacts with IRF6 and HDAC1, which modulate the expression of key genes involved in the inhibition of tumor growth and angiogenesis. Maspin/HDAC1 interaction was confirmed in human prostate tissues and in prostate cancer cell lines. Following a molecular interaction, Maspin inhibits HDAC1 and leads to an increase of its target genes, such as Bax, Cytokeratin 18 (CK18) and $p21^{WAF1/CIP}$, an important regulator of cell cycle progression. [Li et al., 2006].

3 AIM OF THE WORK

The effects of two glucosamine-derivatives have been widely investigated previously in or laboratory. In particular, glucosamine (GlcN) and its glucosamine-derivative, 2-(N-Acetyl)-L-phenylalanylamido-2-deoxy-β-D-glucose (NAPA), designed and synthesized in our laboratory, showed a good anti-inflammatory activity, affecting MAPK pathway by the inhibition of p38 and JNK kinases, and in turn interfering with AP-1 pathway [Giordano et al., 1991; Scotto d'Abusco et al., 2007a, 2007b]. GlcN and mainly NAPA were also able to inhibit NF-κB pathway, particularly by inhibiting IKKα kinase activity and its translocation into the nucleus [Scotto d'Abusco et al., 2010].

Michael Karin, 2009 reported that inflammation enhances tumor promotion through NF-κB dependent mechanisms. NF-κB transcriptional factors are under control of IKK complex. In particular, the nuclear role of IKKα, one of the two catalytic subunits of IKK complex, has been correlated to cancer progression through the inhibition of Maspin expression [Luo et al., 2007].

In order to find a glucosamine derivative more effective than NAPA in inhibiting IKKα nuclear translocation, a new and more hydrophobic glucosamine-derivative, 2-(N-Carbobenzyloxy)-L-phenylalanylamido-2-deoxy-β-D-glucose (NCPA), was tested. As predicted by bioinformatic models, NCPA revealed to be more effective than NAPA in inhibiting IKKα nuclear translocation and it was also able to enhance Maspin production and its nuclear localization in an osteosarcoma cell line, 143B [Leopizzi et al., 2017].

Maspin was found to be highly expressed in normal prostatic epithelial cells and its expression is almost completely suppressed in metastatic prostatic cells, such as PC3, LNCaP and DU145. It displays heterogenous functions

depending on its subcellular localization, with an onco-suppressor role when it is located into the nucleus [Berardi et al., 2013; Yu et al., 2006]. Moreover, Maspin has been found to be highly expressed in tumor specimens from patients treated with neoadjuvant androgen ablation therapy [Zou et al., 2002]. On the other hand, patients with Maspin expressing prostate cancer had a significantly longer survival rate, suggesting a role for Maspin expression as a good prognosis biomarker [Berardi et al., 2013].

Thus, Maspin expression and its nuclear localization have been correlated to a more positive evolution of CaP.

Considering the ability of NCPA to stimulate Maspin production both at mRNA and protein level and to enhance its nuclear localization in a very aggressive osteosarcoma model, the aim of the present study was to evaluate the ability of NCPA to interfere with metastatic properties of two prostate cancer cell lines, the hormone-insensitive PC3 cell line and the hormone-sensitive LNCaP cell line, which represent good models to study the progression of CaP, due to their different metastatic origin and aggressiveness features related to their Maspin relative expression.

4 MATERIALS AND METHODS

4.1 Cell culture

Human prostate carcinoma cell lines PC3 and LNCaP, obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), were used. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with 10% fetal bovine serum (FBS), 1% $_{L}$-glutamine, 1% sodium pyruvate, 1% penicillin/streptomycin solution of 10,000 U/mL penicillin and 10 mg/mL streptomycin (Sigma Aldrich, St. Louis, MO, USA). Both cell lines were cultured at 37 °C and 5% CO_2 according to ATCC recommendations. Experiments were performed using 80% confluent monolayer cells.

4.2 Cell treatment

Cells were left untreated (CTL) or treated with 0.5 mM 2 - (N - Carbobenzyloxy) - $_{L}$ - phenylalanylamido - 2 - deoxy - β - $_{D}$-glucose (NCPA), a glucosamine-derivative synthesized in our laboratory [Giordano et al., 1991]. For AR inhibition, cells were pre-treated overnight with 120 μM Bicalutamide (Sigma Aldrich, St. Louis, MO, USA) and then treated with 0.5 mM NCPA plus 40 μM Bicalutamide for the required time points.

4.3 Cell viability

To assess a potential cytotoxic effect of NCPA on PC3 and LNCaP cells at different concentration and time points, MTS (3 - [4,5 - dimethylthiazol - 2 - yl] - 5 - [3-carboxymethoxyphenyl] - 2 - [4 - sulfophenyl] - 2H-tetrazolium) based colorimetric assay was performed (Promega Corporation, Madison, WI, USA). Briefly, 1×10^3 PC3 cells and 3×10^3 LNCaP cells per well, according

to growth rate, were seeded in a 96-well plate. The day after seeding, cells were starved overnight in reduced serum medium, in order to align cell cycle progression. Cells were then left untreated (CTL) and NCPA or Bicalutamide plus NCPA treated for 24h, 48h and 72h. After each time point, 100 µl MTS solution was added to the wells. Spectrophotometric absorbance was directly measured at 492 nm after 3h incubation. Cells were monitored at 24h, 48h and 72h under optical microscope, by Leica DM IL LED using AF6000 modular Microscope.

4.4 RNA extraction and reverse transcription

Total RNA was extracted using TRIZOL reagent (Invitrogen, Thermo Fisher Scientific), purified using micro TNeasy column (Qiagen, Valencia, CA, USA) and reverse transcribed by TetroReverse transcriptase enzyme, (Bioline, London, UK), according to the manufacturers' instructions.

4.5 Quantitative Real-Time-PCR (q-RT-PCR)

Quantitative-Real Time-PCR analysis was performed using an ABI Prism 7300 (Applied Biosystems, Thermo Fisher Scientific). Amplification was carried out using SensimixPlus SYBR Master mix (Bioline). Primers were designed using Primer Express software (Applied Biosystems) and synthesized by Biofab Research (Rome, Italy) (Table 2). Relative expression levels were normalized with glyceraldheyde-3-phosphate dehydrogenase (*GAPDH*) as housekeeping gene. Data were analyzed by $2^{-\Delta\Delta Ct}$ method [Schmittgen and Livak, 2008] and expressed as fold change compared to CTL.

Accession number	Gene	Primers	Primers 5'-3'
NM_002639	*maspin*	f	GCCAGGAGCACGGATCCT
		r	GTTGTGCCTGATGATGTAAATAAAGG
NM_002046	*GAPDH*	f	GGAGTCAACGGATTTGGTCGTA
		r	GGCAACAATATCCACTTTACCAGAGT

Table 2. GeneBank accession number and sequence of primers used to analyze gene expression by q-RT-PCR

4.6 Protein extraction, SDS-PAGE and Western blotting

Cells, untreated and treated as previously described, were washed with phosphate buffer saline (PBS) and lysated by Nuclear Extract Kit (Active Motif, CA, USA), which allowed to separate the cytosolic from nuclear extract, in accordance with manufacturer's instructions. Extracts were resolved on Mini-protean TGX precast gels by SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in 3% Bovine Serum Albumine (BSA) solution in Tween Tris Buffer Saline (TTBS), under gentle shaking, for 1h and then probed with specific antibodies, anti-Maspin, purchased from BD Pharmingen (BD Biosciences, San Jose, CA, USA) and anti-Fibrillarin from Santa Cruz (Santa Cruz Biotechnology Inc., CA, USA), according to the manufacturers' instructions. Nuclear proteins were detected by Chemi-Doc system (Bio-Rad) and signal quantification was performed by ImageLab software.

4.7 Immunocytochemistry

Immunostaining was performed by an automated staining system (Ventana BenchMark ULTRA, Roche diagnostic, Monza-Italy) in accordance with the manufacturer's instructions. Antigen epitopes were retrieved using Ventana Benchmark CC1 standard program. The antibody anti-cyclin D1 (rabbit monoclonal clone SP4-R; Ventana Medical Systems, Inc.) was incubated for 60 minutes at 37 °C. The slides were counterstained with hematoxylin, mounted and analyzed with Leica LAS V3.8 Software (Leica Microsystems, Switzerland) associated with the Leica DMRB microscope.

4.8 Fluorescence in situ hybridization (FISH)

FISH analysis for CCND1 (Cyclin D1) gene copy number was performed using SPEC CCND1/CEN 11 Dual Color Probe ZytoLight (ZytoVision GmbH, Germany). CCND1 gene was labeled with green fluorophore, and CEN11 (centromere) was labeled with orange fluorophore, following the manufacturer's protocol. For each specimen, at least 100 intact non-overlapping cells with good signals were analyzed. FISH images were acquired at 63x magnification utilizing Leica DM 6000B fluorescence microscope (Leica Microsystems, Switzerland) equipped with DAPI, SpectrumGreen™, SpectrumOrange™ filters. Images were processed by Leica LAS V3.8 Software (Leica Microsystems, Switzerland) to obtain a quantitative analysis of genes.

4.9 Transmission electron microscopy (TEM)

Cells, after each treatment, were immediately fixed in 2.5% cacodylate-buffered glutaraldehyde, pH 7.3, for 2h at 4°C and pelleted by centrifugation

at 1200 rpm for 5 minutes at room temperature. Samples were washed overnight in cacodylate buffer, post-fixed in buffered 1% osmium tetroxide for 1h, dehydrated through a graded series of acetone and embedded in Epoxy resin (Araldite, Fluka). Semithin sections (1μm), were cut with a glass knife on Leica EM Uc7 Ultratome, stained with toluidine blue and examined by light microscope for a general evaluation of cells morphology. Ultrathin sections were cut with a diamond knife (Diatome) at 60-80 nm, retrieved onto copper grids, double stained with Uranyl Acetate Replacement Stain (UAR-EMS) and lead citrate and examined at 100 kV with a Philips EM208 S electron microscope.

