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ASSESSMENT OF ENOXACIN EFFECT ON CANCER GROWTH AND MICRORNA EXPRESSION IN PROSTATE CELL LINES

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Dissertation to a Master's Degree in Oncology

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Dissertation for applying to a Master's degree in Oncology – Specialization in Molecular Oncology submitted to the Institute of Biomedical Sciences Abel Salazar, University of Porto.

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RELEVANT ABBREVIATIONS

A	Adenine
AMO	Anti-miRNA Oligonucleotide
AS	Active Surveillance
BCA	Bicinchoninic Acid
BPH	Benign Prostatic Hyperplasia
BSA	Bovine Serum Albumin
C	Cytosine
CASP3	Caspase 3
cDNA	Complementary DNA
CpG	Cytosine-phosphate-Guanine
CRPC	Castration-Resistant Prostate Cancer
DAB	3,3-Diaminobenzidine
DGCR8	Di George Syndrome Critical Region 8
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
DRE	Digital Rectal Examination
dsRNA	Double-stranded RNA
EMT	Epithelial-to-Mesenchymal Transition
FBS	Fetal Bovine Serum
FI	Fluorescence Index
G	Guanine
GS	Gleason Score
GUSB	Beta-Glucuronidase
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HDAC1	Histone Deacetylase 1
HDM	Histone Demethylase
HGPIN	High-Grade Prostatic Intraepithelial Neoplasia
HMT	Histone Methyltransferase
LNA	Locked Nucleic Acid
miRNA	MicroRNA
mRNA	Messenger RNA

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide
ncRNA	Non-coding RNA
OD	Optical Density
PBS	Phosphate-Buffered Saline
PCa	Prostate Cancer
PCR	Polymerase Chain Reaction
PIA	Proliferative Inflammatory Atrophy
PIN	Prostatic Intraepithelial Neoplasia
Pre-miRNA	Precursor-miRNA
Pri-miRNA	Primary-miRNA
PSA	Prostate-Specific Antigen
RIPA	Radio Immuno Precipitation Assay
RISC	RNA-induced Silencing Complex
RNA	Ribonucleic Acid
RNAi	RNA interference
RP	Radical Prostatectomy
SDS	Sodium Dodecyl Sulfate
SNP	Single Nucleotide Polymorphism
T	Timine
TARBP2	Trans-Activation-Responsive RNA-Binding Protein 2
TBS	Tris-Buffered Saline
TRUS	Transrectal Ultrasound
UTR	Untranslated Region
XPO5	Exportin-5

SUMMARY

Background: Prostate cancer (PCa) is one of the most incident malignancies worldwide and represents a leading cause of cancer-related morbidity and mortality. Although efficient therapy is available for early-stage PCa, treatment of advanced disease is mainly ineffective and remains a clinical challenge. Thus, new therapeutic strategies, based on the biology of PCa, are urgently needed. MicroRNA (miRNA) dysregulation is associated with PCa development and progression. In fact, several studies have reported a widespread downregulation of miRNAs in this disease, which highlights the importance of studying compounds with the ability to restore the global miRNA expression.

Aims: The main aim of this study was to define the usefulness of enoxacin as an anti-tumoral agent in PCa, due to its ability to induce miRNA biogenesis in a Trans-activator RNA-binding protein (TRBP)-mediated manner.

Material and Methods: A panel of five PCa cell lines was screened for *TARBP2* mutations by direct sequencing and the protein levels of TRBP were evaluated by Western Blot. Immunohistochemistry was performed to assess the protein levels of TRBP in primary prostate carcinomas. After exposure of PCa cell lines to enoxacin, cell viability, apoptosis, cell cycle, and cell invasion assays were carried out to evaluate the effects of the drug. A miRCURY LNA™ array was used to determine the impact of enoxacin on the expression profile of miRNAs. Then, the protein levels of histone deacetylase 1 (HDAC1), a direct target of one of the overexpressed miRNAs, were assessed by Western Blot.

Results and Discussion: All PCa cell lines analyzed were *TARBP2* wild-type and expressed TRBP protein. Furthermore, primary prostate carcinomas displayed normal levels of TRBP protein, rendering them sensitive to restoration of normal miRNA biogenesis by enoxacin. Remarkably, enoxacin was able to decrease cell viability, induce apoptosis, lead to cell cycle arrest, and inhibit the invasive potential of PCa cell lines. Enoxacin was also effective in restoring the global expression of miRNAs, enhancing the expression of tumor-suppressor miRNAs in PCa. Moreover, the overexpression of miR-449a, one of the tumor-suppressor miRNAs implicated in PCa, was associated with the downregulation of its direct target oncoprotein, HDAC1.

Conclusions: These results demonstrated that PCa cells are highly responsive to the anti-tumoral effects of enoxacin. Therefore, enoxacin constitutes a promising therapeutic agent and *in vivo* studies should be performed to further support the potential of enoxacin for PCa treatment.

RESUMO

Introdução: O cancro da próstata (CaP) é uma das neoplasias malignas mais incidentes no mundo, representando uma causa importante de morbilidade e mortalidade. Embora estejam já disponíveis terapias eficazes para a fase inicial da doença, o tratamento da doença avançada apresenta limitações importantes, constituindo um desafio clínico. Assim, torna-se imperativo o desenvolvimento de novas estratégias terapêuticas, baseadas nas características biológicas do CaP. A desregulação dos microRNAs (miRNAs) está associada ao desenvolvimento e progressão do CaP. De facto, diversos estudos têm descrito uma sub-expressão global dos miRNAs nesta neoplasia, destacando a importância de investigar compostos com a capacidade de restabelecer a expressão global dos miRNAs.

Objetivos: O principal objetivo deste estudo foi investigar a utilidade da enoxacina como agente anti-tumoral no CaP, tendo como base a sua capacidade de promover a biogénese dos miRNAs de forma dependente da TRBP.

Material e Métodos: Foram pesquisadas mutações no gene *TARBP2* por sequenciação direta e avaliados os níveis proteicos da TRBP por Western Blot em cinco linhas celulares de CaP. A expressão proteica da TRBP em adenocarcinomas primários da próstata foi determinada por imunohistoquímica. Após exposição das linhas celulares à enoxacina, realizaram-se ensaios para avaliar os efeitos do fármaco na viabilidade celular, apoptose, ciclo celular e invasão. O impacto da enoxacina no perfil de expressão dos miRNAs foi determinado através da análise de *arrays* miRCURY LNA™. De seguida, os níveis proteicos da HDAC1, alvo direto de um dos miRNAs sobre-expressos, foram avaliados por Western Blot.

Resultados e Discussão: Não foram encontradas mutações no gene *TARBP2* em nenhuma das linhas celulares e todas expressaram a proteína TRBP. Adicionalmente, os tumores prostáticos primários exibiram níveis proteicos normais de TRBP, o que os torna sensíveis ao restabelecimento da biogénese dos miRNAs pela ação da enoxacina. Este fármaco foi capaz de reduzir a viabilidade celular, induzir apoptose, levar a uma paragem do ciclo celular e inibir o potencial invasor em linhas celulares de CaP. A enoxacina foi igualmente eficaz na restituição da expressão global dos miRNAs, promovendo a expressão dos miRNAs supressores tumorais no CaP. Adicionalmente, a sobre-expressão do miR-449a, um dos miRNAs supressores tumorais implicados no CaP, induziu a sub-expressão da sua oncoproteína alvo, a HDAC1.

Conclusão: Estes resultados demonstraram que as linhas celulares de CaP respondem significativamente aos efeitos anti-tumorais da enoxacina. Assim, a enoxacina constitui um agente terapêutico promissor para o tratamento do adenocarcinoma da próstata, sendo ainda necessária a realização de estudos *in vivo* para confirmar o seu potencial clínico.

TABLE OF CONTENTS

FIGURES INDEX

TABLES INDEX

INTRODUCTION

1. Prostate Cancer

1.1. Anatomy and Histology of the Prostate

The prostate is a walnut-shaped gland located deep in the pelvis between the bladder neck and the urogenital diaphragm and is part of the male reproductive system [1, 2]. A normal adult prostate measures approximately 25 cm^3 and weighs about 20 g [1, 3]. This gland is responsible for producing a secretion that makes up part of the seminal fluid [1].

As described by McNeal, the prostate presents a specific architecture being composed by four distinct zones: peripheral, transition, and central zones, and anterior fibromuscular stroma (AFMS) (Figure 1) [2, 3]. The peripheral zone represents the bulk of the normal gland and comprises all the prostatic glandular tissue at the apex as well as all of the tissue located posteriorly near the capsule. The transition zone, which normally encompasses only about 5% of the prostatic glandular tissue, is located centrally and surrounds the urethra. The central zone is a conical structure surrounding the ejaculatory ducts that arises from the confluence of the seminal vesicles with the vas deferens on each side. The AFMS accounts for the convexity of the anterior external surface. The apical portion of this area is mainly composed of striated muscle, which blends into the gland and the pelvic diaphragm, whereas smooth muscle cells are predominant at the base, blending into the fibers of the bladder neck. The gland is surrounded by a layer of fibrous tissue usually referred in the literature as a capsule [1, 3].

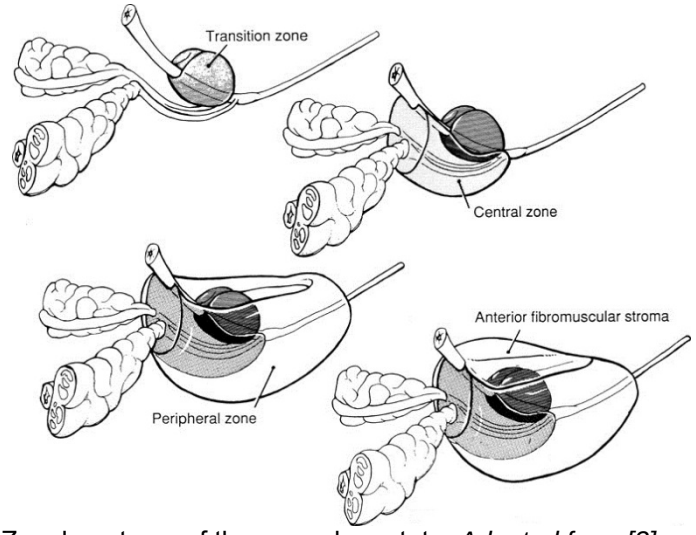

Figure 1. Zonal anatomy of the normal prostate. *Adapted from [3]*.

At histological level, the human prostate is composed of several branched ductal glands. Each gland is delimited by two cell layers, an inner luminal secretory columnar cell layer and an outer basal cell layer. Neuroendocrine cells are also present in normal prostatic epithelium being interspersed between the luminal and basal cells and representing a rare population [3].

1.2. Pathologic Conditions of the Prostate Gland

Benign prostatic hyperplasia (BPH) represents the most common urologic disease among elderly males, affecting about 75% of men over age 50 years [4, 5]. BPH usually develops from the transition zone of the gland and is defined by a hyperplastic growth of both epithelial and stromal components of the prostate [5, 6]. BPH is considered a chronic disease characterized by prostate enlargement accompanied by lower urinary tract symptoms [4, 5]. Despite the high impact of BPH on public health, the pathogenesis of this disease is still largely understood [4, 5].

Prostatic intraepithelial neoplasia (PIN) is characterized by the presence of cytologically atypical epithelial cells and architectural derangement without compromise of the basement membrane [1, 7]. It is subdivided into low-grade and high-grade and the main differences between the two grades are that high-grade prostatic intraepithelial neoplasia (HGPIN) presents cells with large nuclei of relatively uniform size, an increased chromatin content, and prominent nucleoli [8]. Currently, it is widely accepted that only HGPIN represents a precursor lesion to prostatic carcinoma [9]. In fact, the occurrence of areas of HGPIN carries a 30% to 50% risk of finding neoplastic tissue on a subsequent biopsy. Furthermore, it has been shown that these lesions can harbor many molecular abnormalities seen in prostatic carcinoma [1].

Another lesion that has been proposed as a prostatic carcinoma precursor is proliferative inflammatory atrophy (PIA), an atrophic but highly proliferative condition associated with chronic inflammation [1]. It has been postulated that the atrophic epithelial cells in PIA lesions give rise to carcinoma directly or indirectly through the development of HGPIN [10]. Indeed, there is some evidence in the literature supporting that a significant fraction of PIN and/or prostatic adenocarcinoma may originate in these atrophic lesions [11].

Finally, prostate adenocarcinoma, which accounts for over 95% of prostatic neoplasms [2], has a variable natural history, ranging from indolent, with a long preclinical phase, to strikingly aggressive [12]. Most tumors arise in the peripheral zone of the prostate, but a significant minority of prostate adenocarcinoma foci arise in the transition

and central zones [3]. A feature common to almost all prostate adenocarcinomas is the presence of only a single cell type without a basal cell layer [13].

1.3. Epidemiology

Prostate cancer (PCa) is the second most incident cancer among men worldwide, only behind lung cancer, and ranks fifth overall. An estimated 914,000 new cases occurred in 2008, accounting for 14% of the total cancer cases [14]. In Europe, PCa was reported as the most common non-skin cancer neoplasm in men, with an estimated 370,733 cases occurring in 2008 (Figure 2). In Portugal, the estimated number of newly diagnosed cases in 2008 was 5,140 being also the leading cancer among male population (Figure 2) [14].

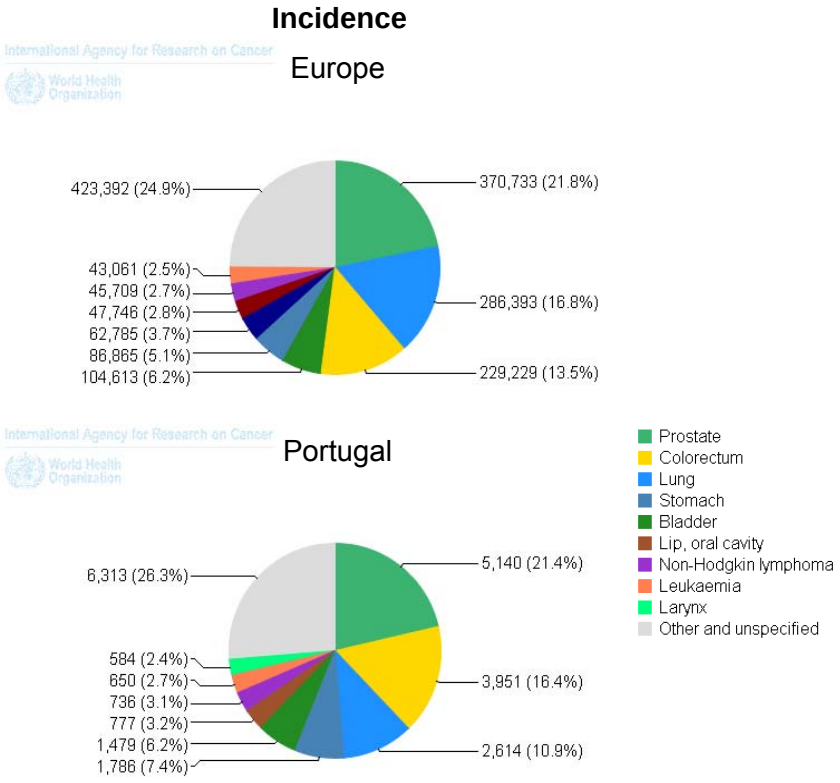

Figure 2. Incidence of different types of cancer in Europe and Portugal, in males. *Adapted from [14]*.

The trends in PCa incidence are quite heterogeneous across countries being estimated a 25-fold variation in occurrence worldwide. Highest incidence rates are estimated in Oceania, Western and Northern Europe, and Northern America, mainly because of the wide utilization of prostate-specific antigen (PSA) testing in those regions,

while the lowest are found in South-Central Asia [14]. Indeed, about three-quarters of the registered cases occur in more developed countries (Figure 3) [14]. Over the past decades, incidence of PCa has noticeably changed due to several reasons depending on the country [15]. Interestingly, temporal trends in incident rates in countries with higher PSA testing, such as the United States, Australia, Canada and the Nordic countries, followed similar patterns [16, 17]. There was a rapid rise in incidence in the early 1990s, soon after the introduction of PSA testing, followed by a sharp reduction in rates. In contrast, in countries with a lower prevalence of PSA screening, such as the United Kingdom (England and Wales) and Japan, incidence rates are slightly increasing, and a peak has not been observed yet [17].