4.10 Immunofluorescence

The distribution of mitotic spindles and centrosomes in untreated (CTL) and NCPA-treated PC3 and LNCaP cells was detected using a monoclonal antibody to acetylated α-tubulin and a polyclonal antibody to γ-tubulin, respectively. Samples were fixed with 4% paraformaldehyde for 10 min at 4°C, washed with PBS, permeabilized with 0.3% Triton X-100 for 30 min at room temperature, blocked with NGS for 1 hour at room temperature. For double labeling, the mouse anti-acetylated α-tubulin (clone 6-11B-1, 1:800; Sigma-Aldrich, Milano, Italy) and the rabbit anti–γ-tubulin (1:800; Sigma-Aldrich, Milano, Italy) antibodies were concomitantly incubated overnight at 4°C. After thorough washing, samples were incubated with goat anti-mouse conjugated to Alexa 594 and goat anti-rabbit conjugated to Alexa 488 (Alexa Fluor SFX Kit, Molecular Probe, Invitrogen, Carlsbad, CA, USA) for 1h at room temperature. After a final wash step in PBS, cells were stained with DAPI and observed with a Leica DMRB microscope, associated with Leica LAS V3.8 Software (Leica Microsystems, Switzerland).

4.11 Flow cytometry

Cell cycle progression and apoptosis/necrosis pattern were analyzed by flow cytometry. Cells were digested with trypsin and harvested. Then, cells were pelleted at 800 g for 10 minutes at 25°C, resuspended in PBS and fixed in PBS plus 70% ethanol for 10 min at 4°C. After washing, cells were incubated with RNase A (50 µg/mL) for 30 minutes at 37 °C, sequentially stained with propidium iodide (100 µg/mL) for 1h and analyzed by BD Accuri C6 flow cytometer (BD Bioscience, Milano, Italy), to evaluate the cell cycle progression. The apoptosis/necrosis assay was performed using Annexin V-FITC kits (Immunological Sciences), according to the manufacturer's instructions.

4.12 Statistics

All data were obtained from at least three independent experiments, each performed either in duplicate or in triplicate. Data were statistically analyzed with two-way repeated measures analysis of variance (ANOVA) with Bonferroni's multiple comparison test, using Prism 5.0 software (GraphPad Software, San Diego, CA, USA). P value < 0.05 was considered significant.

5 RESULTS

5.1 Effect of NCPA on cell viability

NCPA dose-response and time-course effect on cell viability was determined after exposure of PC3 and LNCaP cells to 0.1, 0.5 and 1 mM NCPA concentrations for 24h, 48h and 72h. Cell viability was measured performing an MTS assay. NCPA showed a cytotoxic effect on PC3 cell viability at 1mM concentration at all time points analyzed; it showed a mild detrimental effect at 0.5 mM both at 24h and 48h, and a major detrimental effect at 72h; finally, it resulted unable to induce any cytotoxic effect at 0.1 mM concentration at any time point. NCPA did not affect LNCaP cell viability at any time point and concentration used (Figure 9A). Furthermore, PC3 cells treated with 0.5 mM NCPA and observed under optical microscope, showed a decrease in cell number and a morphology change, whereas, in the same condition, LNCaP showed any change in cell number or morphology (Figure 9B). For further analyses, we decided to use 0.5 mM concentration, which is the concentration that induced approximately 50% reduction in cell viability in PC3 after 72h treatment and it is also the same concentration used in previous studies on osteosarcoma cell line [Leopizzi et al., 2017].

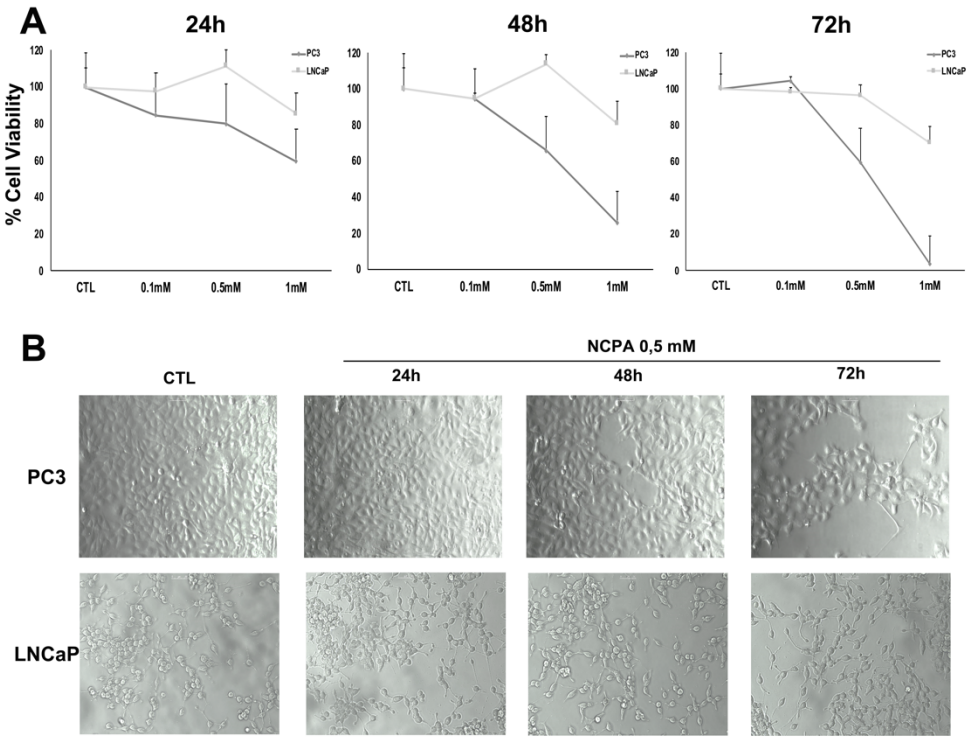

Figure 9. Effect of NCPA on cell viability. **A**: Cell viability was assessed by MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H- tetrazolium) based colorimetric assay. Cells were left untreated (CTL) or treated with 0.1, 0.5 and 1 mM NCPA, for 24h, 48h and 72h; **B**: Representative images of cells untreated (CTL) or treated with 0.5 mM NCPA for 24h, 48h and 72h. Experiments were repeated at least three times. Photographs were taken with Leica DM IL LED using AF6000 modular Miscroscope (original magnification 20x).

5.2 Effect of NCPA on Maspin production

Maspin has been described to play an important role as tumor suppressor protein when it is localized into the nucleus. To determine the ability of NCPA to enhance Maspin production and nuclear localization in PC3 and LNCaP, we analyzed cells treated with 0.5 mM NCPA at different time points by Western Blot. Nuclear extracts from NCPA-treated samples showed a significant increase of Maspin production after 24h treatment ($p<0.05$), which restored to control level after 48h and 72h treatment, in PC3 cell line (Figure 10A). NCPA showed no effect on Maspin production and nuclear localization in LNCaP cell line at any time point (Figure 10B).

The increase in Maspin protein level in PC3 was consistent with its transcriptional up-regulation. In fact, the abundance of *maspin* was being significant greater after 24h treatment, compared with CTL ($*p<0.05$) but did not differ from 48h and 72h (Figure 10C). According to protein expression, in LNCaP cells, NCPA was not able to induce *maspin* mRNA expression level at any time point analyzed (Figure 10D).

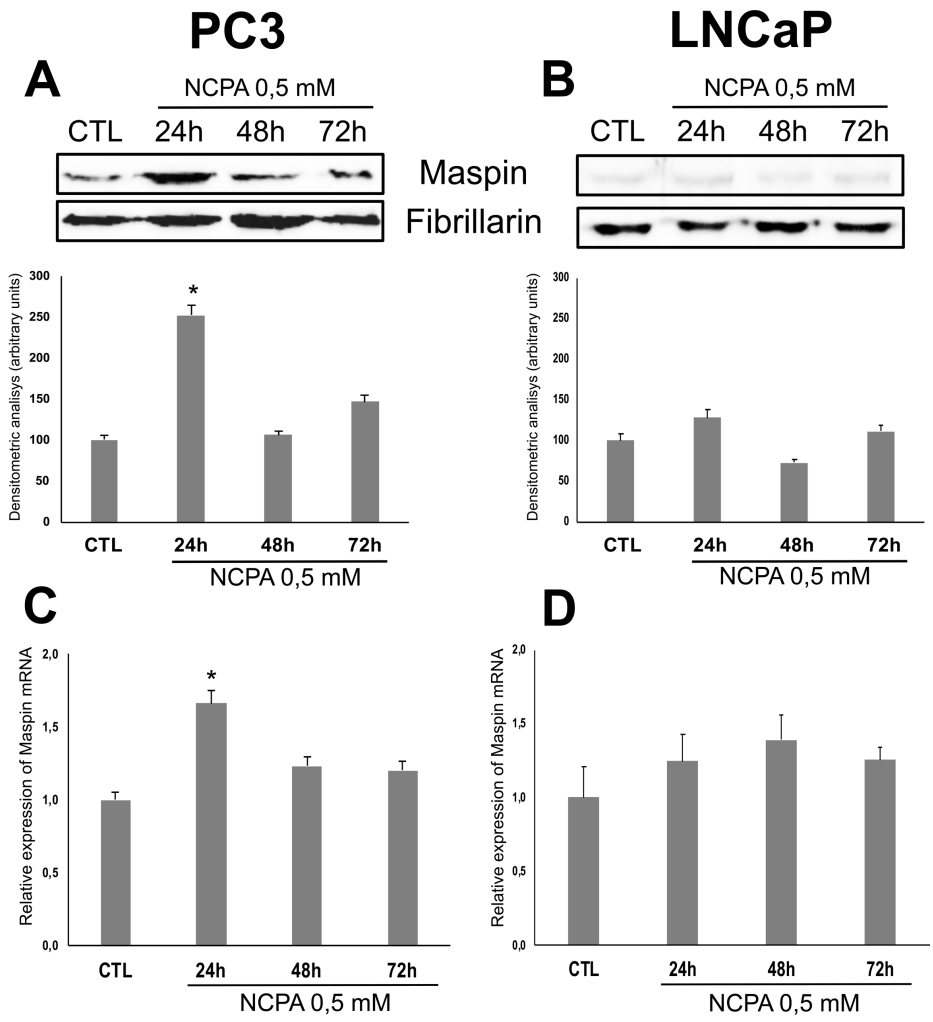

Figure 10. NCPA enhances Maspin production in PC3 cells. **A**: Proteins from PC3 cells, untreated (CTL) or treated with 0.5 mM NCPA for 24h, 48h and 72h, were extracted and nuclear extract was analyzed by Western blot, using anti-Maspin antibody and anti-Fibrillarin as loading control. Bar graph represents the densitometric analysis of positive bands normalized using the amount of fibrillarin; **B**: LNCaP cells were treated, extracted and analyzed as described in **A**; **C**: PC3 cells and **D**: LNCaP cells, treated as described in **A**, were harvested and mRNA was extracted and analyzed by RT-PCR. Data are expressed as mRNA level calculated by $2^{-\Delta\Delta Ct}$ method with respect to *GAPDH* mRNA. Results represent the mean ± S.E.M. of data obtained by three independent experiments. Statistical significance was: *$p < 0.05$.

5.3 Effect of NCPA on cell cycle progression and cell proliferation

Tumor suppressor molecule Maspin is involved in the inhibition of cell motility, invasion, metastasis and apoptotic process. Considering that NCPA was able to stimulate Maspin production in PC3 and not in LNCaP cell line, we evaluated the effect of this molecule on cell cycle progression in both cell lines by flow cytometry. The percentage of NCPA-treated PC3 cells in subG1 (G0/G1) stage increases progressively at 24h (15.1%), 48h (16.7%) and remained stable at 72h (13.7%), whereas the percentage in G1 increased al all time analyzed, at 24h (59.8%), 48h (65.3%, $p<0.05$) and mainly at 72h (69.8%, $p<0.05$), alongside, percentage of cells in S (12.9% at 24h, 7.4% at 48h and 7.1% at 72h, $p<0.005$ for all time point analyzed) and G2/M (12.2% at 24h, 10.6% at 48h and 9.4% at 72h, $p<0.05$ for 48h and 72h) stages decreased (Figure 11A). The percentage of LNCaP cells was almost the same in untreated and treated samples at each time point, with no detectable subG1 (G0/G1) cells (Figure 11B). The statistical analysis was performed comparing treated cells to untreated ones (Figure 11C).

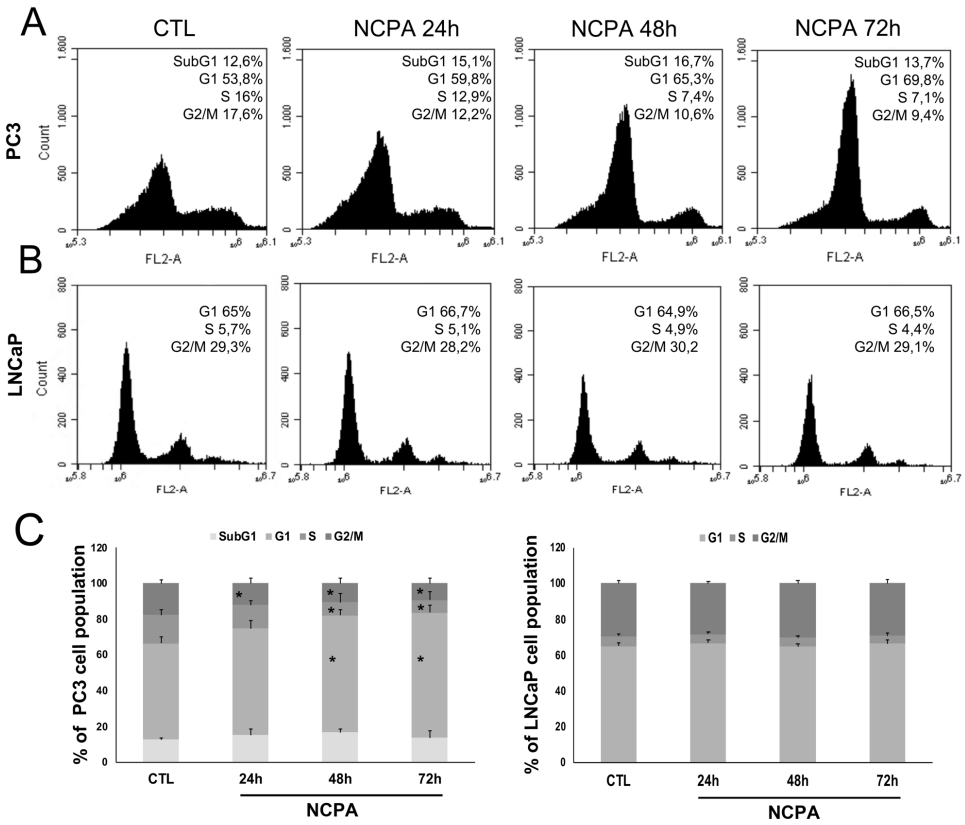

Figure 11. Effects of NCPA on cell cycle progression. **A**: PC3 Cells, untreated (CTL), or treated with 0.5 mM NCPA for 24h, 48h and 72h were analyzed by flow cytometry, after treatment cells were harvested and stained with propidium iodide and analyzed with BD Accuri C6 flow cytometer. The percentage of cells in subG1, G1, S and G2/M stages is indicated; **B**: LNCaP cells were treated and analyzed as in **A**. **C**: Statistical analysis conducted comparing the treated cells to untreated one (* = $p < 0.05$).

5.4 Effect of NCPA on cyclin D1 (CCND1) gene amplification

Cyclin D1 protein is one of the major regulator of cell cycle progression. Thus, we analyzed the presence of this protein in untreated and treated cells, by immunocytochemistry, finding that Cyclin D1 was present in large amount in untreated PC3 cell nuclei, and was decreased in NCPA-treated PC3 cell nuclei, according to cell cycle arrest observed. Consistently with cell cycle analysis, LNCaP showed very low amount of Cyclin D1 levels in untreated and treated cells, respectively (Figure 12A and 12B). Cyclin D1 protein overexpression has been shown to be significantly associated to high CCND1 gene amplification, for this reason we analyzed the CCND1 amplification by FISH [Bubendorf et al., 1999]. Kim et al., 2017a defined "gene amplification" as > 6 gene copies per nucleus in gastric cancer and Turner et al., 2017 found 4–10 CCND1 gene copies per nucleus in PC3 cell line. We analyzed CCND1 gene copy number in 100 nuclei both in untreated and treated PC3 cells or LNCaP cells. A representative image of all analyzed samples is reported (Figure 12C). Comparing the average of CCND1 gene amplification in both cell lines, we observed that CCND1 average is decreased in PC3 treated cells, whereas CCND1 average was unchanged in LNCaP treated cells compared with untreated ones (Figure 12D). Amplification of CCND1 gene was detected in 40% of PC3 untreated cells, whereas in NCPA-treated cells only in 4% of cells. Conversely, in LNCaP untreated cells, amplification of CCND1 was observed in 12% of cells, while in NCPA-treated cells was observed in 7% of cells (Figure 12E).