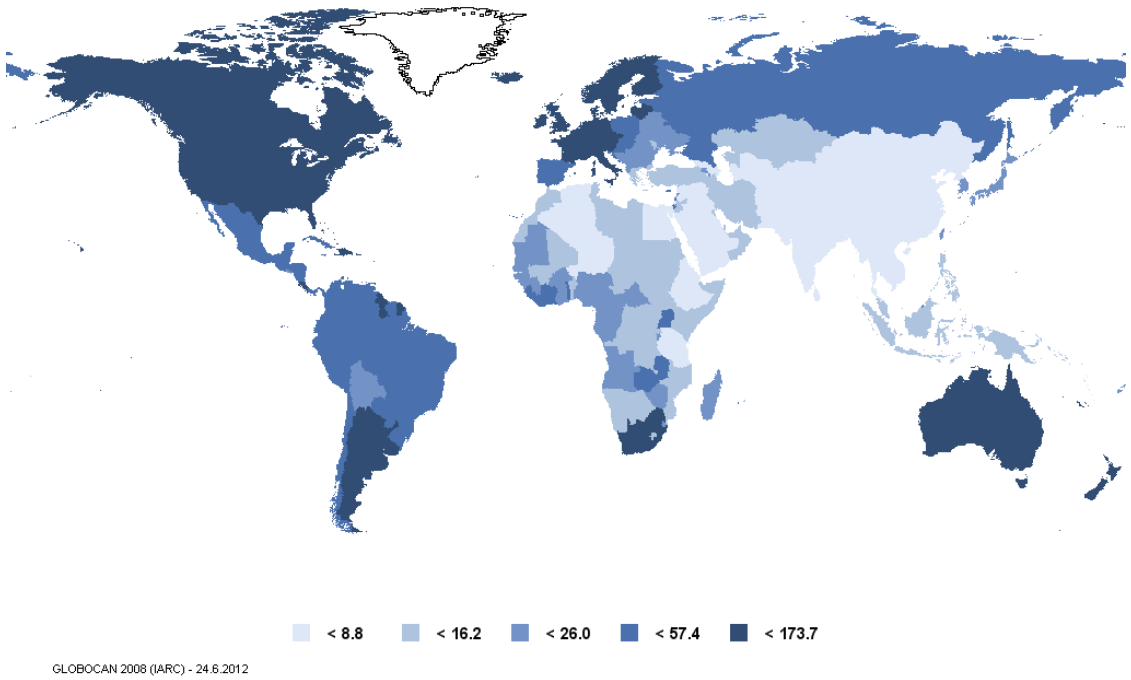

Figure 3. Estimated age-standardized incidence rate per 100,000 worldwide. *Adapted from [14]*.

Regarding mortality, about 258,400 deaths from PCa were estimated to have occurred in 2008 worldwide (6.1% of the total), being the sixth leading cause of death from cancer in men. In Europe as well as in Portugal, PCa is the third most frequent cause of cancer death [14]. Because PSA testing has a stronger effect on incidence than on mortality, there is a less variation in mortality rates worldwide (10-fold) than is observed for incidence. Hence, the difference in the number of deaths between developed and developing regions is less accentuated (136,000 and 122,000, respectively) [14].

1.3.1. Risk Factors

There are only three well-established risk factors for PCa: age, ethnicity, and a positive family history of PCa [18, 19]. In addition to these non-modifiable risk factors, numerous modifiable or behavioral factors, such as high intake of meat or obesity, have been associated with this malignancy [19].

Age

The association between increasing age and PCa risk is very strong [12]. PCa in men under 50 is rare corresponding to less than 0.1% of all patients [20]. After that age, the incidence rate increases sharply, being the mean age of diagnosed patients with this malignancy between 72 and 74 years [19, 20].

Ethnic Origin

Incidence of PCa varies widely between ethnic populations [20]. African-American men in the United States have one of the highest incidences of PCa worldwide, presenting more cases diagnosed at a younger age, more aggressive forms of PCa, and lower survival rates than European-American men [1, 17]. In addition, several studies in African and Caribbean men have suggested that Sub-Saharan African ancestry may be more relevant than location [1]. The lowest rates are observed in Asian men both living in Asia and in the United States [1, 17]. Although the exact reasons for these racial differences remain unclear, they could include genetic susceptibility, exposure to unknown external risk factors, or artifactual reasons, such as differences in cancer registration and in health care across countries [17, 20].

Family History

A family history of PCa is an important risk factor for disease development [12]. Familial clustering of PCa can be explained by genetic susceptibility, exposure to common environmental factors, or chance alone due to the high prevalence of this malignancy [20]. Men with first-degree relatives with PCa are more susceptible to develop this disease. Moreover, with an increase in the number of affected individuals in the family and/or a decrease in the age at diagnosis, the risk of PCa occurrence is even higher [1, 20].

1.4. Diagnosis

Currently, diagnosis of PCa is based on histological examination of prostatic tissue and the standard method to obtain material from the prostatic gland is transrectal ultrasound (TRUS)-guided systemic biopsy [21, 22]. The most common indications for a prostate biopsy are an elevated PSA level and/or a suspicious digital rectal examination (DRE). Furthermore, the patient's age, potential comorbidities, and therapeutic consequences should also be considered [22].

The DRE was the primary diagnostic tool employed by physicians for PCa detection [23]. Nevertheless, its central role in PCa diagnosis was superseded by the widespread application of serum PSA [21]. PSA is a kallikrein-related serine protease that is produced in normal prostate secretions being released into the blood due to the disruption of the normal prostatic membrane structures [24, 25]. Hence, PSA is specific to the prostate but not to PCa. In fact, most men with increased serum PSA do not have PCa since benign prostatic diseases are also responsible for an increased PSA [24]. The availability of a highly accessible blood test for PSA has revolutionized the diagnosis of PCa over the past three decades. Although widely practiced, PSA screening for PCa is still controversial since it has led to an overdetection and therefore overtreatment of indolent tumors [24, 25]. Moreover, it has become increasingly clear that there is no cut-off point below which there is no risk of having a biopsy positive for PCa [1].

Usually, men with PSA levels of 4.0 ng/mL or greater and/or those with abnormal DRE result are candidates to perform a prostatic biopsy [13, 26]. According to the 2011 Guidelines on Prostate Cancer from the European Association of Urology, a minimum of 10 systemic laterally directed cores are recommended and, in patients with prostate volumes greater than 40 mL, more cores should be sampled [22]. One set of repeat biopsies may be necessary in cases with persistent suspicion of PCa [22].

1.5. The Gleason Grading System

The Gleason grading system was first described by Donald F. Gleason in 1966 and it is currently the most commonly used pathologic grading system for PCa. Although many aspects of PCa have changed since the introduction of the Gleason grading system, it has remained timely because of gradual adaptations to accommodate the changing practice of medicine [27].

This system is exclusively based on glandular architecture of the tumor and defines five different histological grades with decreasing differentiation (Figure 4) [27]. As PCa has a marked morphological heterogeneity, usually presenting more than one of the

five patterns defined by Gleason in the same tumor, the Gleason Score (GS) was developed, resulting from the sum of the primary (most prevalent) and the secondary (second most prevalent) grades. Consequently, the GS possibilities range from 2 (1+1) for tumors in which the only histological pattern is 1 to 10 (5+5) for tumors in which the only histological pattern is 5 [3, 13].

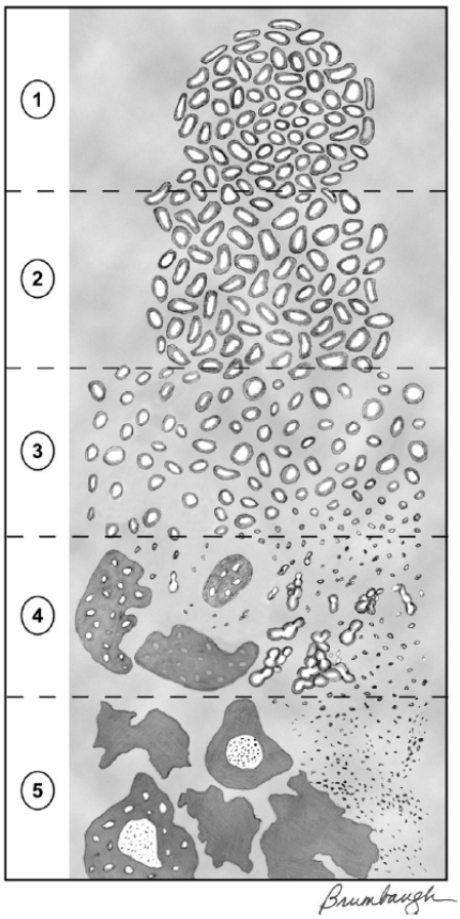

Figure 4. Updated Gleason score for histological grading of prostate tumors. Pattern 1 – Closely-packed, uniform, rounded to oval glands; Pattern 2 – More loosely arranged glands with smooth ends that may minimally invade non-neoplastic tissue; Pattern 3 – Irregular size and shape glands with more infiltrative margins; Pattern 4 – Fused, cribriform or ill-defined glands; Pattern 5 – Essentially no glandular differentiation. *Adapted from [27]*.

Although the Gleason system is now widely accepted, a number of controversial issues have concerning it as a grading system. Most notably, Gleason grading is observer dependent and may vary with the level of experience. Another limitation is that most men diagnosed today fall into the Gleason 6-7 category, an intermediate prognostic range limiting the potential usefulness of a 10-point scale [3]. Nevertheless, GS is a very important prognostic indicator enabling the prediction of the natural history of PCa and the assessment of the risk of recurrence after total prostatectomy or radiotherapy [13].

1.6. Clinical and Pathologic Staging

The most widely used staging system for PCa is the tumor node metastasis (TNM) system (Table 1) which is based on the size and extent of the primary tumor (T), involvement of regional lymph nodes (N), and the presence or absence of distant metastases (M) [3, 28]. Stage may be defined at different points in the care of the cancer patient, including pretreatment or clinical stage, and postsurgical or pathologic stage [28].

Clinical stage refers to the extent of disease defined by diagnostic study before information is available from surgical resection [28]. It is mainly established through the evaluation of the patient by DRE and, less commonly, by transrectal ultrasonography, and magnetic resonance imaging (MRI). Further information can be provided by biopsy histopathological evaluation and serum PSA levels [1, 15, 21].

On the other hand, pathologic stage is determined after surgical removal of the prostate through adequate analysis of the prostatectomy specimen, and enables the prediction of disease recurrence much more accurately [1]. Indeed, prognosis can be estimated with more precision though the combination of several factors in nomograms thus leading to an improvement of biological characterization of a given tumor and helping in clinical decision concerning treatment options. These prognostic factors include extra-prostatic tumor invasion, seminal vesicles involvement, lymph node metastases, and distant metastases, as well as the preoperative serum PSA levels, GS in the prostatectomy specimen, and surgical margins [1]. The gold-standard method to assess N-staging is pelvic lymphadenectomy, whereas for the classification of M-staging, bone scan remains the most sensitive method, but it is only recommended in high-risk patients (i. e., symptomatic or asymptomatic patients with a PSA level > 20 ng/mL and a GS of 8 or higher) [15, 21].

Table 1. The 2010 American Joint Committee on Cancer/International Union Against Cancer TNM Staging Classification for prostate cancer. *Adapted from [28]*.

Primary Tumor	
Clinical	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically inapparent tumor neither palpable nor visible by imaging
T1a	Tumor incidental histologic finding in 5% or less of tissue resected
T1b	Tumor incidental histologic finding in more than 5% of tissue resected
T1c	Tumor identified by needle biopsy
T2	Tumor confined within prostate
T2a	Tumor involves one-half of one lobe or less
T2b	Tumor involves more than one-half of one lobe but not both lobes
T2c	Tumor involves both lobes
T3	Tumor extends through the prostate capsule
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
Pathologic	
pT2	Organ confined
pT2a	Unilateral, one-half of one side or less
pT2b	Unilateral, involving more than one-half of side but not both sides
pT2c	Bilateral disease
pT3	Extraprostatic extension
pT3a	Extraprostatic extension or microscopic invasion of bladder neck
pT3b	Seminal vesicle invasion
pT4	Invasion of rectum, levator muscles, and/or pelvic wall
Regional Lymph Nodes	
Clinical	
NX	Regional lymph nodes were not assessed
N0	No regional lymph node metastasis
N1	Metastasis in regional lymph node(s)
*Pathologic**	
pNX	Regional nodes not sampled
pN0	No positive regional nodes
pN1	Metastasis in regional node(s)
Distant Metastasis	
M0	No distant metastasis
M1	Distant metastasis
M1a	Non-regional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

* There is no pathologic T1 classification.

1.7. Therapy

Therapeutic management of PCa must be based on a multidisciplinary approach, taking into account the TNM classification, GS, preoperative serum PSA level, patient's age, comorbidity, life expectancy, and quality of life [21].

1.7.1. Treatment of Clinically Localized Disease

The main therapeutic options for early-stage PCa are watchful waiting/active surveillance, surgery, and radiotherapy [22]. Traditionally, watchful waiting has meant that no active treatment is administered until a patient develops evidence of symptomatic disease progression, at which time androgen-deprivation therapy is initiated. This approach do not attempt to administer potentially curative treatment, but aims to limit morbidity from the disease and therapy [1]. A more recent concept, termed active surveillance (AS), assumes that delayed treatment will be as curative as immediate treatment and attempts not only to avoid overtreatment in the majority of patients, but also to administer curative therapy to selected cases [1]. Therefore, men with low-risk PCa (*i.e.*, stage T1 to T2, GS of 6 or less and serum PSA lower than 10 ng/mL) and more than 10 years of life expectancy are good candidates for AS [22]. Patients who are offered AS must be followed-up carefully with serial PSA measurements and periodic prostate re-biopsies [21].

Radical prostatectomy (RP), which consists of removing the whole prostate gland and the seminal vesicles, is the only treatment for localized PCa that has shown a cancer-specific survival benefit when compared to AS [21]. This procedure has been refined, resulting in high cure rates with decreased morbidities, such as urinary incontinence or erectile dysfunction, which are frequent in patients submitted to RP [29].

Alternatively to RP, external beam-radiotherapy and brachytherapy have achieved disease-free survival rates comparable to those of the surgical procedure in the treatment of early-stage PCa, but with a different spectrum of side effects [29]. Because it is non-invasive and has no anesthesia risk, external beam-radiotherapy may be offered to a wide range of patients with PCa [29]. Patients with local failure after prostatectomy may also be submitted to external-beam radiotherapy [15]. Brachytherapy, which involves placement of the radiation source directly into the region of interest, is offered to patients with clinically localized, low-volume and low-grade disease [15].

More recently, hormonal therapy has been evaluated as adjuvant therapy in combination with RP or radiotherapy in the treatment of early-stage PCa. However, only in combination with radiotherapy it was shown an improvement in survival [29].

Besides these conventional procedures, cryosurgical ablation of the prostate and high-intensity focused ultrasound have emerged as alternative therapeutic options in patients with clinically localized PCa [21].

1.7.2. Treatment of Advanced and Castration-Resistant PCa

Androgen-deprivation therapy has long been the mainstay for management of advanced PCa [15], and is usually performed by administration of gonadotropin-releasing hormone analogs and/or surgical castration (orchiectomy), often in combination with anti-androgens such as flutamide or bicatulamide [2]. Although this therapeutic strategy reduces symptoms in about 70-80% of patients [15], most tumors progress to castration-resistant disease after a median duration of response of 18 to 24 months [30].

Castration-resistant PCa (CRPC) has been essentially untreatable, with the most effective standard chemotherapeutic regimens (*i.e.*, docetaxel in combination with either prednisone or estramustine) demonstrating a limited survival benefit of approximately 2 months [2, 30]. Moreover, many patients develop painful metastases, usually osseous, and are not amenable to chemotherapy. Metastatic PCa is incurable and all therapy available is merely for palliative purposes [31, 32].

Given this scenario, it is clear that new therapeutic strategies are urgently needed.

2. Epigenetics

The word “Epigenetics” was coined by Conrad Waddington in the early 1940s [33]. Derived from the word “epigenesis”, the term “Epigenetics” was first introduced to describe “the causal interactions between genes and their products, which bring the phenotype into being” [34]. This definition was originally used in the context of embryonic development but it is now superseded since epigenetics have been implicated in a wide variety of biological processes [35]. Therefore, in 1996 Arthur Riggs and colleagues defined epigenetics as “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence” [36]. As used today the term “Epigenetics” encompasses both heritable and transient changes in gene expression that do not involve a change in primary DNA sequence [36, 37].

The mammalian genome is organized and packaged into chromatin, a highly compact and structured complex consisting of DNA and histone proteins [38]. Epigenetic mechanisms that modify chromatin structure can be divided into three main categories: DNA methylation, post-translational histone modifications, and expression of non-coding RNAs [39]. The continuous interplay of these mechanisms creates what Waddington called the “epigenetic landscape” and today is designated by the “epigenome” – the epigenetic status that determines the way the mammalian genome manifests itself in different cell types, developmental stages and disease [33, 35].

DNA Methylation

DNA methylation is perhaps the most studied epigenetic mechanism [35]. Although this modification is found in the genome of almost all living organisms, the patterns and levels are variable across species [40, 41].

In mammals, DNA methylation is mostly confined to cytosines that precede a guanine, in the so-called CpG dinucleotides. This process is catalyzed by DNA methyltransferases (DNMTs), which add a methyl group to the fifth carbon position of a cytosine residue ring, resulting in the formation of a new DNA base – 5-methylcytosine (m^5C). The DNMTs use S-adenosyl-L-methionine (SAM) as a donor of methyl groups [42, 43].