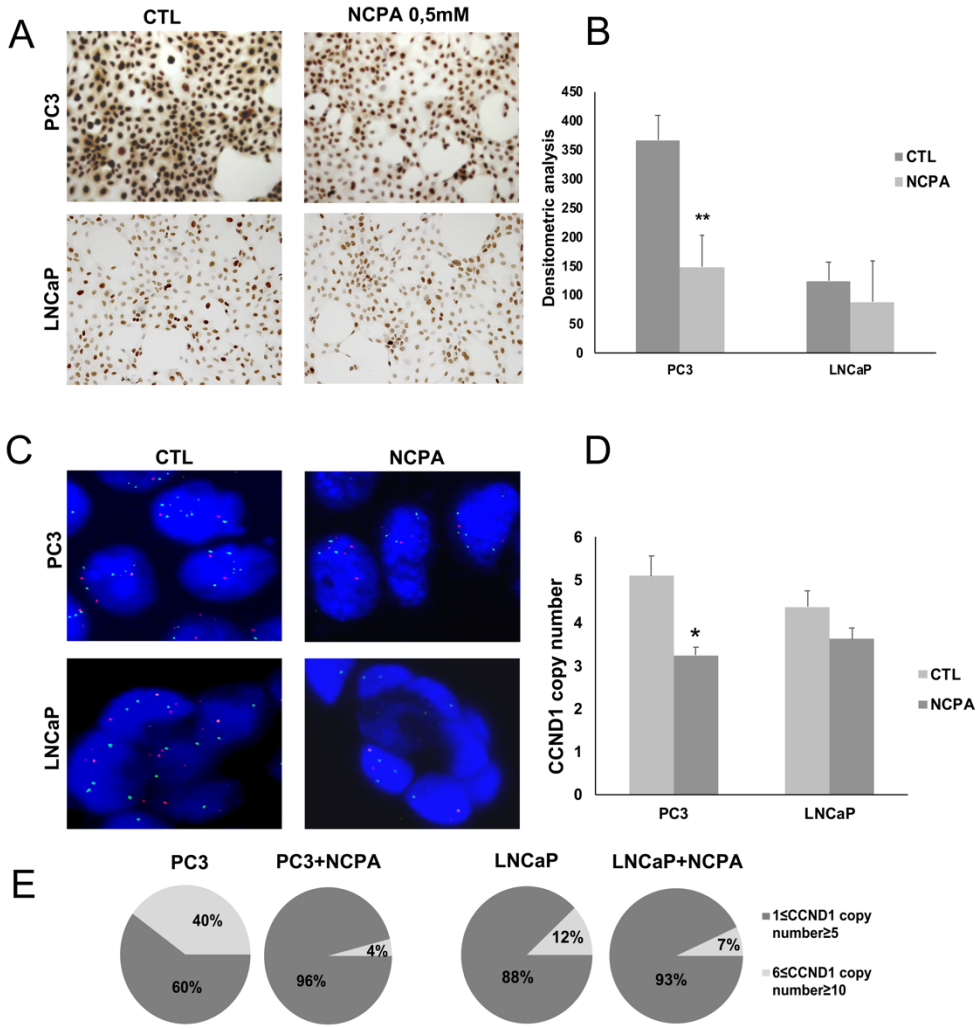

Figure 12. NCPA affects Cyclin D1 production and CCND1 gene amplification. **A**: PC3 and LNCaP cells untreated (CTL), or treated with 0.5 mM NCPA for 48 h were analyzed by immunocytochemistry using anti-Cyclin D1 rabbit monoclonal antibody (original magnification 20x, scale bar = 100 µm). **B**: Bar graph representing the densitometric analysis of positive nuclei in PC3 and LNCaP cells, analyzed by ImageJ software; **C**: PC3 and LNCaP cells, treated as described in A, were analyzed by FISH using SPEC CCND1/CEN 11 Dual Color Probe ZytoLight, CCND1 labeled with green fluorophore the cyclin D1 gene; CEN11 labeled the centromeres with orange fluorophore (representative image, original magnification 63x, scale bar=10µm). **D**: Bar graph representing the CCND1 copy number average in both cell lines; **E**: Graphic representation of low-level CCND1 gene amplification (light grey) or non-amplified (dark grey) in PC3 and LNCaP cell lines.

5.5 Effect of NCPA on mitotic spindle assembly in late-stage PC3 cell line

Tubulin, the major constituent of microtubules, is involved in cell division. In preparation of M phase, chromosome undergoes to condensation, a mitotic spindle is assembled under the control of the two centrosomes and spindle microtubules interact specifically with kinetocore proteins located at the site of the active chromosomal centromeres [Mandelkow and Mandelkow, 1995]. To determine if NCPA, affecting cell cycle progression, had a detrimental effect on cell division, PC3 and LNCaP cells were processed for immunofluorescence analysis regarding the distribution and orientation of microtubule structures and mitotic spindles. PC3 cells, untreated (CTL) and NCPA-treated for 72h showed a large amount of mitotic spindles in CTL compared with NCPA-treated cells. Moreover, the basal amount of mitotic spindles was lower in untreated LNCaP compared with untreated PC3 and NCPA did not show any effect on mitotic spindles assembly (Figure 13).

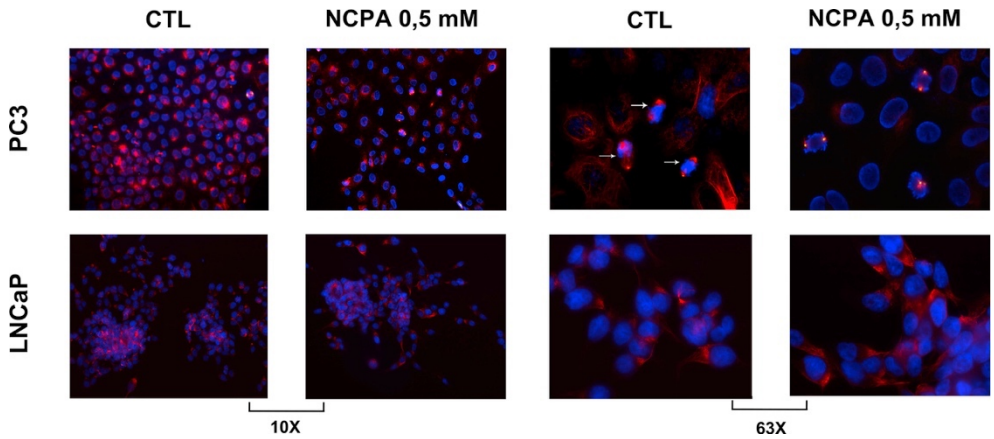

Figure 13. Analysis of tubulin in PC3 and LNCaP cell lines. **A**: Cells, untreated (CTL), or treated with 0.5 mM NCPA for 48 h were analyzed by immunofluorescence. Cells were double-stained using mouse anti-acetylated α-tubulin conjugated to Alexa Fluor 594 and rabbit anti-γ-tubulin conjugated to Alexa Fluor 488, nuclei were stained with DAPI (original magnification 10x and 63x). As can be observed, the analysis of tubulin filaments showed a large amount and a better organized mitotic spindles (arrows) in untreated PC3 compared to NCPA-treated PC3. Moreover, the number of mitotic spindles was undetectable both in untreated and treated LNCaP cells compared to untreated PC3 ones.

5.6 Effect of NCPA on apoptosis in PC3 and LNCaP

Considering the results obtained from cell cycle analysis and CCND1 quantification, we verified the ability of NCPA to induce apoptosis, performing an apoptosis/necrosis assay by flow cytometry. The current finding revealed that NCPA treatment induced apoptosis, both after 48h and mainly after 72h. The percentage of early apoptosis was 1.2% and 1.3% after 48h and 72h respectively, and late apoptosis 2.0% and 3.4% after 48h and 72h, respectively, whereas, it was 0.1% early and 0.4% late apoptosis in untreated cells. As observed in Figure 14A, compared with the untreated cells, a statistically significant increase in the ratio of apoptotic cells was observed at 48h and 72h for treated cells. LNCaP cells did not show any apoptosis feature at any time point in accordance with cell cycle analysis (Figure 14A and 14B). To further demonstrate that cell death monitored by flow cytometry was due to apoptosis we analyzed cells by TEM and we observed apoptotic nuclei in NCPA-treated PC3 cells, whereas apoptotic nuclei were not observed in LNCaP neither in untreated nor in treated cells (Figure 14C).

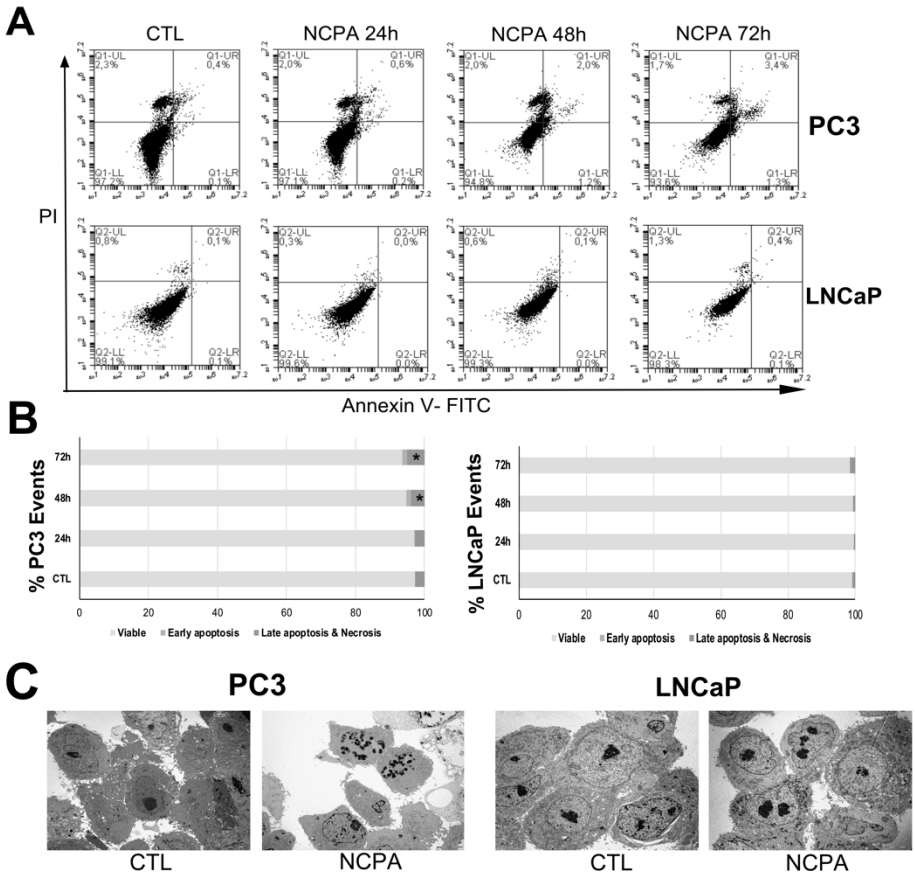

Figure 14. NCPA induces apoptosis in PC3 cell line. **A**: PC3 and LNCaP cells, untreated (CTL), or treated with 0.5 mM NCPA for 24h, 48h and 72h were analyzed by flow cytometry, after treatment, cells were harvested and the apoptosis/necrosis assay was performed using Annexin V-FITC kit for analysis with BD Accuri C6 flow cytometer, the experiments were repeated at least three times. **B**: Statistical analysis conducted comparing early apoptosis, late apoptosis and necrosis stages in treated cells with corresponding stages in untreated cells, (* = $p < 0.05$). **C**: Representative Transmission Electron Microscope images of untreated (CTL) and NCPA-treated PC3 and LNCaP cells (0.5 mM for 72 h) (scale bar = 5 µm).