The DNMTs fit into two main categories based on their preferred DNA substrate. The *de novo* methyltransferases DNMT3a and DNMT3b are able to methylate previously unmethylated CpG sequences, whereas the maintenance methyltransferase DNMT1 has preferential activity for hemi-methylated DNA, copying the pre-existing methylation marks onto the new DNA strand during replication [41, 44, 45]. The DNA methyltransferases family includes other two members: DNMT2 and DNMT3L. DNMT2 is the smallest

mammalian DNMT and has shown weak DNA methyltransferase activity. DNMT3L is a DNMT-related protein that does not contain intrinsic DNA methyltransferase activity, but interacts with *de novo* DNMTs and modulates their catalytic activity [44].

DNA methylation is associated with a repressed chromatin state and inhibition of gene expression. In fact, there are two general mechanisms by which DNA methylation inhibits gene expression. Although it can inhibit the association of some DNA-binding factors with their cognate DNA recognition sequences, repression seems to occur largely indirectly, via recruitment of methyl-CpG binding proteins (MBPs) that induce chromatin changes. MBPs use transcriptional co-repressor molecules to silence transcription and to modify surrounding chromatin, providing a link between DNA methylation and chromatin remodeling and modification [44, 46].

In human somatic cells, m^5C accounts for nearly 1% of total DNA bases and affects 60% to 90% of all CpG dinucleotides in the mammalian genome. The exceptions are CpG islands, CG-rich sequences that frequently coincide with gene promoter regions and are normally unmethylated [40, 45]. Nonetheless, a small but significant proportion of all CpG islands become methylated during development, which results in stable silencing of the associated promoter. Developmentally programmed CpG island methylation of this kind is involved in genomic imprinting and X-chromosome inactivation [40, 43]. Moreover, alterations in DNA methylation are linked to many human diseases, including cancer [46].

Histone Modifications

Histones are small basic proteins around which DNA is packaged within the chromatin, and consist of a globular C-terminal domain and a flexible unstructured N-terminal tail [47]. The N-terminal tail of histones can undergo a variety of post-translational covalent modifications, among which the most studied are acetylation and methylation [48]. These modifications are known to play a critical role in chromatin packaging and key cellular processes, such as replication, transcription, and DNA repair [49].

In general, acetylation of lysine residues decreases the affinity of histones for DNA creating an “open” chromatin conformation that enables gene transcription. This modification is catalyzed by histone acetyltransferases (HATs) and can be reversed by the enzymatic action of histone deacetylases (HDACs) [50]. Thus, the interplay between HATs and HDACs activities regulates cellular histone acetylation levels through a dynamic equilibrium [51].

Contrarily to acetylation, histone methylation can lead to either transcriptional activation or repression depending on the altered residue and its position. For instance, methylation of lysines 4, 36, and 79 of histone 3 (H3K4me3, H3K36me, and H3K79me) is associated with transcriptionally active regions, whereas methylation of lysines 9 and 27

of histone 3 (H3K9 and H3K27) and of lysine 20 of histone 4 (H4K20) is generally linked to heterochromatin formation and the presence of inactive promoters [52]. Histone methylation-modifying enzymes include histone methyltransferases (HMTs) and histone demethylases (HDMs), which present high substrate specificity [49].

Remarkably, histone modifications are also implicated in the previously mentioned epigenetic mechanisms present in normal cells, namely genomic imprinting and X-chromosome inactivation, as well as in embryonic stem cell development, and differentiation [49, 52]. In malignant cells, genome-wide histone modifications are also altered in concert with changes in DNA methylation [35].

Non-coding RNA

Non-coding RNAs (ncRNAs) are a growing class of transcripts that does not encode for proteins, but is biologically functional [53, 54]. Recently, multiple ncRNAs have been demonstrated to play a role in transcriptional regulation through their interaction with several transcription factors. This class of transcripts also interacts with chromatin-modifying enzymes demonstrating its relevance in maintaining a proper chromatin conformation [55, 56]. Indeed, these molecules have been recognized as an important component in the epigenetic modification mechanisms, as shown by silencing of transposable elements, X-chromosome inactivation, and DNA imprinting [57]. Therefore, it has been suggested that ncRNA transcripts are crucial players in eukaryotic molecular biology and that their dysregulation might be involved in the development of many different human diseases [54, 57].

NcRNAs comprise several different classes, such as microRNAs (miRNAs), transcribed ultraconserved regions (T-UCRs), small nucleolar RNAs (snoRNAs), PIWI-interacting RNAs (piRNAs), large intergenic non-coding RNAs (lincRNAs), and the heterogeneous group of long non-coding RNAs (lncRNAs) [54]. The functional relevance of the non-coding genome for normal development and physiology and for disease have been particularly evident for miRNAs [54].

2.1. MicroRNA Biogenesis and Mode of Action

MiRNAs have been the most widely investigated class of ncRNAs characterized by only having approximately 18 to 25 nucleotides in length [54, 58]. The first miRNA to be identified was *lin-4*, in 1993, from a study of developmental timing in the nematode *Caenorhabditis elegans* [59, 60]. Since then, hundreds of miRNAs have been identified and currently, 2,042 human miRNAs are registered in the miRBase database (release 19, August 2012).

The canonical miRNA biogenesis pathway begins in the nucleus with the synthesis by RNA polymerase II of a long capped and polyadenylated RNA with hairpin structures known as primary transcript (pri-miRNA) (Figure 5) [58, 59]. Following transcription, a pri-miRNA forms a stem-loop structure with a double-stranded RNA (dsRNA) of approximately 33 nucleotides [61]. This dsRNA intermediate is then recognized and excised by a complex known as Microprocessor which is composed of RNase III Drosha and its dsRNA-binding partner Di George syndrome critical region 8 (DGCR8). The cleavage produces a hairpin RNA of about 65 nucleotides that is termed a precursor-miRNA (pre-miRNA) [58, 61]. Alternatively, some miRNAs (termed mirtrons), which are located in short introns of host genes, bypass the Drosha processing step and get processed using the splicing machinery. After splicing, the excised intron is debranched and trimmed by lariat-debranching enzyme to generate the pre-miRNA [54, 58, 60]. At this point, the mirtron pathway merges with the canonical miRNA pathway [58], and pre-miRNAs are actively transported to the cytoplasm by the nuclear export factors Exportin-5 (XPO5) and RanGTPase [61, 62]. In the cytoplasm, the loop of miRNA is cleaved off by another RNase III named Dicer, which acts in complex with the dsRNA-binding protein Trans-activator RNA-binding protein 2, TARBP2 (TRBP) [18, 60, 62]. The cleavage results in the production of a short RNA duplex, which consists of a functional miRNA (called the *guide* strand) and its partially complementary *passenger* strand (denoted as miRNA*) [62]. The strand with the 5' segment more loosely paired with the opposite strand is chosen as the functional one and is loaded into an Argonaute protein to form the RNA-induced silencing complex (RISC), whereas the *passenger* strand is subjected to degradation [59, 62].

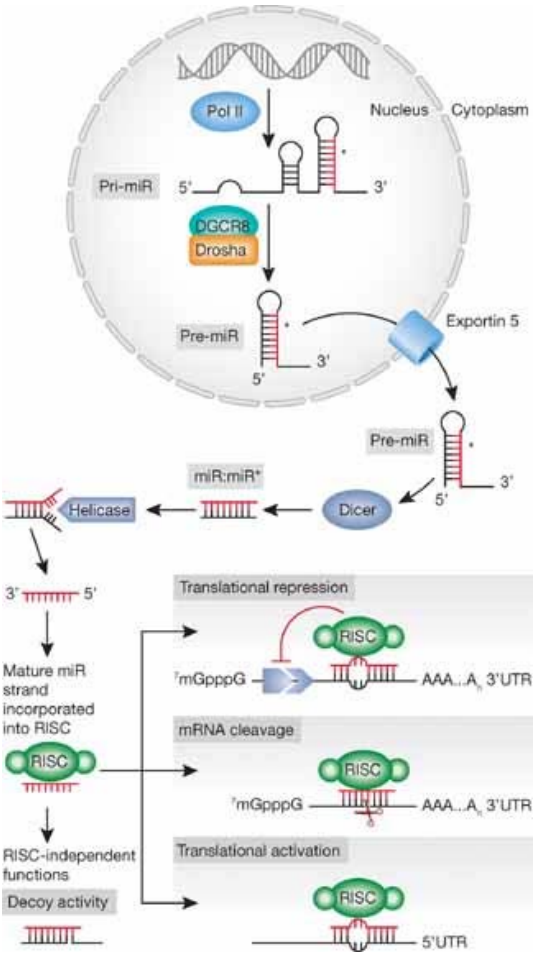

Figure 5. miRNA biogenesis and mechanism of action. *Adapted from [60].*

RISC directs the regulation of gene expression at post-transcriptional level, binding mostly through partial complementarity to a sequence in the target mRNA generally located at the 3'-untranslated region (3'UTR) [54, 60]. This regulation is performed by two main ways depending on level of complementarity between miRNA and its target mRNA sequence: if perfect or near-perfect complementarity is established, the RISC induces mRNA degradation; if they exhibit an imperfect pairing, mRNA translation into a protein is blocked (Figure 5) [60, 63]. More recent studies have reported that miRNAs do not only bind to 3'UTR but also to the open reading frame or to the 5'UTR of the target mRNAs [64, 65]. Additionally, they can bind to ribonucleoproteins in a seed sequence in a RISC-independent manner interfering with their RNA binding functions [66]. Furthermore, there are three reports that describe miRNAs as being able to regulate gene expression at transcriptional level by direct binding to the DNA [67-69]. Surprisingly, it was recently shown that miRNAs can activate, rather than inhibit gene expression [70]. Given the complexity of mechanisms regulating the interaction between a miRNA and its target mRNA, it is accepted that each miRNA has the potential to target several different mRNAs. Conversely, a single mRNA can be targeted by multiple miRNAs [59, 60].

In mammals, miRNAs are predicted to regulate the translation of more than 60% of protein-coding genes and play crucial roles in the regulation of multiple pathways and processes, including proliferation, differentiation, apoptosis, development, and metabolism [54, 71]. Due to their involvement in almost every biological processes, the abnormal expression or alteration of miRNAs contributes to a range of human diseases, including cancer [54, 58, 72].

2.2. MicroRNAs and Cancer

The first evidence of the association between aberrant miRNA expression and human cancer derived from studies on B-cell chronic lymphocytic leukemia. In 2002, Calin and colleagues showed that miR-15-a and miR-16-1 were downregulated or deleted in a majority of patients with B-cell chronic lymphocytic leukemia due to chromosomal deletion at the 13q14 locus [73]. Since then, several studies have identified miRNA expression signatures that distinguish between normal and cancer tissues [74, 75]. Additionally, miRNA profiles might also discriminate different subtypes of a particular cancer [76-78], or even specific oncogenic abnormalities [79].

Alterations of miRNA expression have been described in most cancers and can arise from either genetic or epigenetic means (Figure 6) [80]. Chromosomal abnormalities represent an important mechanism of alteration, as suggested by the evidence that miRNAs are frequently located within fragile chromosomal sites which are often deleted or rearranged in cancer [73, 81, 82]. Mutations and single nucleotide polymorphisms (SNPs) within miRNAs have also been described in different types of cancer [83-85]. Moreover, miRNA dysregulation may result from increased or decreased transcription activity of a transcription factor at the promoter [86]. Concerning epigenetic regulation, between 20% and 40% of miRNA genes are located close to CpG islands, which suggests that they represent candidate targets of the DNA methylation machinery being susceptible to epigenetic silencing [87]. Conversely, methylation is not the only epigenetic mechanism that might affect miRNA expression, since it has been shown that HDAC inhibitors have the potential to promote downregulation of some miRNAs [71]. In addition to genetic and epigenetic alterations targeting miRNA genes, miRNA expression can also be modulated by defects in the machinery implicated in miRNA biogenesis [60]. Indeed, changes in miRNA levels might be a consequence of genetic alterations in different molecules involved in miRNA processing, such as Drosha [88], XPO5 [89], Dicer [90], and TARBP2 [91]. These alterations affect the production of the pri-miRNA, their processing to mature miRNA form and/or interaction with mRNA targets, and have been already implicated in neoplastic transformation [54].

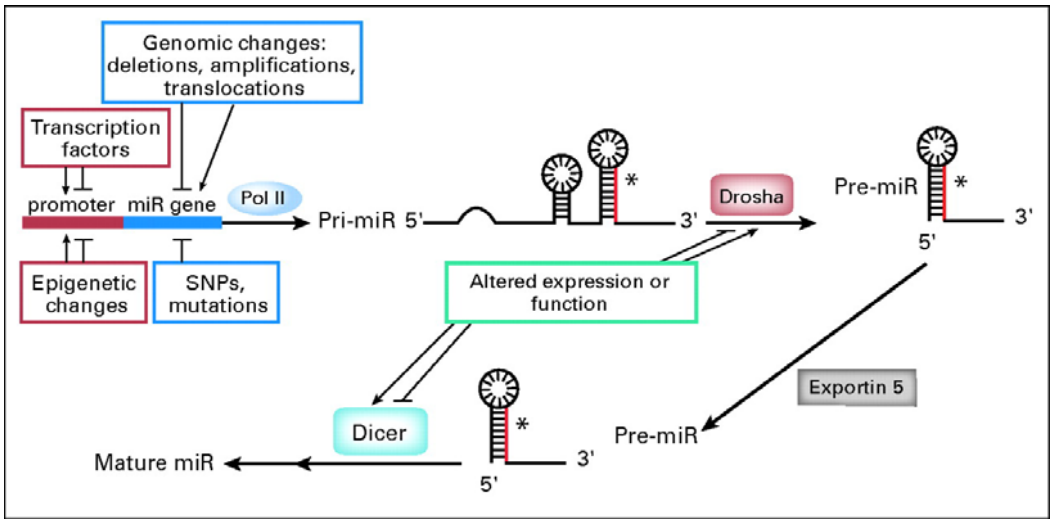

Figure 6. Mechanisms of miRNA dysregulation. *Adapted from [80].*

As same as for protein-coding genes, miRNAs have been proposed to function as oncogenes or tumor-suppressor genes, depending on the target genes and the tissue context in which they are expressed [92]. Although it has been known that both overexpression and depletion of specific miRNAs play pathogenic roles in tumor progression, most human tumors are characterized by a general defect in miRNA production that results in global downregulation of miRNA expression [74, 93].

Taken together, these findings have suggested the promising potential of miRNAs as cancer biomarkers, for prognosis and/or prediction of response to specific therapies [94-97]. The translational applications for miRNAs also include their use as novel therapeutic targets [98].

2.3. MicroRNA-based Therapies

The association of aberrant miRNA expression with cellular transformation and maintenance of the malignant state placed miRNAs as attractive targets for cancer treatment [98]. The identification of their dual role in carcinogenesis has led to the development of two different therapeutic approaches: inhibition of oncogenic miRNAs and restoration of tumor-suppressor miRNAs expression [99].

MiRNAs with oncogenic functions might be inhibited by several RNA interference-type strategies, namely anti-miRNA oligonucleotides (AMOs), miRNA sponges and miRNA masking [100]. AMOs competitively block the interaction between miRNAs and target mRNAs and have been chemically modified in a variety of ways to improve their stability [99, 100]. One example of these chemically altered AMOs is locked nucleic acids (LNAs) which have many advantages over traditional AMOs, since they do not require a

vector and present higher thermal stability and lower toxicity [99, 100]. Alternatively, it is possible to use a sponge vector containing multiple artificial miRNA binding sites that are placed under the control of strong promoters to produce large quantities of transcript. They act as sponges for cognate miRNAs, preventing their association with natural targets [54]. Finally, a miR-mask, which is synthesized as a single-stranded 2′-O-methylmodified oligoribonucleotide, has perfect complementarity to an endogenous miRNA binding site in the 3′UTR of a protein-coding gene [100].

Regarding tumor-suppressor miRNAs that are downregulated in cancer, the fundamental principle of miRNA-based therapy relies on restoring their expression to normal levels [99, 100]. This can be achieved through miRNA mimics or viral vector-based miRNA replacement [100, 101]. MiRNA mimics are small chemically modified dsRNA molecules that imitate endogenous miRNAs, whereas viral vectors encoding miRNAs function as miRNA deliver vehicles [100]. However, because most human tumors show a general downregulation of miRNA expression, a miRNAome-based approach may be much more effective for therapy than strategies that aim to regulate a single miRNA. Therefore, compounds with the ability to restore the global miRNA expression might be an excellent therapeutic option for cancer.

2.3.1. Enoxacin

Enoxacin is a member of a family of synthetic antibacterial compounds based on a fluoroquinolone skeleton (Figure 7). It has been used to treat a wide variety of bacterial infections such as gonorrhea and urinary tract infections [102, 103]. Clinically, side effects have been minimal in adults, with an incidence of adverse events ranging from 0% to 24% [103].

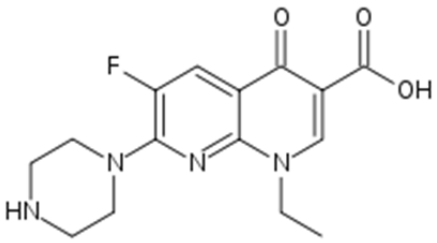

Figure 7. Chemical structure of enoxacin. *Adapted from [104].*

In 2008, the small molecule enoxacin was found to promote RNA interference (RNAi), the mechanism that uses small ncRNAs to post-transcriptionally silence gene expression. This finding was achieved through a chemical screen of a collection of 2,000 US Food and Drug Administration (FDA)-approved compounds and natural products to

identify small molecules that enhance RNAi [105]. Additionally, of 10 fluoroquinolones analyzed, enoxacin was the only one capable of enhancing RNAi, suggesting that this ability of enoxacin does not depend on general fluoroquinolone activity, but rather on the unique chemical structure of the molecule [105]. Recently, enoxacin was shown to be effective in the inhibition of tumor cell growth *in vitro* and *in vivo* by enhancing the production of miRNAs with tumor-suppressor functions [106]. Remarkably, the drug did not affect normal cells and was not associated with toxicity in mice models [106]. Moreover, both studies reported that enoxacin promotes RNAi and miRNA biogenesis by facilitating the interaction between TRBP and miRNA precursors [105, 106]. Thus, it has been demonstrated that enoxacin acts in a TRBP-dependent manner [105, 106] and, consequently, *TARBP2*-mutant cells are less responsive to enoxacin [91, 106].

3. MicroRNA Dysregulation in PCa

Currently, there are more than 100 published studies investigating miRNA expression in PCa and a vast number of miRNAs have been reported to be abnormally expressed in this malignancy [107, 108]. Although still controversial, several studies have reported a widespread downregulation of miRNAs in human PCa [93, 109]. Nonetheless, only a few miRNAs have been experimentally proven to contribute to prostate carcinogenesis and the knowledge about their respective target genes is still limited [107]. Despite the vast number of differentially expressed miRNAs in PCa, they mostly converge on key cellular pathways which are often dysregulated in cancer, specifically in epigenetic reprogramming, apoptosis, cell cycle, migration, invasion, and androgen signaling [110].

The relationship between miRNAs and androgen signaling is indicative of their role in PCa progression, with miRNAs modulating the androgen pathway or being regulated by androgens [111]. Indeed, miR-146a expression has been found to be lower in androgen-independent PCa cell lines than in androgen-dependent PCa cell lines [112]. The contribution of miR-146a to PCa progression has been supported by its capacity to repress Rho-activated protein kinase 1 (ROCK1) [112], an enzyme involved in the activation of hyaluronan-mediated hormone-refractory PCa transition [113]. On the other hand, miR-141, which presents higher levels in PCa, is an androgen-responsive miRNA and it has been recently demonstrated that its expression was affected by the integrity of the androgen pathway [114]. Moreover, it has been reported that overexpression of miR-141 in LNCaP cells increased cell proliferation, suggesting a role in the progression of PCa [114]. Recently, different studies have also shown the value of miR-141 expression as a noninvasive biomarker for tumor progression and therapeutic response [115, 116].

The two closely related miR-221 and miR-222 were also described as massively overexpressed in CRPC cells [117]. Moreover, the induced overexpression of miR-221 and miR-222 in androgen-dependent LNCaP cells altered cells' responsiveness to dihydrotestosterone, assessed by PSA expression, and increased androgen-independent growth. Hence, these findings suggest the importance of specific miRNAs in maintaining the castration-resistant phenotype [117]. Interestingly, miRNAs also regulate androgen signaling through the cross-talk between androgen receptor (AR) and v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (HER2) signaling pathways. Sun *et al.* reported that reduced levels of miR-331-3p in PCa promoted high *HER2* expression, which increased AR signaling [118]. Moreover, transfection of PCa cell lines with miR-331-3p reduced *HER2* expression, as well as, downstream PI3K/AKT signaling, and blocked AR signaling [118].

In addition to their role in androgen signaling, miRNAs are also involved in apoptosis avoidance during carcinogenesis [107]. For instance, the miR-34 family has been implicated in the p53 tumor-suppressor network which is an important mediator of apoptosis [119, 120]. MiR-34a expression was found to be lost in androgen-independent cell lines and its reconstitution in PC-3 cells resulted in cell-cycle arrest and apoptosis [121]. Furthermore, it was demonstrated that miR-34a inhibits silent information regulator 1 (*SIRT1*) [122], a gene that suppresses p53-dependent apoptosis [123]. Likewise, miR-34c, which negatively regulates the oncogenes E2F transcription factor 3 (*E2F3*) and B-cell CLL/Lymphoma 2 (*BCL2*), was found to be downregulated in PCa, further contributing to apoptosis evasion by neoplastic cells [124]. Another illustrative example is provided by miR-449a which presents decreased expression levels in PCa compared to normal prostate tissues. In fact, its reintroduction in PC-3 cells resulted in cell-cycle arrest, apoptosis, and a senescent-like phenotype [125]. Additionally, it has been established that miR-449a modulates the expression of histone deacetylase 1 (*HDAC1*) [125], which is overexpressed in about 70% of PCa [126]. Therefore, it has been proposed that miR-449a regulates cell growth and viability in part by repressing the expression of *HDAC1* in PCa cells [125].

Remarkably, miRNAs might function either as metastatic inducers or suppressors by regulating multiple critical steps in the metastatic cascade. Several miRNAs have also been implicated in the regulation of the multistep metastatic cascade by targeting various proteins that play major roles in this process [127]. So far, only a few studies have investigated miRNAs associated with metastatic PCa. A recent study, using next generation sequencing, has demonstrated that the miRNA profile of a transplantable metastasis is rather different from a non-metastatic PCa xenograft [128]. Moreover, miR-205, which plays a crucial role in epithelial organization maintenance in human prostatic

tissue mainly through the suppression of protein kinase Cε, has been shown to be downregulated in PCa [129]. Interestingly, miR-205 restoration in aggressive cancer cells induced marked morphological changes consistent with a reverse transition from a mesenchymal to an epithelial state [129]. Finally, miR-29b which acts as an antimetastatic miRNA by modulating the expression of multiple proteins involved in metastasis formation, including metalloproteinase-2 (MMP2), E-cadherin, N-cadherin, Snail and Twist, has been also found to be decreased in PCa [130, 131]. Remarkably, its ability to diminish migration and invasiveness has been confirmed by both *in vitro* and *in vivo* functional assays [131].

Therefore, the involvement of miRNAs dysregulation in PCa not only strongly supports its importance in PCa carcinogenesis, but it is also indicative of its putative value as tumor biomarkers for diagnosis and prognosis assessment. Ultimately, it may also open new avenues for innovative PCa treatment approaches.

AIMS OF THE STUDY

PCa is a complex disease and when it reaches advanced stages there are no effective treatment options available. Therefore, new therapeutic strategies, based on the knowledge of its biology, are urgently needed. Since it is well known that epigenetic changes, namely miRNA dysregulation, are implicated in PCa development and progression, miRNAs might be a promising therapeutic target for this disease. Therefore, the main objective of this Thesis was to define the usefulness of enoxacin, an antibacterial compound which promotes miRNA biogenesis in a TRBP-mediated manner, as an anti-tumoral agent, especially in the inhibition of the cell growth and in the restoration of normal miRNA levels in PCa cells.

Specifically, the aims of this study were:

- ✓ Analyze the mutational status of *TARBP2* and the protein levels of TRBP in PCa cell lines.
- ✓ Assess the expression levels of TRBP in primary tumor tissues to evaluate the clinical usefulness of enoxacin in PCa treatment.
- ✓ Evaluate the phenotypic effects of enoxacin on PCa cell lines.
- ✓ Test the effect of enoxacin on the expression of miRNAs involved in prostate carcinogenesis.

MATERIAL AND METHODS

1. Cell Culture

In this study, five cell lines derived from human metastatic PCa were selected: LNCaP, 22Rv1, VCaP, DU145, and PC-3. These PCa cell lines are representative of different relevant features of prostatic adenocarcinoma: LNCaP, 22Rv1 and VCaP are hormone-sensitive cell lines, whilst DU145 and PC-3 are castration-resistant cell lines. DU145 was obtained from the American Type Culture Collection (ATCC, Lockville, MD, USA), whereas LNCaP, VCaP and PC-3 were kindly provided by Prof. Ragnhild A. Lothe from the Department of Cancer Prevention at The Institute for Cancer Research, Oslo, Norway, and 22Rv1 by Dr. David Sidransky from the Johns Hopkins University School of Medicine, Baltimore, MD, USA. For control purposes, we used the human colon carcinoma-derived cell line Co115 and the human thyroid papillary carcinoma-derived cell line TPC-1. Co115 was kindly provided by Prof. Fátima Baltazar from the Life and Health Sciences Research Institute at the University of Minho, Braga, Portugal and TPC-1 by Prof. Paula Soares from the Institute of Molecular Pathology and Immunology of the University of Porto, Porto, Portugal.

All cell lines were cultured in the recommended medium (Table 2), supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO, Invitrogen, Carlsbad, CA, USA) and 1% Penicillin-Streptomycin (GIBCO). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cell lines were subcultured, using the dissociation reagent TrypLE™ Express (GIBCO) to harvest them as many times as necessary in order to obtain the desired number of 75 cm^3 cell culture flasks.

All PCa cell lines were routinely tested for Mycoplasma spp. contamination.

Table 2. Characteristics of cultured cell lines.

Cell Line	Cancer Type	Growth Medium
LNCaP	Prostatic Carcinoma	RPMI-1640
22Rv1	Prostatic Carcinoma	RPMI-1640
VCaP	Prostatic Carcinoma	DMEM
DU145	Prostatic Carcinoma	MEM
PC-3	Prostatic Carcinoma	F-12 Nutrient Mixture (Ham) + RPMI-1640
Co115	Colon Carcinoma	RPMI-1640
TPC-1	Thyroid Papillary Carcinoma	RPMI-1640

2. *TARBP2* Analysis

Since the mechanism of action of enoxacin is TRBP-dependent, PCa cell lines were screened for *TARBP2* mutations and the mutational status was confirmed through the evaluation of TRBP protein levels. As control, we used the colon carcinoma cell line Co115. We also interrogated primary PCa cases using an immunohistochemistry assay for TRBP.

2.1. Mutational Status Evaluation of PCa Cell Lines

2.1.1. DNA Extraction

DNA from cell lines was extracted by the phenol-chloroform conventional method as previously described [132]. Briefly, the samples were digested by adding 500 µL of buffer solution SE (75 mM NaCl; 25 mM EDTA), 30 µL of 10% sodium dodecyl sulfate (SDS) and 15 µL of proteinase K (20 mg/mL) (Sigma-Aldrich, Schnelldorf, Germany) to each sample, which were then incubated for 2 to 3 days in a bath at 55ºC until total digestion. Proteinase K was added twice a day during this period. After digestion, DNA extraction was performed with 500 µL of phenol-chloroform solution at pH 8 (Sigma-Aldrich; Merck, Darmstadt, Germany) in Phase Lock Gel™ Light tubes (5 PRIME, Hamburg, Germany). After centrifuging the samples for 15 minutes at 13,000 rpm, the upper aqueous phase containing DNA was transferred to a new 1.5 mL-tube. Then, 2

volumes (of original amount of this phase) of 100% cold ethanol (approximately 1,000 µL), 1/3 volume of 7.5 M ammonium acetate (approximately 165 µL) and 2 µL of glycogen (5 mg/mL) (Sigma-Aldrich) were added to each sample. Samples were incubated overnight at -20ºC to precipitate DNA, subsequently centrifuged for 20 minutes at 13,000 rpm and washed twice with 70% ethanol. Pellets were air dried and eluted in sterile bidistilled water (B. Braun, Melsungen, Germany). DNA concentration and purity were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA was stored at -20ºC until further use.

2.1.2. Direct Sequencing

Mutational evaluation of *TARBP2* in PCa cell lines was performed by direct sequencing. We used the primers previously described in Melo *et al.* [91], which were used to screen several cancer cell lines for the presence of mutations in all the exonic mononucleotide repeats present in the coding sequences of *TARBP2* [91]. The location of the corresponding repeats, the PCR primers and the amplicon lengths are shown in Table 3. Primers were provided by Eurofins MWG Operon (Ebersberg, Germany).

Table 3. Sequences of the primers used in sequencing analysis.

Repeat	Repeat location	Primers	Amplicon size (bp)
6G	Exon 3	F:5'TTTCCCGTCCTTTCAGTGAC3' R:5'GGTTCCTTGAAGGGCTTCTG3'	196
7C and 5C	Exon 5	F:5'CGGGAGATGGTAGTCAGGAA3' R:5'AAATGAGGATGGGACACACC3'	199
6A	Exon 7	F:5'ATAACCCAGCAGCCCTCTCT3' R:5'GTCATCATCAGGCTCCACCT3'	146

F – forward; **R** – reverse; **bp** – base pairs

PCR was performed using 2 µL of template DNA, 0.2 mM of dNTPs mix (Fermentas, Ontario, Canada), 250 nM of each primer (forward and reverse), 2.5 µL of 10x *Taq* Buffer with $(NH_4)_2SO_4$ (Fermentas), 0.2 µL of *Taq* DNA Polymerase (5U/µL) (Fermentas) and sterile bidistilled water (B. Braun) in a total reaction volume of 25 µL. PCR amplifications were performed as follows: a 10-minute 95ºC incubation step followed by 35 cycles of 95ºC for 30 seconds, 55ºC (annealing temperature) for 30 seconds, and 72ºC for 30 seconds. A 10-minute elongation step at 72ºC completed the PCR amplification program. The efficiency of PCR reaction was assessed by loading 3 µL of the PCR product onto nondenaturing 2% agarose gel, stained with ethidium bromide, and visualized under an ultraviolet transilluminator. To remove excess primers and dNTPs

from this PCR, its product was submitted to Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacture's protocol. Briefly, 500 µL of Capture Buffer type 3 were added to each GFX MicroSpin column previously placed in a Collection Tube. The PCR product was then transferred into the column and mixed with Capture Buffer type 3 by pipetting. The assembled column and Collection tube were centrifuged at 12,000 rpm for 1 minute. After discarding the flow-through, 500 µL of Wash Buffer type 1 were added to the column and a new centrifugation was performed. The column was transferred into a new 1.5 mL-tube and the PCR product was eluted by incubation with 30 µL of sterile bidistilled water (B. Braun) for 5 minutes at room temperature. After centrifuging the tubes at 12,000 rpm for 5 minutes, 3 µL of purified PCR product were then run in a 2% agarose gel stained with ethidium bromide to determine which volume of product (1 to 3 µL) should be used in sequencing reaction.

The sequencing reaction was performed using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The reaction was prepared using 350 nM of forward or reverse primers, 1 µL of BigDye® Terminator v1.1 Ready Reaction Mix (Applied Biosystems), 1.9 µL of BigDye® Terminator v1.1 Sequencing Buffer, 1 to 3 µL of purified PCR product, in a total volume of 10 µL completed with sterile bidistilled water (B. Braun). The reaction was performed according to the following conditions: 96ºC for 2 minutes and 30 cycles of 96ºC for 15 seconds, 50ºC for 15 seconds and 60ºC for 4 minutes.

Sequencing reaction products were purified prior to sequencing to remove free fluorescent ddNTPs using the Illustra Sephadex™ G-50 fine (GE Healthcare). After purification, 12 µL of Hi-Di™ Formamide (Applied Biosystems) were added to the purified sequencing reaction product and the mixture was then run on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The electropherograms were analyzed using Sequencing Analysis Software v5.2 (Applied Biosystems). All electropherograms were read manually.

2.2. Assessment of Protein Levels of PCa Cell Lines

2.2.1. Protein Extraction

Protein extraction from whole-cell lysates was obtained using the Radio Immuno Precipitation Assay (RIPA) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). In brief, the growth medium was removed from the 75 cm^3 cell culture flask by aspiration and the cells were washed with 1x phosphate-buffered saline (PBS) to remove residual medium. Then, an appropriate volume of complete RIPA buffer (10 µL of PMSF solution, 10 µL of sodium orthovanadate solution and 20 µL of protease inhibitor cocktail solution

per mL of 1x RIPA Lysis Buffer), depending on cellular confluence, was added and the flasks were quickly scrapped to promote cell lyses and removal. The cell lysate was then transferred into a 1.5 mL-tube and incubated for 15 minutes on ice. After incubation, the samples were centrifuged for 30 minutes at 13,000 rpm at 4ºC and supernatant was collected into a new tube.

The protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific Inc., Bremen, Germany). This assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) used for the colorimetric detection and quantitation of total protein. The purple-colored reaction product of this assay exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range. Protein concentrations were determined with reference to standards of bovine serum albumin (BSA). A series of dilutions of known concentration were prepared from the protein and assayed alongside with the samples with unknown concentration. Briefly, 25 µL of each standard or sample and 200 µL of the working reagent (50:1, Reagent A:B) were added to a 1.5 mL-tube and subsequently incubated at 37°C for 30 minutes. The content of the tubes was then transferred into a 96-well plate (Ratiolab, Dreieich, Germany) and the absorbance was measured at 562 nm on a microplate reader (Fluostar Omega, BMG Labtech, Offenburg, Germany). Proteins were stored at -20ºC until further use.