5.7 Effect of NCPA on LNCaP cells treated with AR inhibitor

In order to confirm the stimulation of Maspin production in PC3 cell line was directly involved in the induction of apoptosis and consequently in cell death, we evaluated if the stimulation of Maspin production in LNCaP could induce the same effects. LNCaP are hormone-sensible cells and in the promoter region of *maspin*, a Hormone Responsive Element that negatively controls the Maspin production is present [Zhang et al., 1997]. We treated the LNCaP cells with Bicalutamide, an AR inhibitor and then with NCPA, finding that it induced a significant cell death compared with untreated cells, but the co-treatment with NCPA further induced cell death significantly higher than Bicalutamide alone, with the highest effect after 72 h (Figure 15A). In order to verify if the treatment with Bicalutamide could induce the production of Maspin, we performed a Western Blot experiment, analyzing the nuclear extract. From this latter we found that the treatment with Bicalutamide alone stimulated Maspin production, but the treatment with Bicalutamide plus NCPA induced a very large amount and nuclear accumulation of Maspin (Figure 15B). Finally, we analyzed cell cycle progression and apoptosis/necrosis induction where the pre-treatment with Bicalutamide and treatment with NCPA induced the increase of cell number in subG1 stage 65.4% Bicalutamide alone at 72h, 61% at 24h, 79.8% at 48h and 79.5% at 72h after NCPA treatment (Figure 15C). The increase of cell number in subG1 in Bicalutamide plus NCPA at 72h was statistically significant compared with the apoptosis rate in Bicalutamide alone at 72h ($p<0.05$). The apoptosis/necrosis assay showed that Bicalutamide treatment induced early apoptosis, 7.8% at 72h, whereas Bicalutamide plus NCPA induced an increased percentage of cells in early apoptosis, 4.1%, 15.5% and 20.2% at 24h, 48h and 72h, respectively (Bicalutamide plus NCPA vs Bicalutamide alone at 72h was statistically significant, $p < 0.01$). Same

result was obtained for late apoptosis, Bicalutamide alone 4.6% at 72h, Bicalutamide plus NCPA 5.1%, 28.6% and 51.3% at 24h, 48h and 72h, respectively (Bicalutamide plus NCPA vs Bicalutamide alone at 72h, $p < 0.001$) (Figure 15D).

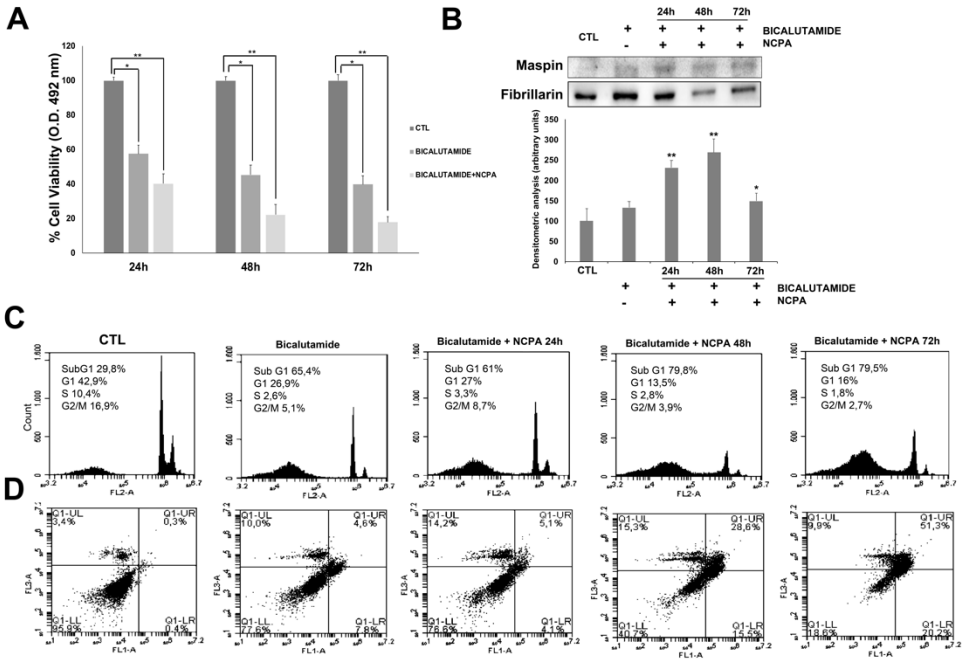

Figure 15. Effects of NCPA on LNCaP pre-treated with hormone receptor inhibitor Bicalutamide. **A**: Cell viability was assessed by MTS (3-[4,5-dimethylthiazol-2-yl]-5- [3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) based colorimetric assay. Cells were left untreated (CTL) or treated with Bicalutamide for 72h or pre-treated ON with Bicalutamide and treated with 0.5 mM NCPA, for 24h, 48h and 72h. Statistical significance: *$p < 0.05$, **$p < 0.01$; **B**: Proteins from LNCaP cells treated as in **A**, were extracted and nuclear extract was analyzed by Western blot, using anti-Maspin antibody and anti-Fibrillarin as loading control. Bar graph represents the densitometric analysis of positive bands normalized using the amount of fibrillarin. Statistical significance: *$p < 0.05$, **$p < 0.01$; **C**: LNCaP cells treated as in **A,** were stained with propidium iodide and cycle progression was analyzed with BD Accuri C6 flow cytometer. The experiments were repeated three times. The percentage of cells in subG1, G1, S and G2/M stages is indicated; **D**: LNCaP cells treated as in **A,** were harvested and the apoptosis/necrosis assay was performed using Annexin V-FITC kit for analysis with BD Accuri C6 flow cytometer. The experiments were repeated three times, a statistical analysis has been conducted comparing the sample treated with Bicalutamide alone with sample treated with Bicalutamide plus NCPA, the statistical significance has been reported in Results section.

6 DISCUSSION

Maspin is an unusual non-inhibitory member of the SERine Protease INhibitor superfamily, Serpins, characterized as a class II tumor suppressor gene in many cancer types, such as breast, lung, epithelial and prostate cancer [Hall et al., 2008; Lonardo et al., 2006; Streuli, 2002; Zou et al., 1994], due to its ability to inhibit cell invasiveness, angiogenesis and metastasis by increasing apoptosis [Bodenstine et al., 2012; Shankar et al., 2016]. Maspin was found to be highly expressed in normal prostate epithelial cells. In normal cells it localizes heterogeneously in cytoplasm, nucleus, secretory vesicles and occasionally at cell surface and displays different biological functions according to its subcellular localization [Berardi et al., 2013; Yu et al., 2006]. In prostate cancer cell lines, PC3, LNCaP and DU145, its expression is almost completely suppressed [Bernardo et al., 2011]. Moreover, Maspin has been found highly expressed in tumor specimens from patients treated with neoadjuvant androgen ablation therapy [Zou et al., 2002]. On the other hand, patients with Maspin expressing prostate cancer had a significantly longer survival rate, suggesting a role for Maspin expression as good prognosis biomarker [Berardi et al., 2013].

Previously, in our laboratory, glucosamine (GlcN) and a glucosamine-derivative, NAPA, have been studied, finding that both molecules showed a good anti-inflammatory activity through the inhibition of p38 and JNK kinases, thus interfering with AP-1 pathway [Scotto d'Abusco et al., 2007a, 2007b]. GlcN and mainly NAPA were also able to inhibit NF-κB pathway, particularly by inhibiting IKKα migration into the nucleus and its kinase activity [Scotto d'Abusco et al., 2010].

Inflammation enhances tumor promotion through NF-κB dependent mechanisms. NF-κB affects both apoptosis and proliferation, processes involved in cancer progression [Karin, 2009]. NF-κB transcriptional factors are under control of IKK complex, characterized by two cataliyic subunits IKKα and IKKβ and a regulatory subunit IKKγ. In particular, considering that nuclear IKKα enhances tumor promotion by repressing, among other mechanisms, *maspin* promoter [Luo et al., 2007], our group decided to investigate if glucosamine-derivative molecules could be able to interfere with Maspin production. In order to obtain a more effective IKKα inhibitor, it was synthesized a new glucosamine-derivative, 2-(N-Carbobenzyloxy)-L-phenylalanylamido-2-deoxy-β-D-glucose, or NCPA, which revealed to be very effective in inhibiting IKKα nuclear translocation and in stimulating Maspin production in osteosarcoma cell lines, 143B and Saos-2 [Leopizzi et al., 2017]. During my PhD period we decided to analyze the effectiveness of NCPA in two human prostate cancer cell lines, PC3 and LNCaP, which represent suitable models to study the progression of prostate cancer, due to their different metastatic origin and aggressiveness features related to their Maspin relative expression. LNCaP cell line is considered an early stage of CaP, whose development is based on androgen-sensitive cell growth, and progression has been shown to be based on an AR-independent cell growth [Dozmorov et al., 2009; Lin et al., 2003; Nunlist et al., 2004]. On the other hand, PC3 cell line is considered representative of a late-stage malignancy, due to its source of origin and to androgen-insensitive features [Cocchiola et al., 2017; Tai et al., 2011]. PC3 lack in AR expression, and this cell line shows a more aggressive phenotype compared to LNCaP. Considering the differences between LNCaP and PC3 we decided to analyze the effects of NCPA on both cell lines, focusing the attention on the ability to stimulate Maspin production.

The first difference we found was that NCPA was able to inhibit cell viability only in PC3 and not in LNCaP cells. Moreover, it enhanced the production of Maspin only in PC3, with a prevalent subcellular localization in the nucleus. This finding is remarkable, considering that several authors described Maspin nuclear localization as essential for its onco-suppressor activity [Goulet et al., 2011; Lonardo et al., 2006; Machowska et al., 2014; Solomon et al., 2006]. Goulet et al., 2011 demonstrated that nuclear Maspin is physically associated to target gene promoters, suggesting that its metastasis suppressor functions could involve, at least in part, this mechanism. It is associated with chromatin and it is recruited to the promoter of the gene encoding the cytokine colony-stimulating factor-1 (CSF-1), an important cytokine altered during metastatic process, significantly inhibiting its expression. Maspin interacts with IRF6 and HDAC1, which modulate the expression of key genes involved in the inhibition of tumor growth and angiogenesis. In particular, Maspin/HDAC1 interaction was confirmed in human prostate tissues and in prostate cancer cell lines. Following a molecular interaction, Maspin inhibits HDAC1 and leads to an increase of its target genes, such as Bax, Cytokeratin 18 (CK18) and $p21^{WAF1/CIP}$, an important regulator of cell cycle progression [Li et al., 2006]. Thus, the tumor-suppressing and anti-metastatic activities of Maspin have been also attributed to its ability to inhibit cell cycle progression and to stimulate apoptosis of tumor cells [Berardi et al., 2013; Schaefer and Zhang, 2005; Yu et al., 2006]. Therefore, we analyzed the effects of NCPA in PC3 and LNCaP cell lines on cell cycle progress, finding that the compound caused a cell cycle inhibition in PC3, whereas NCPA did not altered cell cycle progression in LNCaP cells. Cyclin D1 is considered a key regulator of the progression of cell cycle through G1 phase and its overexpression is also been related to the evolution through an androgen-independent CaP phenotype [Drobnjak et al.,

2000]. Analyzing the expression level of Cyclin D1 both in PC3 and LNCaP, we found that Cyclin D1 was up-regulated in PC3 and not in LNCaP and that after treatment with NCPA in PC3 Cyclin D1 expression level decreased. Our data are in accordance with those of Lee et al., 2016, who found that the arrest in G1 phase was related to decrease of Cyclin D1 and to up-regulation of Maspin in Oral Squamous Cell Carcinoma Cells. The overexpression of Cyclin D1 has been associated to gene amplification mainly in hormone-insensitive and metastatic CaP samples, whereas, the amplification rate is very low in early stage CaP samples [Bubendorf et al., 1999]. According to these findings, we found that CCND1 gene is largely amplified in PC3 cell line, representative of an advanced and hormone-insensitive CaP, and is slightly amplified in LNCaP, representative of an early stage and hormone-sensitive CaP. Interestingly, NCPA was effective mainly in PC3 cells on decreasing cell number presenting amplified CCND1 from 40% to 4%. In LNCaP, the percentage of cells with CCND1 gene amplification was only 12% and was lowered to 7% after treatment, which was not statistically significant. Turner et al., 2017 found that the amplification of some common oncogenes, among them CCND1, can be attributed to extra chromosomal DNA (ecDNA). However, they found that CCND1 amplification in PC3 cell line is not due to ecDNA, but resides on chromosomal loci. We speculate that NCPA could be effective mainly in cells with CCND1 chromosomal loci amplification. The data on CCND1 amplification are in accordance with analysis of tubulin filaments, performed by immunofluorescence, showing a large amount of mitotic spindles in untreated PC3 compared to NCPA-treated PC3. Moreover, the number of mitotic spindles was lower in untreated LNCaP compared to untreated PC3.

Finally, we analyzed the ability of NCPA to induce apoptosis in PC3 cells, finding that progressively the percentage of both early and late apoptotic cells was increased, with the higher amount at 72h. This result agrees with the increase of nuclear Maspin at 24h, suggesting that the nuclear localization of Maspin enhances the production of molecules involved in apoptosis processes. Noteworthy, the amount of Maspin was slightly increased again at 72h suggesting that NCPA could support the apoptotic process.

Interestingly, Coulson-Thomas et al., 2013 demonstrated that, in the early stages of CaP development, tumor cells are surrounded by lumican, a Small Leucine-rich proteoglycan (SLRP), which inhibits tumor progression and invasion. During cancer progression, cancer cells increase the production of MMPs degrading the deposited stromal lumican. Moreover, Vuillermoz et al., 2004 showed that the over-expression of lumican in a mouse cancer model induced the decrease of Cyclin D1 production, increased apoptosis rate and in turn significantly decreased subcutaneous tumor formation in vivo. Moreover, Dzinic et al., 2014 observed that Maspin is involved in the production of extracellular matrix in prostate cancer cell lines. Other potential Maspin binding proteins, whose regulation is involved in metastatic process, include type I, III and IV collagens and laminin [Blacque and Margaret Worrall, 2002; Ngamkitidechakul et al., 2001]. Previously, it was demonstrated that GlcN and glucosamine-derivative NAPA were able to stimulate extracellular matrix components, among them also the SLRP lumican and collagens [Stoppoloni et al., 2015; Veronesi et al., 2017]. Considering that NCPA is a glucosamine-derivative, very similar to NAPA, we hypothesize that also NCPA induces lumican and collagens production, strengthening the anti-metastatic effects on cell cycle and apoptosis that we observed.

A hormone negative regulatory element is present in the promoter region of *maspin* gene [Zhang et al., 1997]. Taking into account this finding, Zou et al., 2002 demonstrated that LNCaP cultured in androgen-depleted medium exhibit induction of Maspin expression. We treated LNCaP with AR inhibitor Bicalutamide and with Bicalutamide plus NCPA finding that our molecule, in AR-inhibition condition, was able to induce the expression of Maspin also in LNCaP cells and in turn to promote the apoptosis. In particular, Bicalutamide plus NCPA induced a higher accumulation of Maspin in the nucleus compared to Bicalutamide alone, and consequently also the apoptosis rate was higher in Bicalutamide plus NCPA compared to Bicalutamide alone treatment. Considering that Bicalutamide induced a mild effect on apoptosis and NCPA was not effective at all on LNCaP cells, whereas the contemporary administration of Bicalutamide plus NCPA induced 51,3% late apoptosis rate, we suggest that the combination of two molecules results in a synergistic effect on LNCaP cell line.

In conclusion, we demonstrated that NCPA was able to stimulate Maspin production in PC3 cell line and in LNCaP cell line pre-treated with a hormone inhibitor, affecting metastatic activity by the induction of apoptosis. The increase of autologous Maspin, would be a desirable therapeutic strategy, considering that the delivery of recombinant Maspin by adenoviral construct or liposomal DNA complexes is accompanied with safety and efficacy issues [Shi et al., 2002; Zou et al., 1994]. These evidences highlight the possibility to use NCPA as a chemo sensitizing agent.

7 LIST OF ABBREVIATIONS

ACT α_1-antichymotrypsin

AF2 activation function 2

AMG α_2-macroglobulin

ANOVA analysis of variance

AP1 activator protein 1

APAf1 apoptotic protease activating factor-1

AR androgen receptor

AR-DBD androgen receptor-dimerization binding domain

AR-Vs androgen receptor variants

ARA70 activation of androgen receptor- associated protein 70

ARE androgen responsive element

Bak1 Bcl-2 homologous antagonist/killer 1

Bax Bcl-2-like protein 4

Bcl B-cell lymphoma 2

BPH benign prostatic hypertrophy

BSA bovine serum albumine

CaP cancer prostate

CBP CREB-binding protein

CCND1 cyclind1

CDKN1B cyclin-dependent kinase inhibitor 1B

CK cytokeratin

CREB cAMP response element-binding protein

CRPC castration resistant prostate cancer

CSF1 colony-stimulating factor-1

CTCs circulating tumor cells

DHT dihydrotestosterone

ECM extracellular matrix

EGF epidermal growth factor

EGF-R epidermal growth factor receptor

Egr1 early growth response protein 1

EMT epithelial-mesenchymal transition

ERG *ets*-related gene

ETS erythroblastosis virus E26 transforming specific

ETV *ets* variant

FBS fetal bovine serum

FISH fluorescence in situ hybridization

FOXA1 forkhead box protein A1

GAPDH glyceraldheyde-3- phosphate dehydrogenase

GCF2 GCrich-binding factor 2

GLcN glucosamine

GSH glutathione

GST glutathione S-transferase

GTF general transcription factor

HAT histone acetyltransferase

HDAC1 histone deacetylase 1

HGPIN high-grade prostatic intraepithelial neoplasia

hK Human kallikrein

HRE hormonal responsive element

HSP heat shock protein

IGF-1 Insulin Growth Factor 1

IKK IκB kinase

IRF6 interferon regulatory factor 6

ISUP International Society of Urologic Pathology

KLK3 kallikrein-related peptidase 3

LBD ligand-binding domain

LNCaP lymph node cancer prostate cell line

MASPIN MAmmary Serine Protease INhibitor

miRNAs microRNAs

MMPs matrix metalloproteinases

mTOR mechanistic target of rapamycin kinase

MTS 3 [4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium)