2.2.2. Western Blot

Protein expression of TRBP was evaluated by Western Blot using a specific antibody. Briefly, 30 µg of protein from each cell line were resuspended in loading buffer and denatured at 95ºC for 5 minutes. Proteins were separated by SDS-PAGE on 10% polyacrylamide gels at 120 V at room temperature, and subsequently blotted onto Protran nitrocellulose transfer membranes (Whatman, Dassel, Germany) at 50 V for 1 hour at 4ºC. After electroblotting, the membranes were incubated in blocking buffer (5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 – TBST) for 1 hour at room temperature with agitation. The membranes were then incubated overnight at 4ºC with the primary polyclonal rabbit antibody for TRBP (Abcam, Cambridge, UK) diluted 1:1,000 in blocking buffer. After three washing steps with TBST, the membranes were incubated for 1 hour at room temperature with a horseradish peroxidase conjugated secondary anti-rabbit antibody (Bio-Rad, München, Germany) diluted 1:3,000 in blocking buffer. After washing with TBST, the membranes were developed using Immun-Star WesternC Chemiluminescent Kit (Bio-Rad) and exposed to Amersham Hyperfilm (GE Healthcare).

To ascertain equal loading of protein, the membranes were stripped and reprobed with an antibody against the loading control (β-Actin). The membranes were incubated for 45 minutes in a bath at 55ºC in a solution containing 8 mL of 10% SDS, 5 mL of 0.5 M Tris-HCl pH 6.8 (Bio-Rad) and 320 µL of β-mercaptoethanol (Sigma-Aldrich) in a total volume of 40 mL completed with sterile bidistilled water (B. Braun). The membranes were then washed under running water for 2 hours and subsequently blocked for 30 minutes. After blocking, they were incubated with a monoclonal mouse antibody against β-Actin (Sigma-Aldrich) diluted 1:8,000 in blocking buffer for 30 minutes at room temperature. After washing, the membranes were incubated for 15 minutes at room temperature with a horseradish peroxidase conjugated secondary anti-mouse antibody (Bio-Rad) diluted 1:3,000 in blocking buffer.

To relate the protein band intensity with the loading control, protein band intensities were determined using Quantity One software (Bio-Rad).

2.3. Assessment of Protein Levels of PCa Tumor Samples

2.3.1. Patients and Sample Collection

Fifty prostate tumor samples from patients with clinically localized PCa (clinical stage II: T1cN0M0 or T2N0M0, according to the TNM staging system) consecutively diagnosed and primarily treated with radical prostatectomy at the Portuguese Oncology Institute, Porto, Portugal, were prospectively collected from 2001 to 2006. Tumor tissue was routinely fixed in buffered formalin and paraffin-embedded. All patients were enrolled after informed consent. This study was approved by the Institutional Review Board (Comissão de Ética) of Portuguese Oncology Institute, Porto, Portugal.

2.3.2. Immunohistochemistry

TRBP expression in tumor tissue samples was assessed by immunohistochemistry using the Novolink™Polymer Detection System (Novocastra, Newcastle, UK). Sections 4 µm thick from the representative paraffin-embedded samples were placed in StarFrost® adhesive slides (Knittel-Gläser, Braunschweig, Germany). The slides were deparaffinized in xylene and rehydrated with a graded ethanol series (100%, 90%, and 70% ethanol solutions). After washing the slides in running water, antigen retrieval was accomplished by microwaving the specimens at 700 W for 20 minutes in a 1x EDTA buffer solution. At the end of this process, slides were cooled for 10 minutes in running water. Endogenous

peroxidase activity was blocked by incubating the slides in a 0.6% H_2O_2 solution for 20 minutes. After washing in distilled water and TBST, the slides were incubated with Protein Block (Novocastra) for 5 minutes. The slides were washed twice for 3 minutes and the primary rabbit polyclonal antibody against TRBP (Abcam) was applied in a 1:100 dilution for 1 hour in a humid chamber, at room temperature. The slides were then rinsed in TBST and incubated with Post Primary Block (Novocastra) for 30 minutes followed by incubation for 30 minutes with the Novolink™ Polymer (Novocastra). After washing, the slides were incubated for 7 minutes with 3,3-diaminobenzidine (DAB; Sigma-Aldrich) dissolved in 50 mL of PBS and activated by 0.05% H_2O_2. After rinsing the slides in water, they were counterstained with hematoxylin (Harris Modified Hematoxylin Stain; Fisher Scientific, Fair Lawn, NJ, USA) for 20 seconds and washed in tap water for 5 minutes. Finally, the slides were mounted with Entellan® (Merck) after dehydration with increasing ethanol concentrations (70%, 90%, and 100%) and diaphanization with xylene. Colorectal cancer tissues showing intense immunoreactivity for TRBP protein, was used as positive control. The negative control consisted on the omission of the primary antibody.

The assessment of immunostaining results was performed by an experienced pathologist and was expressed in a semiquantitative way according to the estimated percentage of positive tumor cells. Immunostaining of more than 10% of the tumor cells was required for scoring a case as positive.

3. Evaluation of the Effect of Enoxacin on PCa Cell Lines

3.1. Enoxacin Exposure

The stock solution of enoxacin (Sigma-Aldrich) was prepared at a concentration of 8 mg/mL in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at -80ºC until further use. The five selected PCa cell lines were exposed to enoxacin diluted in the respective culture medium at a final concentration of 40 µg/mL (124 µM), for five days. For control purposes, cell lines were exposed to the vehicle of the drug (DMSO). Enoxacin or DMSO diluted in culture medium was renewed every day. Three independent experiments were performed for each condition.

3.2. Cell Viability Assay

The effect of enoxacin on the viability of PCa cell lines was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay. The principle of the MTT assay is that for most viable cells mitochondrial activity is constant and thereby an increase or decrease in the number of viable cells is linearly related to mitochondrial activity. So, the mitochondrial activity of the cells is reflected by the conversion of the tetrazolium salt MTT into purple formazan crystals (Figure 8) [133, 134].

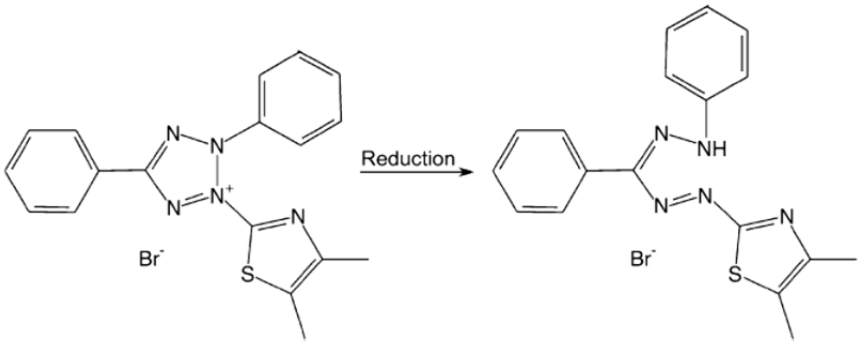

Figure 8. Reduction of MTT: the chemical basis of the cell viability assay. *Adapted from [134].*

PCa cells were seeded onto 96-well plates (Sarstedt, Numbrecht, Germany) at 2 x 10^3 cells per well in 200 µL of complete medium and incubated in a humidified chamber (37ºC, 5% CO_2). Cells were allowed to adhere overnight and then exposed to 40 µg/mL of enoxacin or DMSO for 5 days with fresh media being added every 24 hours. Subsequently, cell viability was measured by MTT assay at days 1, 2, 3, 4, and 5. Briefly, 20 µL of 5 mg/mL MTT (Sigma-Aldrich) diluted in culture medium at a final concentration of 0.5 mg/mL were added to each well and incubated in a humidified chamber (37ºC, 5% CO_2) for 3 hours. After removal of MTT solution, the formazan crystals formed in viable cells were solubilized in 100 µL of DMSO (Sigma-Aldrich) and plates were subjected to gentle shaking for 10 minutes to achieve complete dissolution.

The absorbance was measured using a microplate reader (Fluostar Omega) at a wavelength of 540 nm with background subtraction at 630 nm. Three independent experiments were performed, using triplicates for each experiment. Nine blanks (DMSO) were included in each plate and all optical density (OD) values were blank corrected. The number of cells was calculated using the formula: [(OD experiment x Number of cells at day 0) / Mean OD at day 0].

3.3. Apoptosis Assay

Apoptosis evaluation was performed using the APOPercentage Assay Kit (Biocolor Ltd., Belfast, Northern Ireland). This assay is based on phosphatidylserine transmembrane movements which result in the uptake of the APOPercentage dye by apoptosis-commited cells (Figure 9).

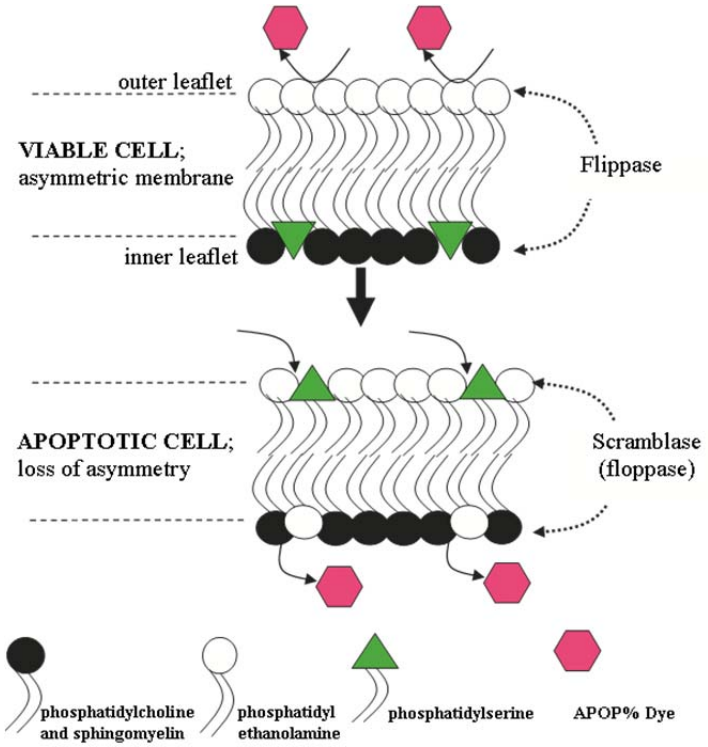

Figure 9. Apoptotic cells staining using APOPercentage Assay Kit. *Adapted from www.biocolor.co.uk.*

Cells were seeded onto 24-well plates (Sarstedt) at 5 x 10^4 cells per well in 1,000 µL of complete medium and incubated in a humidified chamber (37ºC, 5% CO_2). Cells were allowed to adhere overnight and then exposed to 40 µg/mL of enoxacin or DMSO, continuously for 5 days. Subsequently, apoptosis levels were assessed at days 2 and 5. Briefly, 300 µL of culture medium containing 5% APOPercentage dye were added to each well and the cells were incubated at 37ºC and 5% CO_2 for approximately 20 minutes. After medium removal, cells were gently washed twice with 1x PBS to remove non-cell bound dye and 50 µL of dissociation reagent TrypLE™ Express (GIBCO) were added. After incubation in a humidified chamber (37ºC, 5% CO_2) for about 15 minutes, 200 µL of APOPercentage dye releasing reagent, an alkali solution used to disrupt the cell membrane and release intracellular accumulated dye, were added to each well. Plates

were then subjected to shaking for 10 minutes and the content of each well was transferred into a 96 well-plate (Ratiolab).

The absorbance was determined using a microplate reader (Fluostar Omega) at a wavelength of 550 nm with background subtraction at 620 nm. Three independent experiments were carried out in duplicates. Hydrogen peroxide (5 mM) was used as positive control for apoptosis. Six blanks (APOPercentage dye releasing reagent) were included in each plate and all ODs were blank corrected. To normalize the OD measured in the apoptosis test, according to the cell number, the OD of the apoptosis assay was divided by the OD of the cell viability assay. The results were expressed as the ratio of the OD of the cells exposed to enoxacin to that of vehicle cells (set as 1).

3.4. Assessment of *CASP3* mRNA Expression

To evaluate caspase 3, apoptosis-related cysteine peptidase (*CASP3*) mRNA expression, PCa cell lines were seeded into 75 cm^3 cell culture flasks in 12 mL of culture medium. Cells were allowed to attach overnight before medium was replaced with 40 µg/mL of enoxacin or DMSO diluted in culture medium. After 5 days of exposure, cells were harvested by trypsinization and washed in 1x PBS to allow for RNA collection.

3.4.1. RNA Extraction

Total RNA was extracted from cell lines pellets using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, cell pellets were thawed on ice and resuspended in 1 mL of TRIzol® Reagent using a syringe with a 0.9 mm needle. The homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. After incubation, all content of the tubes was transferred into 1.5 mL RNAse-free tubes and 200 µL of chloroform (Merck) were added. Samples were then vortexed for 15 seconds and incubated for 3 minutes at room temperature followed by a centrifugation at 10,600 rpm for 15 minutes at 4ºC. After centrifugation, the mixture consisted of a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase, so it was collected into a fresh 1.5 mL RNAse-free tube placed on ice. Then, 500 µL of 100% isopropanol were added to the aqueous phase, the tubes were vigorously inverted by hand and incubated at room temperature for 10 minutes to precipitate RNA. Samples were centrifuged at 10,600 rpm for 10 minutes at 4ºC and the supernatant was removed from the tube, leaving only the RNA pellet. To wash the

pellets, 1 mL of 75% ethanol were added and the samples were vortexed and centrifuged at 8,400 rpm for 5 minutes at 4ºC. This last step was performed twice. Supernatant was discarded and the RNA pellets were air dried for 15 to 20 minutes. RNA pellets were eluted in a variable volume of RNA Storage Solution (1mM Sodium Citrate, pH 6.4) (Ambion, Heidelberg, Germany) according to pellet size and placed on ice for at least 30 minutes before evaluation of RNA concentration and quality using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Eluted samples were stored at -80ºC until further use.

3.4.2. cDNA Synthesis

cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. The following components were added to an RNAse-free PCR tube on ice: 1 µg of template RNA, 2 µL of 10x RT Buffer, 0.8 µL of 25x dNTP Mix (100 mM), 2 µL of 10x RT Random Primers, 1 µL of RNAse Inhibitor, 1 µL of MultiScribe™ Reverse Transcriptase, and the appropriate volume of DEPC-treated water (deionized, nuclease-free water) (MP Biomedicals LLC, Solon, OH, USA) to complete a total volume of 20 µL. All the components were gently mixed and reverse transcription reaction was performed in Veriti® Thermal Cycler (Applied Biosystems) during the following incubation: 25ºC for 10 minutes, 37ºC for 120 minutes and 85ºC for 5 minutes. The newly synthesized cDNA samples were diluted in 190 µL of DEPC-treated water (MP Biomedicals). All this procedure was also applied to Stratagene® QPCR Human Reference Total RNA (containing a mixture of RNA from ten different cell lines) (Stratagene, La Jolla, CA, USA) that was used as control for the Gene Expression Assays described below. In this case, cDNA was diluted in 100 µL of DEPC-treated water (MP Biomedicals). All samples were stored at -20ºC.

3.4.3. Quantitative Reverse Transcription PCR

cDNA from all cell lines was used as template for quantitative reverse transcription PCR (qRT-PCR) reaction to quantify the *CASP3* transcript. The assay was performed using the *CASP3* gene expression assay (Hs00234387_m1 from Applied Biosystems) and the endogenous control assay *GUSB* (Hs99999908_m1 from Applied Biosystems).

The qRT-PCR reaction is based on the Taqman® technology (Applied Biosystems). A TaqMan® MGB probe contains a reporter dye linked to the 5'-end and a minor groove binder (MGB) at the 3'-end. Besides MGB, there is also a non-fluorescent quencher at the 3'-end of the probe. During PCR, the TaqMan® MGB probe anneals specifically to a

complementary sequence between the forward and reverse primer sites. The DNA polymerase cleaves only probes that are hybridized to the target separating the reporter dye from the quencher dye and the separation results in increased fluorescence by the reporter (Figure 10). This fluorescence is read by Real-Time PCR System and it is proportional to the amount of PCR product.

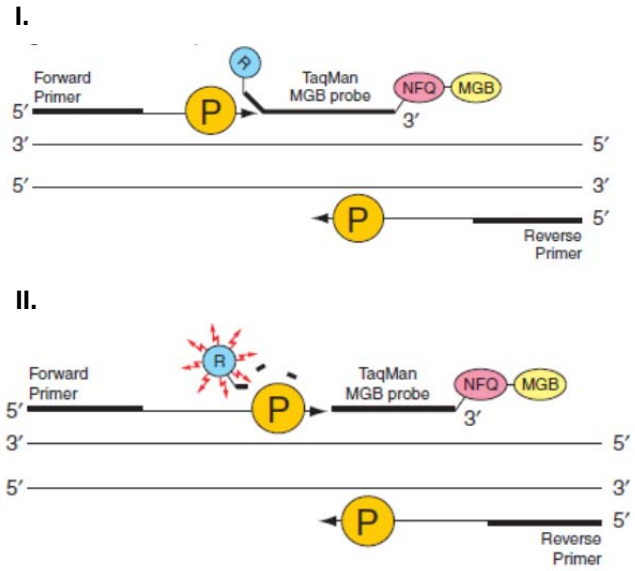

Figure 10. TaqMan® technology chemistry. MGB – minor groove binder; NFQ – non-fluorescent quencher; R – reporter; P – DNA polymerase. *Adapted from TaqMan® Gene Expression Assay Protocol provided by Applied Biosystems.*

Briefly, in each well 9 µL of previously synthesized cDNA, 1 µL of Taqman® Gene Expression Assay and 10 µL of Taqman® Gene Expression Master Mix (Applied Biosystems) were added. The reactions were performed in 96-well plates in a 7500 Real-Time PCR System (Applied Biosystems) using the conditions predefined by the manufacturer: 50ºC for 2 minutes, 95ºC for 10 minutes, 45 cycles at 95ºC for 15 seconds and 60ºC for 1 minute. All samples were run in triplicate and two negative controls were included in each plate. The standard curve method was used for quantitation. cDNA synthesized from Stratagene® QPCR Human Reference total RNA (Agilent Technologies) was used to prepare five consecutive cDNA dilutions (dilution factor of 10x) used as standards on each plate.