NAPA 2-(N-Acetyl)-L-phenylalanylamido-2-deoxy-β-D-glucose

NCPA 2-(N-Carbobenzyloxy)-L-phenylalanylamido-2-deoxy-β-D-glucose

NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells

NTD N-terminal transactivation domain

P/CAF P300/CBP-associated factor

p27/Kip1 Cyclin-dependent kinase inhibitor 1B

PBS phosphate buffer saline

PC3 prostate cancer 3 cell line

PCA3 prostate cancer antigen 3

PDGF-A platelet-derived growth factor-A

PI3K phosphoinositide 3-kinase

PIN prostatic intraepithelial neoplasia

PSA prostate specific antigen

PTEN phosphatase and tensin homolog

RCL reactive center loop

ROS reactive oxygen species

RPMI Roswell Park Memorial Institute

RSL reactive site loop

SCCA squamous cell carcinoma antigen

SDS-PAGE sodium dodecyl sulphate - polyacrylamide gel electrophoresis

Serpin SERine Protease INhibitor

SLRP small leucine-rich proteoglycan

Smac/DIABLO second mitochondria-derived activator of caspases/ direct IAP-binding protein with low PI

SRC/p160 p160 steroid receptor coactivator

TBP TATA binding protein

TEM transmission electron microscopy

TGF-α transforming Growth Factor

TMPRSS2:ERG transmembrane protease serine 2:erytrhoblastosis virus E26 related gene

TP53 tumor protein 53

TRAMP transgenic adenocarcinoma of mouse prostate

UGE urogenital sinus epithelium

UGS urogenital sinus

uPA urokinase plasminogen activator

uPAR urokinase plasminogen activator receptor

VACURG Veterans Administration Cooperative Urological Research Group

WHO World Health Organization

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9 ATTACHMENTS

In the following section are listed the first pages of papers published during my PhD course.

Colloids and Surfaces B: Biointerfaces 158 (2017) 643–649

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Protocols

Taurine grafting and collagen adsorption on PLLA films improve human primary chondrocyte adhesion and growth

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ABSTRACT

Biocompatible and degradable poly(α-hydroxy acids) are one of the most widely used materials in scaffolds for tissue engineering. Nevertheless, they often need surface modification to improve interaction with cells. Aminolysis is a common method to increase the polymer hydrophilicity and to introduce surface functional groups, able to covalently link or absorb, through electrostatic interaction, bioactive molecules or macromolecules. For this purpose, multi-functional amines, such as diethylenediamine or hexamethylenediamine are used. However, common drawbacks are their toxicity and the introduction of positive charges on the surface. Thus, these kind of modified surfaces are unable to link directly proteins, such as collagens, a promising substrate for many cell types, in particular chondrocytes and osteoblasts. In this work, poly(L-lactide) (PLLA) film surface was labelled with negatively charged sulfonate groups by grafting taurine (TAU) through an aminolysis reaction. The novel modified PLLA film (PLLA-TAU) was able to interact directly with collagen. The reaction was carried out in mild conditions by using a solution of tetrabutylammonium salt of TAU in methanol. ATR-FTIR, XPS and contact angle measurements were used to verify the outcome of the reaction. After the exchange of tetrabutylamonium cation with Na^+, collagen was absorbed on the TAU grafted PLLA film (PLLA-TAU-COLL). In vitro biological tests with human primary chondrocytes showed that PLLA-TAU and PLLA-TAU-COLL improved cell viability and adhesion, compared to the unmodified polymer, suggesting that these modifications make PLLA substrate suitable for cartilage repair.

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1. Introduction

Adult articular cartilage is a tissue mainly composed by collagens and glycosaminoglycans, which provide tensile strength, as well as proteoglycans, which retain water molecules in the matrix. This tissue presents a unique cellular component, chondrocytes, which are deputed to produce and renew the cartilage extracellular matrix (ECM) [1]. Being the cartilage an avascular tissue, it has a poor repair ability of the damages caused by injury. The damaged cartilage leads to secondary degenerative diseases, such as osteoarthritis (OA), the most common rheumatic disease and a major cause of disability [2]. It is associated with aging and its medical relevance is rising in Western population given the increase of age. OA is characterized by progressive degradation of ECM and, being to date a non-curable disease, its pharmacological treatment is based on anti-inflammatory drugs and structure-modifying drugs [3,4]. Patients with very extensive cartilage damages need to undergo total arthroplasty, whereas the regeneration of limited zones of cartilage can be treated with clinical methods, as transplantation of chondrocytes or osteochondral graft. In these latter methods, a scaffold, which could reproduce cartilage and/or bone structure, able to support the growth and proliferation of transplanted cells would be required.

Among various biomaterials, poly(α-hydroxy acids) (PHAs), which include polylactides, polyglycolides and their copolymers, have been proposed for the preparation of scaffolds for chondrocytes. Because of suitable mechanical properties, good processability and non-toxicity of their degradation products, PHAs are widely used as biomaterials, including nano- or micro-sized carriers for controlled drug or antigen delivery [5–7], bio-absorbable prosthesis and surgical devices [8,9]. However, some drawbacks related to the interaction of biomaterial surface with the biological tissues still survive and are objects of extensive research [10,11]. In particular, aliphatic polyesters are hydrophobic and

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Olive Fruit Blends Modulate Lipid-Sensing Nuclear Receptor PPARγ, Cell Survival, and Oxidative Stress Response in Human Osteoblast Cells

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ABSTRACT

Objective: The aim of the present study was to investigate how different extravirgin olive oils (EVOOs), obtained by blending *Olea europea* cultivars, could influence the cell growth, the response to inflammatory stimuli, and oxidative stress in a culture of the osteosarcoma cell line Saos-2.

Methods: Three different extravirgin olive oils were physicochemically characterized, determining the free acidity, the oxidation status, the polyphenols content, and the antioxidative activity. Moreover, the effects on Saos-2 cell culture were determined, studying the mRNA expression level by real-time polymerase chain reaction (PCR) assays and the antioxidative activity using fluorescent probes.

Results: The cultivars used in the south of Italy, yield extravirgin oils with different amount of fatty acids and polyphenols, which counteract induction of proinflammatory cytokines and regulate free radical production in hydrogen peroxide-stimulated cells. In vitro analysis using the human osteoblast cell line Saos-2 showed that the addition of oils to cell culture simulated a hypoxic stress followed by a reoxygenation period, during which the antioxidant activity of extravirgin olive oils protected cells from oxidative damages. On the other hand, the mRNA expression levels of factors involved in inflammatory processes, cell growth recovery, and antioxidant response, as heme oxygenase-1, were differently stimulated by EVOOs. Moreover, peroxisome proliferator activated receptor γ (PPARγ) was differently modulated by EVOOs.

Conclusion: These findings show that the blending of different extravirgin olive oil can impact an osteoblast cell line, in particular regarding cell growth recovery and oxidative stress.

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Extravirgin olive oil; antioxidant activity; cell growth; osteoblast cell line; gene expression analysis

Introduction

The quality and features of extravirgin olive oils (EVOOs) depend on lipids and minor compounds content, which is typical of each EVOO. The high amount of lipids in EVOOs is considered very important for its contribute to dietary intake of fatty acids (FAs) and in particular polyunsaturated fatty acids (PUFAs) for their effects on bradikinin and prostaglandin synthesis, also having an impact on inflammation and cell life extension (1). Moreover, the EVOO composition of FAs and minor secondary metabolites (SMs) influences flavor, aromas, and nutritional values of EVOO (2). The organoleptic properties of EVOOs are influenced by SMs, whose amount depends on several factors, including genomic species and agronomical practices as seasonality, mineral and organic contents of soil, and sun exposure (3). In addition EVOO features depend on fruit ripening and extraction processes.

In Italy, a high number of olive millers is present and more than 600 Oleaceae species have been classified; moreover, millers blend fruit, having an impact on nutritional properties and health effects of EVOOs (4). In the last decades, an increasing number of studies have been performed on oil components and their physiological effects on health (5). Regarding the in vitro studies, the activity of EVOO compounds on antioxidant and pro-oxidant equilibrium is still debatable; moreover, the impact of EVOO emulsion on cell growth is scarcely studied. Recently, olive oil-based emulsion has been found potentially useful to supplement patients during acute thromboembolic events because it does not influence the expression of the platelet receptor for fibrinogen (6). An *in vitro* study, carried out with olive oil emulsion, showed that the emulsion affected the endothelial relaxation and increased the oxidative stress (7). In the last years, there has been an increasing interest in studying the impact of hypoxic stress in cancer cells as a therapeutic strategy for cancer treatment.

Anaerobic conditions induce the expression of inflammatory, redox, and tumorigenesis genes. Moreover, hypoxia induces, among other genes, the lipogenic factor peroxisome proliferator activated receptor γ (PPARγ) involved in modulation of inflammatory cytokines (8).

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Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/uacn.

Sergio Ammendola is employed at AMBIOTEC SAS.