The mean quantity of *CASP3* expression levels of cell lines samples was normalized against mean quantity of the respective endogenous control (*GUSB*) expression levels [*CASP3* Expression Level = (*CASP3* Mean Quantity / *GUSB* Mean Quantity)].

3.5. Cell Cycle Analysis

Cell cycle distribution was determined by flow cytometry. After 5 days of exposure to 40 µg/mL of enoxacin or DMSO in 75 cm^3 cell culture flasks, cells were harvested and counted with a hemocytometer using trypan blue solution (Sigma-Aldrich) under a bright-field microscope (Nikon, Düsseldorf, Germany). Approximately 5 x 10^5 cells were collected into a 15 mL-tube and centrifuged at 2,000 rpm for 10 minutes. To wash the pellets, 1 mL of 1x PBS were added and the tubes were vigorously inverted by hand and centrifuged at 2,000 rpm for 10 minutes. Cells were fixed in 4 mL of 70% cold ethanol overnight at 4ºC. After washing with cold 1x PBS, cells were stained with 500 µL of Propidium Iodide (Cytognos S.L., Salamanca, Spain) and incubated at room temperature for 30 minutes in the dark. Cell cycle data were collected using Cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) and analyzed with Modfit LT (Verity Software House Inc., Topsham, Maine, USA). Data were obtained from three independent experiments.

3.6. Cell Invasion Assay

The Oris™ Cell Invasion Assay (Platypus Technologies, Madison, WI, USA) was used to investigate the impact of enoxacin on LNCaP and DU145 cell invasion within a 3-dimensional extracellular matrix comprised of a basement membrane extract (BME) of the murine Engelbreth-Holm-Swarm tumor. A 96-well plate (Platypus Technologies) was coated with BME solution at 3.5 mg/mL, allowed to dry for 30 minutes in a humidified chamber (37ºC, 5% CO_2), and a silicone stopper covering the central area of the wells was inserted to create a cell-free gap. After starvation for 18 hours in a serum-free medium, cells were seeded at 5 x 10^4 cells per well and allowed to attach overnight. After the removal of stoppers, cells were overlaid with a BME solution at 12 mg/mL to form the 3-dimensional matrix and the plate was incubated in a humidified chamber (37ºC, 5% CO_2) for 30 minutes. Cells were exposed to 40 µg/mL of enoxacin or DMSO for 8 days. At this time point, cells were labeled with Calcein AM (AnaSpec, Fremont, CA, USA) diluted in 1x PBS at a final concentration of 0.5 µg/mL.

A detection mask was attached to the bottom of the plate to block from view all cells except those that had invaded into the center zone and fluorescence was measured at excitation and emission wavelengths of 492 nm and 530 nm, respectively, using a microplate reader (Fluostar Omega). Three independent experiments were performed in triplicates. The results were expressed as the ratio of the fluorescence index (FI) of the

cells exposed to enoxacin to that of vehicle cells (set as 1). The thyroid cancer-derived cell line TPC-1 was used as positive control for cell invasion [135].

3.7. Assessment of miRNA Expression by Microarray

The impact of exposure to enoxacin on the expression profile of miRNAs was evaluated in LNCaP and DU145 after 5 days of exposure to the drug or vehicle in 75 cm^3 cell culture flasks.

3.7.1. RNA Extraction and cDNA Synthesis

Total RNA was extracted from cell lines pellets using TRIzol® Reagent (Invitrogen) as described above. cDNA synthesis was performed using the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions. Each of the template RNA samples were adjusted to a concentration of 5 ng/µL using nuclease-free water (Exiqon). The following components were added to an RNAse-free PCR tube on ice: 27 µL of nuclease-free water, 12 µL of 5x Reaction Buffer, 3 µL of Synthetic spike in, 6 µL of Enzyme Mix and 12 µL of template RNA. All the components were mixed by gentle vortexing and reverse transcription reaction was performed in Veriti® Thermal Cycler (Applied Biosystems) during the following incubation: 42ºC for 60 minutes and 95ºC for 5 minutes. All samples were stored at -20ºC.

3.7.2. Quantitative Reverse Transcription PCR

Quantitative reverse transcription PCR was performed using the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon). This system is a miRNA-specific, LNA™-based system designed for sensitive and accurate detection of miRNA by quantitative Real-Time PCR using SYBR® Green (Figure 11).

The reactions were performed using the Ready-to-use microRNA PCR Human Panels (I + II) which consist of 384-well PCR plates containing dried down LNA™ primer sets and enable the quantification of 739 human miRNAs. In brief, 0.05 µL of previously synthesized cDNA, 5 µL of SYBR® Green master mix and 4.95 µL of nuclease-free water (Exiqon) were added to each well. The reactions were carried out in a Roche LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) using the conditions predefined

by the manufacturer: 95ºC for 10 minutes, 45 cycles at 95ºC for 10 seconds and 60ºC for 1 minute. Three independent experiments were carried out.

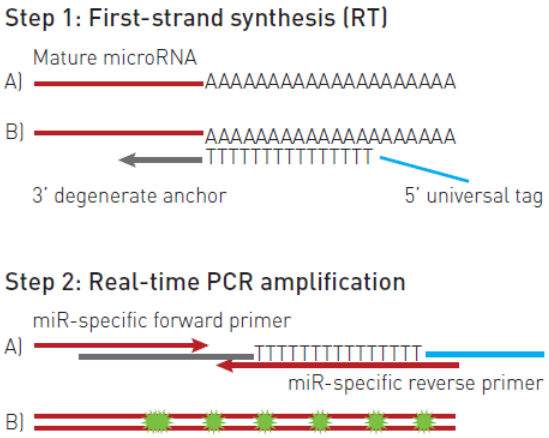

Figure 11. Schematic outline of the miRCURY LNA™ Universal RT microRNA PCR System. *Adapted from miRCURY LNA™ Universal RT microRNA PCR Instruction Manual provided by Exiqon.*

3.7.3. Microarray Analysis

Microarray data were imported into GenEX software (MultiD Analyses AB, Göteborg, Sweden) for subsequent analysis. It was applied a confidence level of 0.95 and a cutoff standard deviation (SD) of 0.25 after which all outliers were deleted. Since the mean expression value of all expressed miRNAs has been considered the ideal method of normalization in large scale miRNA expression profiling studies, global mean normalization was used as a normalization factor. [136]. The relative expression of miRNAs was determined with the $2^{-\Delta C_T}$ method and the values were then converted to logarithmic scale. MiRNAs with fold change above 1.5 or below -1.5 were considered up or downregulated, respectively.

3.8. Protein Expression Evaluation of HDAC1

To analyze protein levels of HDAC1, a direct target of one of the altered miRNAs (miR-449a), PCa cell lines were seeded into 75 cm^3 cell culture flasks and exposed to 40 µg/mL of enoxacin or DMSO alone. At day 5, cells were harvested and washed in 1x PBS to allow for the collection of protein.

3.8.1. Protein Extraction

Protein from cell lines was extracted using RIPA lysis buffer and respective quantification was performed according to the methods described above.

3.8.2. Western Blot

Protein expression of HDAC1 was evaluated by Western Blot as same as described for TRBP. Herein, the membranes were incubated overnight at 4ºC with the primary monoclonal mouse antibody against HDAC1 (Sigma-Aldrich) diluted 1:1,000 in blocking buffer.

4. Statistical analysis

Two-tailed Student's t-test was used to assess differences between the results obtained after exposure to enoxacin and after exposure to vehicle only. Prior to application of the test, all data were transformed to logarithmic scale.

Analysis was performed with the aid of SPSS software for Windows, version 20.0 (IBM-SPSS Inc., Chicago, IL, USA), and the statistical significance level was set at $P<0.05$. Graphics were built using GraphPad Prism 5.0 software.

RESULTS

1. Mutational and Expression Status of TRBP in PCa Cell Lines

In view of the fact that cell lines harboring *TARBP2* mutations are less responsive to enoxacin [91, 106], *TARBP2* mutational status of five PCa cell lines (LNCaP, 22Rv1, VCaP, DU145 and PC-3) was assessed by direct sequencing. Co115, a *TARBP2*-mutant colon carcinoma-derived cell line [91, 106], was used as positive control (Figure 12). No *TARBP2* mutations were found in any of the tested PCa cell lines.

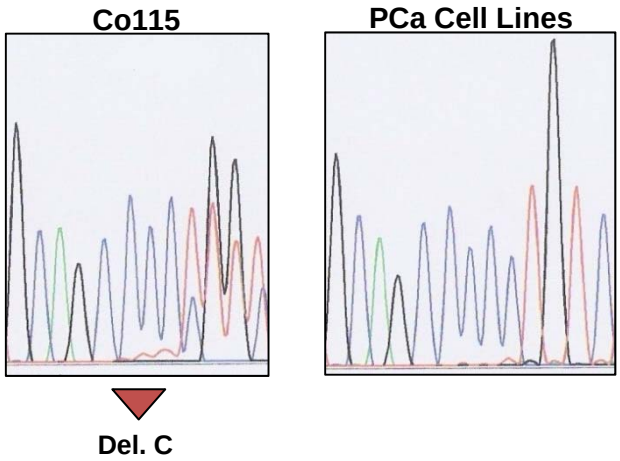

Figure 12. Electropherograms of *TARBP2* mutant (Co115) and wild-type (PCa cell lines) cells. The different colors of the peaks represent the different nucleotides: black – guanine; green – adenine; red – thymine; blue – cytosine.

Subsequently, we analyzed TRBP protein expression in PCa cell lines by Western Blot. As expected, all PCa cell lines expressed higher protein levels of TRBP than Co115 cells, which display very low expression levels (Figure 13).

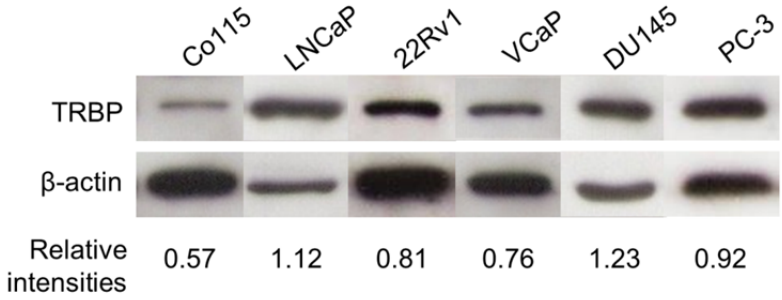

Figure 13. TRBP protein expression analysis by Western Blot in PCa cell lines. The picture is representative of three independent experiments. Mean quantitation values are shown.

2. TRBP Expression in PCa Tumor Samples

To investigate the putative clinical usefulness of enoxacin for PCa therapy, TRBP expression was evaluated in a series of 50 primary PCa tumors by immunohistochemistry. No differences in immunoreactivity for TRBP were apparent between normal and tumoral prostatic tissue (Figure 14).

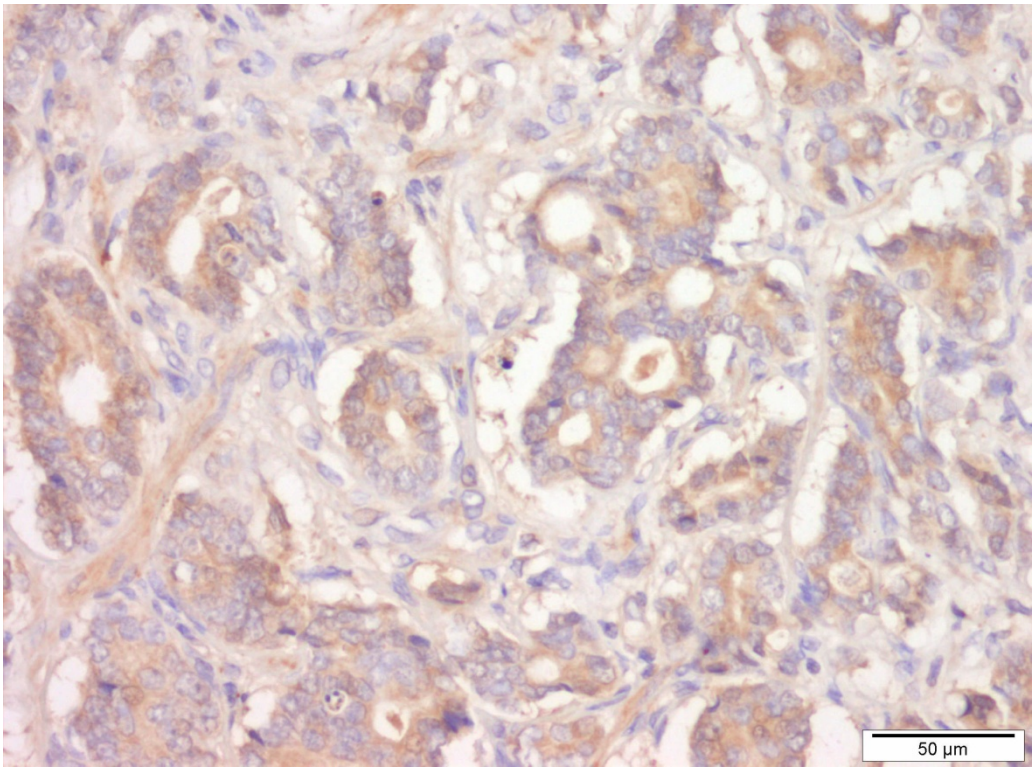

Figure 14. Immunohistochemical stain for TRBP protein expression in glands of prostate adenocarcinoma.

3. Anti-tumoral Effect of Enoxacin on PCa Cell Lines

3.1. Cell Viability

To evaluate the effects of enoxacin on cell viability, five human PCa cell lines were continuously exposed for five days to enoxacin (40 µg/mL). A significant decrease in the number of viable cells was observed after exposure to the drug when compared to the vehicle, DMSO (Figure 15). For LNCaP and 22Rv1 cell lines, the effect was observed from day 1, whereas a significant decrease in the number of viable cells in VCaP, DU145 and PC-3 was found after 2 days of drug exposure.

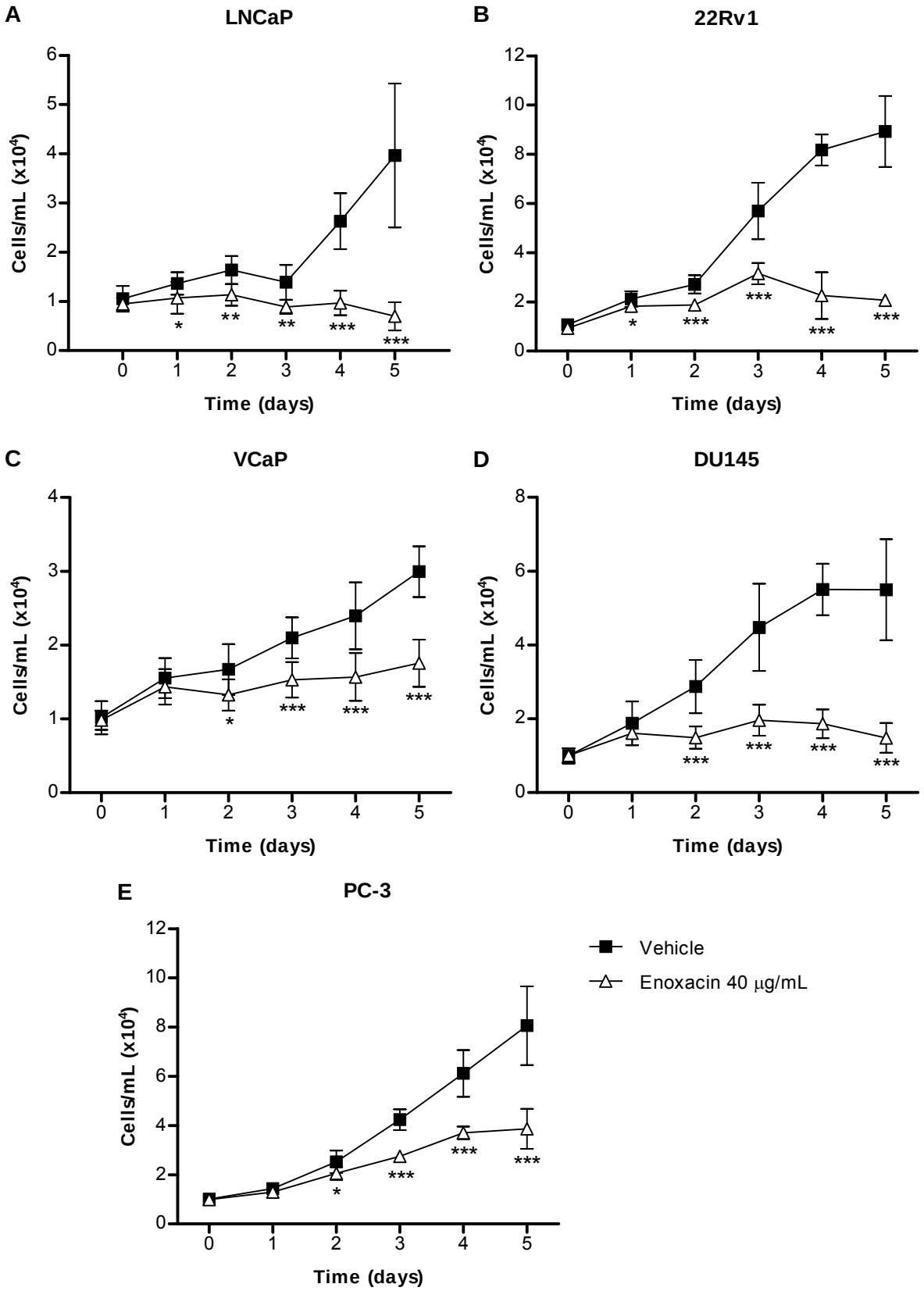

Figure 15. Cell viability evaluations by MTT assay in LNCaP (A), 22Rv1 (B), VCaP (C), DU145 (D), and PC-3 (E) cell lines. The number of cells/mL is shown as mean of three independent experiments performed in triplicates ± s. d. * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$, compared to vehicle group.