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Special Issue: Functional Foods and Nutraceuticals: Their Role in Disease Prevention

Effects of Nutrients, Mainly from Mediterranean Dietary Foods, on Mesenchymal Stem Derived Cells: Growth or Differentiation

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Abstract

During the last decade the interest for the mesenchymal cells is growing due to their possible uses in therapies to treat certain degenerative pathologies. Mesenchymal stem cells have been found in the bone marrow and they have been shown to be responsible for bone repair and fat cells production. Mesenchymal stromal cells can be obtained from a wide variety of tissues in addition to bone marrow and can differentiate into many other cell types. The study of cell differentiation and programming provides new models for drug discovery and cell therapy that now overcomes gene therapy. Senescence, cancer development and degenerative diseases depend on mesenchymal cells contribution to tissue homeostasis. On the other hand, diet and life style are included among risk factors, which can contribute to the success of pharmacological treatments. This review focuses on nutrients from Mediterranean diet and supplements, which have been shown to influence mesenchymal stem cells and cells derived from them. Dietary intake of nutrients impairs both *in vitro* and *in vivo* observations, this review aims to gather the results about the effects of food compounds on mesenchymal cells from which adipocytes and osteoblasts derive. Amino acids and proteins, carbohydrates, lipids, fatty acids and vegetable secondary metabolites, differently act on mesenchymal cells bearing on modulation of gene expression and controlling the fate of cell lineages. Remarkable, the analysis of literature shows that the main effect of nutrients on mesenchymal cells is the stimulation of transcription factors which address the cells toward proliferation or differentiation. For instance, carbohydrates, simple or complex, and lipids appear to stimulate the PPAR receptors, whereas proteins and amino acids result to act on the mTOR system and they can also stimulate the MyoD-1 transcription factor and cooperating proteins. In conclusion, nutrients can promote cell growth and differentiation of mesenchymal cells.

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Mesenchymal cells, Mediterranean foods, Cell growth, Cell differentiation, mTOR pathway, PPARγ pathway, Short Chain Fatty Acids.

Introduction

Mesenchymal stem cells (MSCs) are multipotent stromal cells, *in vivo* isolated by Bone Marrow (BM) that can differentiate into osteoblasts (bone cells), chondrocytes (cartilage cells) and adipocytes (adipose tissue cells) (Figure 1) [1]. Moreover, mesenchymal stromal cells have been described, those cells may originate from several tissue-specific multipotent precursors, that are present in native tissues, and show diverse degrees of plasticity and self-renewal ability [2,3]. These cells, depending on native tissue, can differentiate in osteoblasts and myocytes, under suitable stimulations. Molecules present in foods can interfere with cell differentiation and stimulation. Foods are complex matrix of healthful nutrients and unhealthful anti-nutrients, able to contrast the absorption of healthful compounds, thus, a meal should contain a sufficient amount of synergic nutrients. A food that results efficacious to promote health or prevent damages is considered a functional food. Nutrients in a meal may modulate the expression of some genes (nutrigenomics), on the other hands the body, including microbiota, may modify nutrients (nutrigenetics).

We are interested in cellular models suitable for nutrigenomic studies. Previously we analyzed the effects of nutraceutical compositions in osteoblast cells, studying the gene expression level of several genes by Real Time-PCR (RT-PCR).

Functional nutrients or nutraceuticals can modulate gene expression and chromatin architecture, acting on regulation of key modulators such as the mTOR system, PPAR receptors and HDACs and NAD-dependent mono-ribosyl ATPase (sirtuins), HATs (acetylase) and DNA methylase and DMTs (demethylase) [4,5]. On the other hand, nutrients can indirectly stimulate the expression of transcriptional complexes or modulate the expression of gene coding for regulatory microRNAs.

The aim of the present review is to compare results regarding the effects of some nutrients, especially those present in Mediterranean foods, on osteoblast and adipocyte cells originated from mesenchymal stem cells.

Carbohydrates

Diet carbohydrates are the broadly used nutrients for humans, and their utilization supplies the main energetic source for cells. Moreover, cells utilize carbohydrates also to build many polymers present into tissues, such as glycans. The main carbohydrate utilized by cells is glucose, which enters across specific and regulated transporters, it can be utilized to produce ATP and to synthesize structural molecules. Glucosamine (GlcN) a glucose-derivative, broadly present in living world is utilized to build glycosaminoglycans, the main components of extracellular matrix (ECM) of connective tissues. For instance, GlcN, as N-acetyl glucosamine (GlcNAc), is present in Hyaluronic acid and as N-acetyl glucosamine 6-sulfate (GlcNAc6S) in Keratan sulfate, two glycosaminoglycans that are present in cartilage. For this reason, since several years, GlcN has been orally administered to osteoarthritic (OA) patients, whose articular cartilage is deeply

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The induction of Maspin expression by a glucosamine-derivative has an antiproliferative activity in prostate cancer cell lines

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ABSTRACT

Mammary serine protease inhibitor or Maspin has been characterized as a class II tumor suppressor gene in several cancer types, among them prostate cancer (CaP). Androgen ablation is an effective therapy for CaP, but with short-term effectiveness, thus new therapeutic strategies are actively sought. The present study is aimed to explore the effects of a glucosamine derivative, 2-(N-Carbobenzyloxy)L-phenylalanylamido-2-deoxy-β-D-glucose (NCPA), on two CaP cell lines, PC3 and LNCaP.

In particular we analyzed the impact of NCPA on Maspin production, cell viability and cell cycle progression and apoptosis/necrosis pathway activation in PC3 and LNCaP cell lines.

NCPA is able to stimulate Maspin production in PC3 and not in LNCaP cell lines. NCPA blocks the PC3 cell cycle in G1 phase, by inhibiting Cyclin D1 production and induces the apoptosis, therefore interfering with aggressiveness of this androgen-insensitive cell line. Moreover, NCPA is able to induce the expression of Maspin in LNCaP cell line treated with androgen receptor inhibitor, Bicalutamide, and in turn to stimulate the apoptosis of these cells.

These findings suggest that NCPA, stimulating the endogenous production of a tumor suppressor protein, could be useful in the design of new therapeutic strategies for treatment of CaP.

1. Introduction

Prostate Cancer (CaP) is the most common form of male tumor and is the second leading cause of cancer death. The disease progresses from intra-epithelial neoplasia to locally invasive adenocarcinoma, and then to hormone-refractory metastatic carcinoma [1]. Androgen ablation has proved to be an effective therapy for metastatic prostate cancer, but the regression of metastatic lesions lasts only 18–24 months [2]. The majority of men becomes refractory to androgen blockade, with a median survival time of 9–12 months. In addition to androgen receptor (AR) signaling, it has become evident that other signaling pathways are involved in prostate cancer development and progression [3].

Oncogenic processes, such as mutation of Ras and activation of Bcl2, or loss of function by tumor suppressor genes also contribute to tumor progression. Maspin (*Ma*mmary *S*erine *P*rotease *In*hibitor or SerpinB5) is a tumor-suppressing serine protease inhibitor initially isolated from normal human mammary epithelial cells. Maspin is a 42 kDa protein and is an unusual member of Serpin superfamily, which includes inhibitory members that target proteases, as well as non-inhibitory members that possess a diverse array of functions [4].

Functional studies have demonstrated that Maspin inhibits tumor invasion and motility of human mammary tumor cells in cell culture models [5], as well as tumor growth and metastasis in the nude mice assay [6]. The overexpression of Maspin is a predicting favorable prognosis factor in breast cancer and in lung squamous cell carcinoma [7,8]. However, the exact role of Maspin in tumors is still controversial since conflicting experimental and clinical results are reported. Although the significance of Maspin expression varies in different tumors, the expression of Maspin is frequently absent in prostate cancer [9]. In the prostate, expression of Maspin is regulated by both positive and negative mechanisms at the transcriptional level. It has been identified the Ets site as a positive regulator of Maspin expression, and the hormone responsive element (HRE) sites that bind the androgen receptor as cis elements involved in negatively regulated Maspin transcription

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materials

MDPI

Article

Graphene Oxide Oxygen Content Affects Physical and Biological Properties of Scaffolds Based on Chitosan/Graphene Oxide Conjugates

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Abstract: Tissue engineering is a highly interdisciplinary field of medicine aiming at regenerating damaged tissues by combining cells with porous scaffolds materials. Scaffolds are templates for tissue regeneration and should ensure suitable cell adhesion and mechanical stability throughout the application period. Chitosan (CS) is a biocompatible polymer highly investigated for scaffold preparation but suffers from poor mechanical strength. In this study, graphene oxide (GO) was conjugated to chitosan at two weight ratios 0.3% and 1%, and the resulting conjugates were used to prepare composite scaffolds with improved mechanical strength. To study the effect of GO oxidation degree on scaffold mechanical and biological properties, GO samples at two different oxygen contents were employed. The obtained GO/CS scaffolds were highly porous and showed good swelling in water, though to a lesser extent than pure CS scaffold. In contrast, GO increased scaffold thermal stability and mechanical strength with respect to pure CS, especially when the GO at low oxygen content was used. The scaffold in vitro cytocompatibility using human primary dermal fibroblasts was also affected by the type of used GO. Specifically, the GO with less content of oxygen provided the scaffold with the best biocompatibility.

Keywords: graphene oxide; chitosan; composites; scaffolds; tissue engineering

1. Introduction

Chitosan is a cationic polysaccharide, deriving from chitin deacetylation, which has gained a prominent place in biomedicine for a wide range of applications including drug delivery, wound dressings, bacterial contamination control, fat binding, and tissue engineering [1–5]. The peculiarity of chitosan, compared to other polysaccharides, is that it has been shown to provoke minimal or no foreign-body reaction, including inflammatory response and fibrotic encapsulation when used in hydrogel systems [6,7], polyelectrolyte multilayers [8], biomembranes [9] and as a porous 3-D scaffold [10]. Besides, chitosan has been shown to promote cell adhesion and proliferation in tissue

Accepted Article

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The Glucosamine-derivative NAPA Suppresses MAPK Activation and Restores Collagen Deposition in Human Diploid Fibroblasts Challenged with Environmental Levels of UVB

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