A reduction in the percentage of viable cells was observed for all PCa cell lines, ranging between 17 and 59% at day 5, with LNCaP being the most responsive cell line (Figure 16).

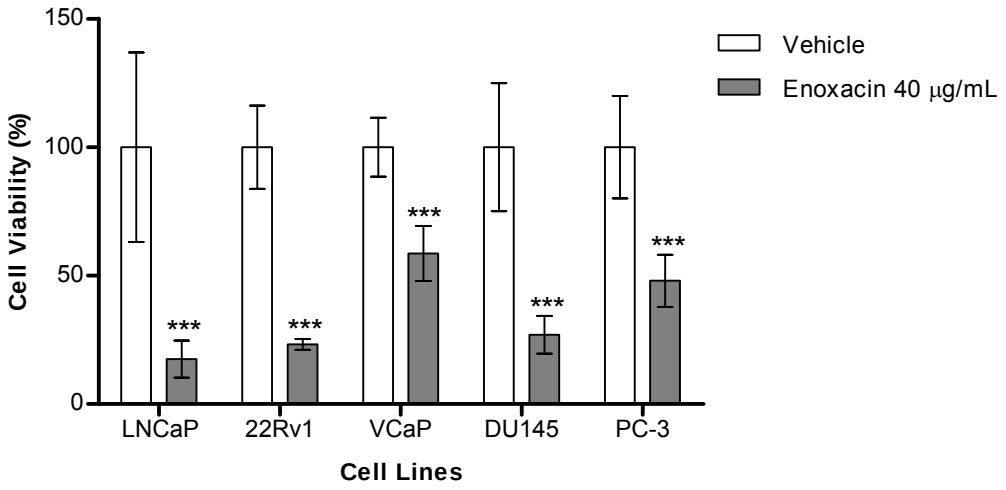

Figure 16. Effect of enoxacin on PCa cell viability measured by MTT assay after 5 days of exposure. Data are presented as mean of three independent experiments performed in triplicates ± s. d. *** $P < 0.001$, compared to vehicle group.

3.2. Apoptosis

To determine whether enoxacin was capable of inducing significant cell death, an apoptosis assay was performed. Indeed, a significant increase in apoptosis was apparent in all tested cell lines at days 2 and 5 (Figure 17), which was also evident when examining stained apoptotic cells (Figure 18). After 5 days of exposure to enoxacin, LNCaP and DU145 displayed the highest levels of apoptotic cells (Figure 17).

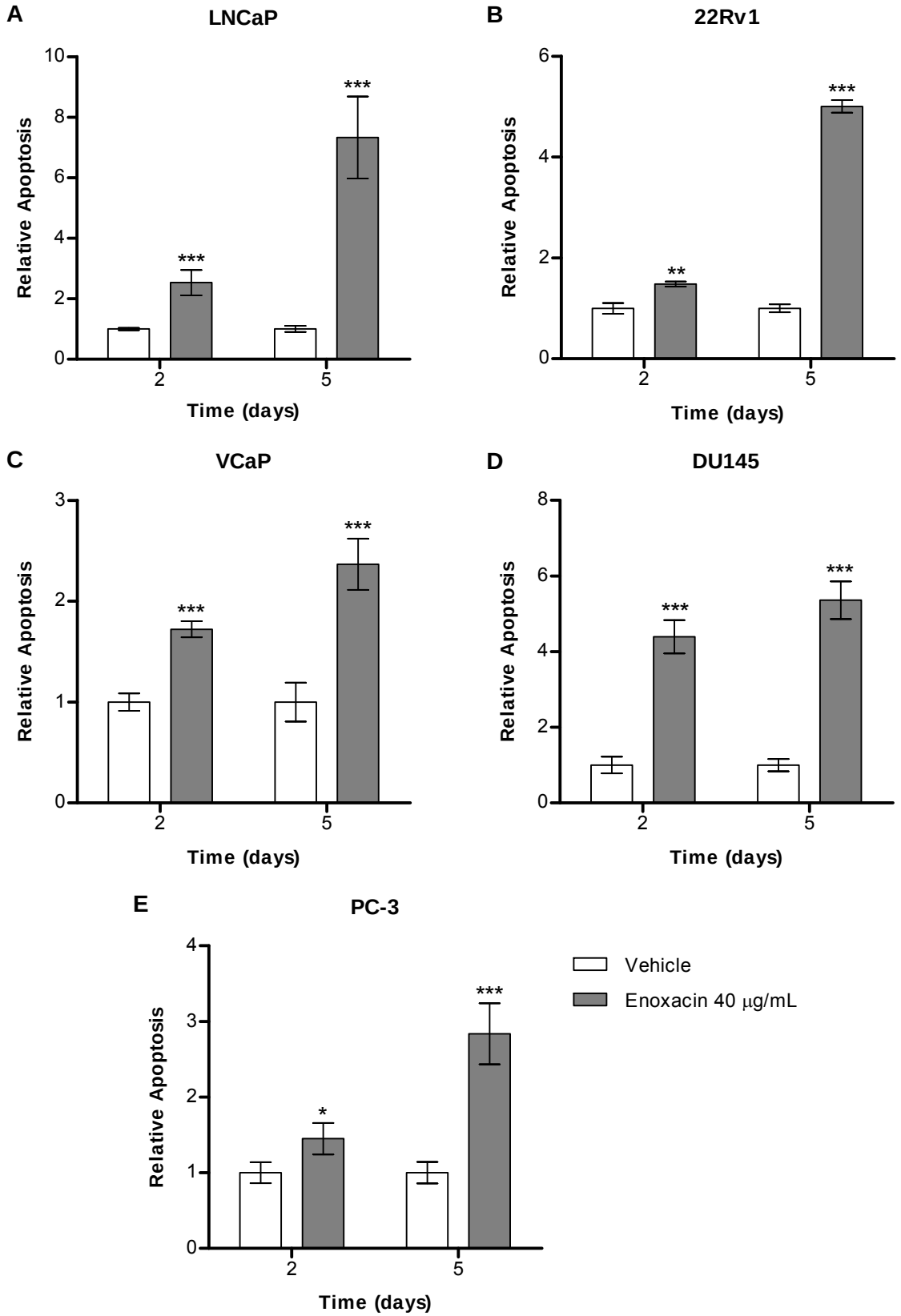

Figure 17. Effect of enoxacin on apoptosis by APOPercentage assay at days 2 and 5 in LNCaP (A), 22Rv1 (B), VCaP (C), DU145 (D), and PC-3 (E) cell lines. Data are shown as mean of three independent experiments carried out in duplicates ± s. d. * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$, compared to vehicle group.

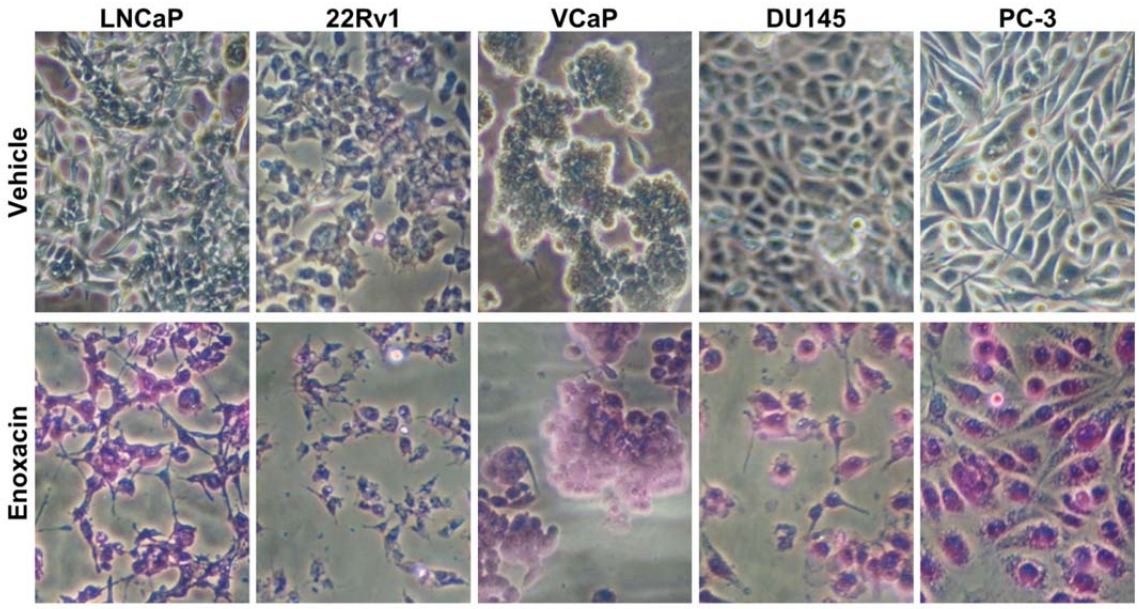

Figure 18. Digital images of PCa cells exposed to enoxacin or vehicle for 5 days (Magnification x10). The induction of apoptosis enable the cells to take up the APOPercentage dye and become pink stained.

Apoptosis was also confirmed at molecular level, through the evaluation of mRNA expression of *CASP3*. Although all cell lines showed an increase in *CASP3* expression levels, statistically significant differences were depicted only for LNCaP, 22Rv1 and DU145 (Figure 19).

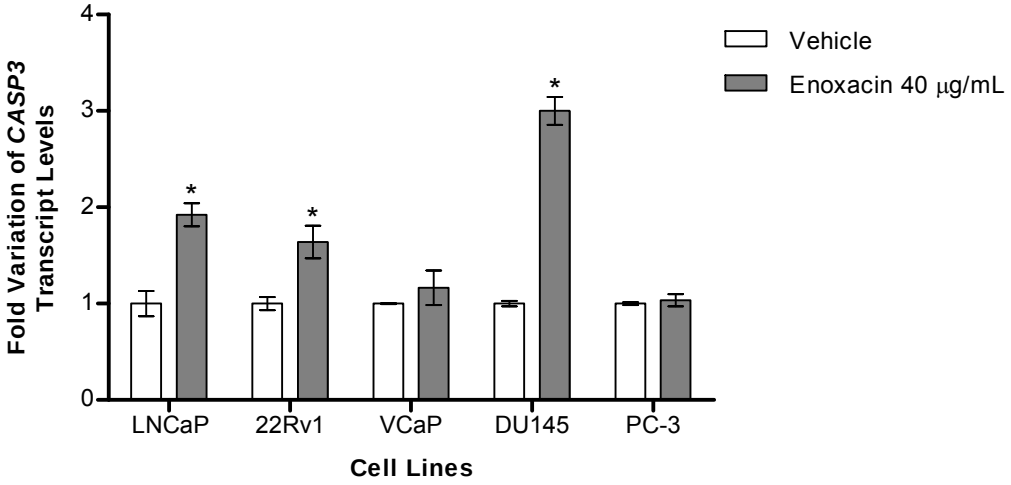

Figure 19. *CASP3* mRNA expression by qRT-PCR at day 5. Data are presented as mean of three independent experiments performed in duplicates ± s. d. * $P < 0.05$, compared to vehicle group.

3.3. Cell Cycle

Cell cycle distribution was evaluated by flow cytometry. Interestingly, 22Rv1 and VCaP, which are hormone-responsive PCa cell lines, showed cell cycle arrest at G2/M, whereas the castration-resistant cell lines DU145 and PC-3 exhibited a significant increase in the percentage of cells in late S and G2/M transition (Figure 20).

Moreover, the percentage of cells in subG1 phase, which is an indirect measure of cell death, increased significantly after enoxacin exposure (Table 4). Thus, both the increase of apoptotic cells and cell cycle arrest support a growth inhibitory effect of enoxacin on PCa cells.

Table 4. Impact of enoxacin on the percentage of cells in SubG1 phase.

Cell Line	Cells in SubG1 Phase (%)		*P* value
	Vehicle	Enoxacin	
LNCaP	1.7	2.0	ns
22Rv1	1.8	13.1	0.000
VCaP	2.5	7.3	0.002
DU145	1.7	7.5	0.000
PC-3	1.0	3.6	0.004

ns – non-significant

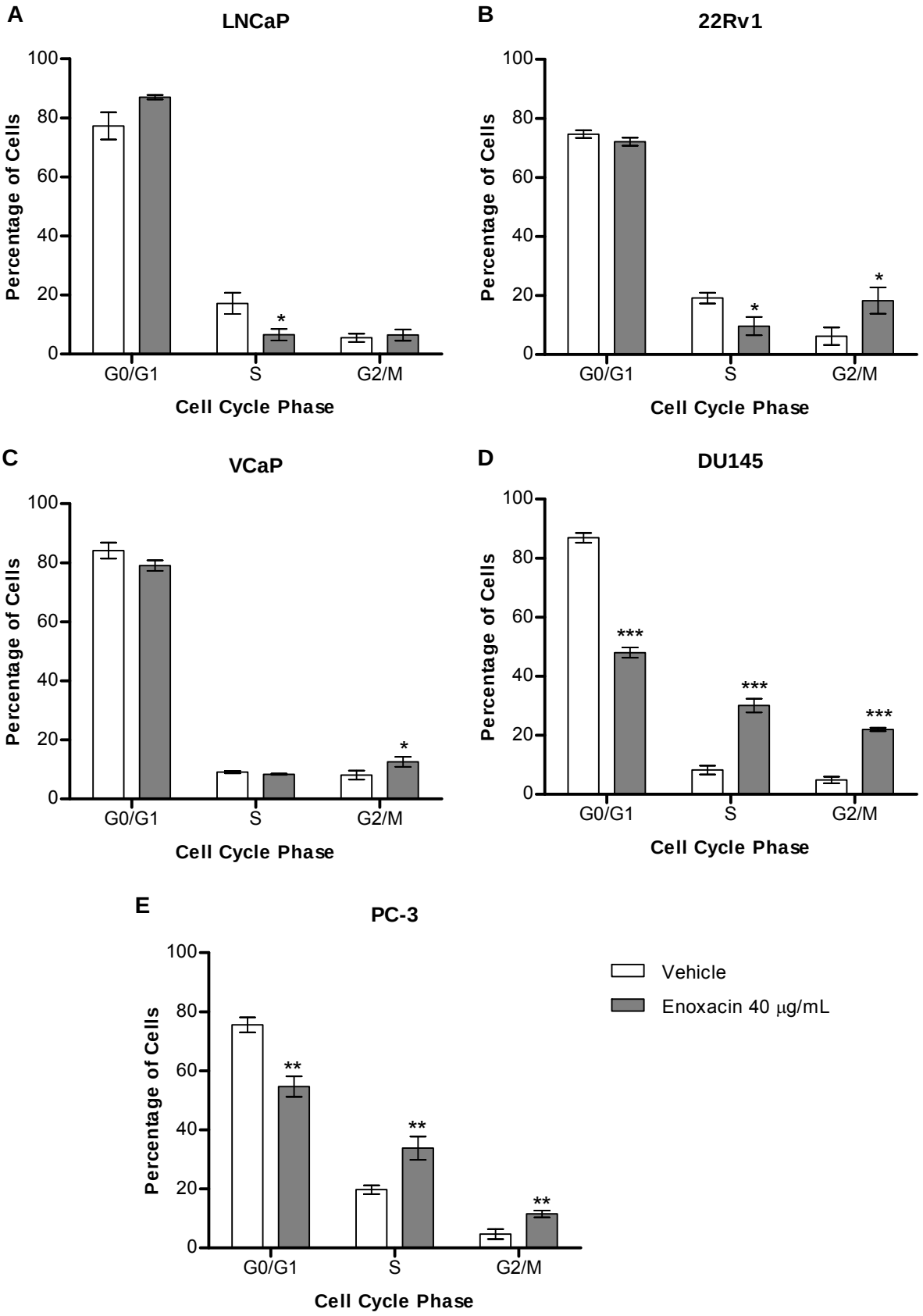

Figure 20. Cell cycle distribution by flow cytometry on LNCaP (A), 22Rv1 (B), VCaP (C), DU145 (D) and PC-3 (E) cell lines. The percentage of cells is shown as mean of three independent experiments ± s. d. * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$, compared to vehicle group.

3.4. Cell Invasion

The effect of enoxacin on the invasiveness of PCa cells was only assessed in LNCaP and DU145 cell lines because, as described in the previous experiments, these cells were the most responsive to enoxacin. We decided to extend enoxacin exposure from 5 to 8 days in order to allow the vehicle cells to invade. In contrast to DU145, LNCaP cells did not show invasive potential in this system. Remarkably, enoxacin significantly reduced the invasiveness of DU145 cells (Figure 21).

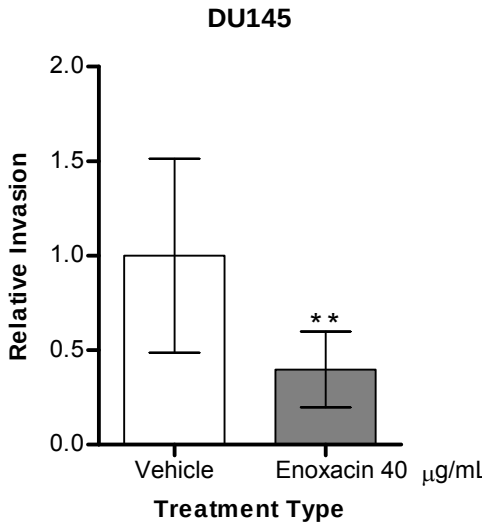

Figure 21. Effect of enoxacin on the invasive potential of PCa cells. Data are presented as mean of three independent experiments performed in triplicates ± s. d. ****** $P < 0.01$, compared to vehicle group.

3.5. MicroRNA Expression

The impact of enoxacin exposure on the expression profile of miRNAs was analyzed in LNCaP and DU145 cell lines, using a panel of 742 miRNAs. MicroRNA analysis demonstrated that enoxacin induced a global upregulation of miRNA expression in both cell lines. Among miRNAs differentially expressed, upregulation was observed in 53% of the miRNAs (65 of 122) for LNCaP cells and in 60% (147 of 247) for DU145 (Figure 22).

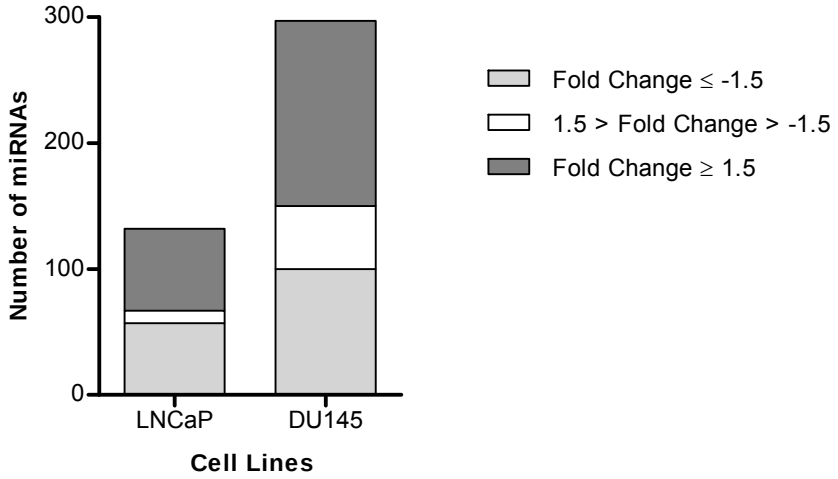

Figure 22. Graphic representation of global alterations induced by enoxacin in miRNA expression. MiRNAs were considered differentially expressed between the cell samples exposed to enoxacin or vehicle when $P < 0.05$. MiRNAs with fold change above 1.5 or below -1.5 were considered upregulated or downregulated, respectively.

Remarkably, enoxacin was able to alter the expression of several miRNAs that have been previously associated with prostate carcinogenesis. Concerning tumor-suppressor miRNAs reported in PCa, miR-17*, miRNA-29b, miR-34a, miR-132, miR-146a, and miR-449a showed increased expression levels following enoxacin exposure. Furthermore, decreased expression of some oncogenic miRNAs was also observed, including miR-141 and miR-191 (Table 5).

Table 5. Effect of enoxacin on the expression of several miRNAs already implicated in PCa.

Cell Line	miRNAs	Fold Change	*P* value
LNCaP	miR-29b	2.9	0.023
	miR-449a	1.8	0.012
	miR-34a	2.3	0.014
	miR-191	-2.3	0.023
DU145	miR-449a	2.2	0.011
	miR-146a	1.8	0.008
	miR-29b	1.7	0.004
	miR-132	1.7	0.003
	miR-17*	1.6	0.008
	miR-141	-1.7	0.002

3.5.1. Protein Expression Status of HDAC1 – a miR-449a target

To confirm the impact of enoxacin on miRNA targets, the protein expression levels of HDAC1, a miR-449a target oncoprotein, were assessed by Western Blot. Both cell lines after drug exposure displayed decreased protein levels of HDAC1 (Figure 23).

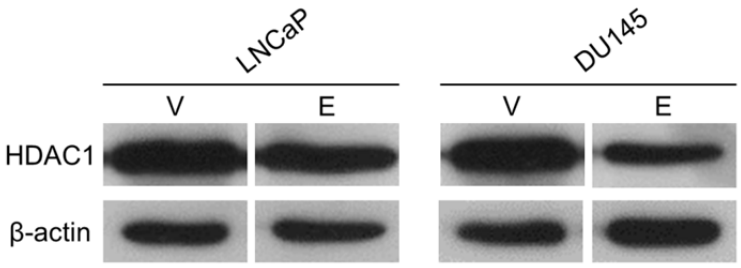

Figure 23. HDAC1 protein expression analysis by Western Blot in PCa cell lines exposed to enoxacin (E) *versus* vehicle (V). The picture is representative of three independent experiments.

DISCUSSION

PCa is one of the leading causes of cancer-related death worldwide [14], and almost all of those deaths occur as a result of the emergence of castration-resistant disease [30]. Although PCa patients initially respond to androgen-deprivation therapy, about 18-24 months after treatment initiation, most patients develop CRPC, which results in progressive clinical deterioration and, ultimately, death [15, 30]. For patients with CRPC, docetaxel remains the only FDA-approved treatment with a proven, but limited, survival benefit [30]. Therefore, new therapeutic options are urgently needed.

Recently, several miRNA microarray profiles demonstrated that miRNAs are commonly dysregulated in PCa when compared to normal prostate tissue and that they are also differentially expressed in different stages of PCa [93, 109]. Hence, miRNAs might be used not only as diagnostic and prognostic biomarkers but also as therapeutic targets in PCa. In recent years, efforts have been made to find effective miRNA-based therapeutic strategies for cancer. Indeed, artificial miRNAs that might act as potential anti-tumoral agents are the most studied so far, with most reports focusing on oncogenic miRNAs inhibition [99, 100]. Until now, few studies have reported the use of these synthetic miRNAs as tumor-suppressors, and, additionally, the effective technology for delivery of these oligonucleotide-based therapies remains a problem [100]. Because most human cancers exhibit global miRNA downregulation [74, 93, 109], the search for compounds able to globally restore the expression of tumor-suppressor miRNAs remains a priority in miRNA research. Herein, we report for the first time the anti-cancer effect of enoxacin, one of such compounds, on PCa cell lines.

Enoxacin, which has been used as a broad-spectrum antibiotic to treat bacterial infections (*e.g.,* urinary tract infections) [102, 103], was recently reported as being capable of enhancing RNAi and consequently induce miRNA expression [105, 106]. However, the mechanism of action of enoxacin is dependent of TRBP as it has been shown that this compound is less effective in cells harboring alterations in this protein caused by *TARBP2* gene mutations [91, 106]. Thus, we initially screened PCa cell lines for *TARBP2* mutations and none was found, although we were able to confirm a *TARBP2* mutation in the control, colorectal cancer cell line Co115, as previously reported [91, 106]. To further validate our results, we performed Western blot for TRBP and confirmed that all PCa cell lines tested displayed higher protein levels than Co115, in agreement with previous studies [91, 106]. We then interrogated primary PCa cases using an immunohistochemistry assay for TRBP and we found that protein immunoexpression in tumor cells was similar to that of normal

epithelial cells. Thus, we may infer that primary prostate carcinomas do not harbor deleterious mutations at the *TARBP2* locus and display normal levels of TRBP protein, rendering them sensitive to restoration of normal miRNA biogenesis by enoxacin.

To demonstrate the growth-inhibitory effect of enoxacin on PCa cell lines, we assessed cell viability, apoptosis and cell cycle characteristics following five-day exposure. Remarkably, in all tested cell lines, exposure to enoxacin resulted in a significant decrease in cell viability and induction of cell death by apoptosis, as previously demonstrated for other cancer cell lines [106]. These results were further confirmed at the molecular level through the observed statistically significant increase in *CASP3* mRNA expression for three of the five cell lines analyzed (LNCaP, 22Rv1 and DU145). Interestingly, LNCaP and DU145, the cell lines that presented the greatest reduction in cell viability and increase in apoptosis, also showed the highest protein levels of TRBP. These findings support the previously reported proposal that enoxacin exerts its anti-tumoral effects on cancer cells in a TRBP-mediated manner [105, 106].

Concerning cell cycle distribution, it was observed that 22Rv1 and VCaP, which are hormone-responsive PCa cell lines, presented cell cycle arrest at G2/M, whereas the castration-resistant cell lines DU145 and PC-3 exhibited an increase in the percentage of cells at late S and G2/M transition. Therefore, we hypothesize that these differences might be explained by the fact that castration-resistant cell lines are more proliferative than the hormone-responsive ones.

Furthermore, we have showed for the first time that enoxacin significantly reduce the invasive potential of PCa cells. As metastasis is the major cause of morbidity and mortality in PCa patients [2], the development of new treatment regimens that would reduce tumor dissemination is extremely important for PCa therapy.

This ability to disrupt pathways of cancer cell survival has been already reported for a broad spectrum of cancer cells, both *in vitro* and *in vivo*, through the enhancement of the miRNA-processing machinery [106].

In this study, we also demonstrated that enoxacin was effective in globally restoring the expression of miRNAs. Nevertheless, we found a decrease in the expression of a significant number of miRNAs, which is not totally in agreement with the mechanism of action purposed for enoxacin [105, 106]. According to previous studies, the presence of enoxacin increases the binding affinity of TRBP for miRNA precursors promoting miRNA biogenesis [105, 106]. Hence, it is tempting to speculate that besides this mechanism there might be other pathways through which enoxacin exerts its action.

Importantly, upregulation of several tumor-suppressor miRNAs known to be involved in PCa development and progression was observed, including miR-29b [130, 131], miR-449a [125], miR-146a [112], miR-17* [137], and miR-34a [121, 122]. For

instance, miRNA-29b was reported to be a negative regulator of PCa cell growth by modulating the expression of multiple proteins implicated in metastasis formation, including MMP2, E-cadherin, N-cadherin, Snail and Twist [130, 131]. MiR-146a is downregulated in CRPC cell lines, and targets ROCK1 and EGFR, which are implicated in the development of CRPC [112]. MiR-17* also suppresses tumorigenicity of PCa cells through inhibition of mitochondrial antioxidant enzymes [137]. On the other hand, miR-34a presents tumor-suppressor functions being implicated in p53 network [121, 122]. Finally, miR-449a, which is underexpressed in PCa, regulates cell growth and viability by repressing HDAC1 [125]. Remarkably, the upregulation of miR-449 resulted in the downregulation of HDAC1, an oncoprotein expressed at significantly higher levels in PCa than in normal prostate [126, 138]. In spite of globally upregulating the expression of miRNAs, enoxacin also caused a decrease in the expression of some oncogenic miRNAs, including miR-141 [114] and miR-191 [139], reported as oncomirs in PCa. MiR-141 is a target of androgen regulation and it has been suggested that its upregulation may enhance the growth of CRPC cells [114]. Finally, although less studied, miR-191 has also been reported as being overexpressed in PCa [139]. Thus, the simultaneous upregulation of tumor-suppressor miRNAs and downregulation of oncomirs by enoxacin in PCa cells highlights the therapeutic relevance of this drug in PCa. Notwithstanding, enoxacin also affected the expression levels of several other miRNAs, which play a role in different types of cancer, but with an unknown function in PCa.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this study, we reported for the first time the potential of enoxacin as an anti-tumoral agent in the treatment of PCa, using PCa cell lines as a model. Remarkably, enoxacin demonstrated the ability to reduce cell viability, induce apoptosis, and provoke cell cycle alterations in PCa cells, thus attenuating the neoplastic phenotype. We also provide evidence that enoxacin exerts an inhibitory effect on the invasiveness of PCa cells. To complement these results, additional studies should be carried out using other PCa cell lines, namely PC-3, which presents a greater invasive potential than DU145.

Moreover, enoxacin was able to promote the expression of tumor-suppressor miRNAs and decrease oncomirs expression, which might constitute a cause of phenotypic alterations observed in PCa cells. To confirm the impact of the altered miRNAs on PCa development and, consequently, their involvement on the phenotypic alterations after enoxacin exposure, it would be necessary to study the effect of the drug on the expression of their targets at protein level. Hence, in addition to HDAC1, more targets of the altered miRNAs should be assessed. Enoxacin also affected the expression levels of several other miRNAs with an unknown role in PCa. Thus, further studies are mandatory to disclose the biological function of these miRNAs in PCa.

An important finding of this study was that primary prostate carcinomas displayed normal levels of TRBP protein, which underlines the fact that enoxacin might have a great impact on the treatment of PCa patients.

Taken together, these results demonstrate that enoxacin constitutes a promising therapeutic agent and *in vivo* studies should be conducted to further support the potential of enoxacin for therapy of PCa patients.

REFERENCES

1. DeVita, V.T., Lawrence, T.S., and Rosenberg, S.A. (2008) *Cancer: Principles and Practice of Oncology*. 8th edn Lippincott Williams & Wilkins.
2. Shen, M.M. and Abate-Shen, C. (2010) Molecular genetics of prostate cancer: new prospects for old challenges. *Genes & Development*, **24**, 1967-2000.
3. Hricak, H. and Scardino, P. (2009) *Prostate Cancer*. Cambridge University Press, Cambridge.
4. Briganti, A., Capitanio, U., Suardi, N., Gallina, A., Salonia, A., Bianchi, M., Tutolo, M., Di Girolamo, V., Guazzoni, G., Rigatti, P., and Montorsi, F. (2009) Benign Prostatic Hyperplasia and Its Aetiologies. *European Urology Supplements*, **8**, 865-871.
5. De Nunzio, C., Kramer, G., Marberger, M., Montironi, R., Nelson, W., Schroder, F., Sciarra, A., and Tubaro, A. (2011) The controversial relationship between benign prostatic hyperplasia and prostate cancer: the role of inflammation. *Eur Urol*, **60**, 106-17.
6. Alcaraz, A., Hammerer, P., Tubaro, A., Schroder, F.H., and Castro, R. (2009) Is there evidence of a relationship between benign prostatic hyperplasia and prostate cancer? Findings of a literature review. *Eur Urol*, **55**, 864-73.
7. Epstein, J.I. (2009) Precursor lesions to prostatic adenocarcinoma. *Virchows Arch*, **454**, 1-16.
8. Montironi, R., Mazzucchelli, R., Lopez-Beltran, A., Cheng, L., and Scarpelli, M. (2007) Mechanisms of disease: high-grade prostatic intraepithelial neoplasia and other proposed preneoplastic lesions in the prostate. *Nat Clin Pract Urol*, **4**, 321-32.
9. Zynger, D.L. and Yang, X. (2009) High-grade prostatic intraepithelial neoplasia of the prostate: the precursor lesion of prostate cancer. *Int J Clin Exp Pathol*, **2**, 327-38.
10. Wang, W., Bergh, A., and Damber, J.E. (2009) Morphological transition of proliferative inflammatory atrophy to high-grade intraepithelial neoplasia and cancer in human prostate. *Prostate*, **69**, 1378-86.
11. Sfanos, K.S. and De Marzo, A.M. (2012) Prostate cancer and inflammation: the evidence. *Histopathology*, **60**, 199-215.
12. Zhu, X., Albertsen, P.C., Andriole, G.L., Roobol, M.J., Schroder, F.H., and Vickers, A.J. (2012) Risk-based prostate cancer screening. *Eur Urol*, **61**, 652-61.

13. Eble, J.L., Sauter, G., Epstein, J.I., and Sesterhenn, I.A. (2004) *Pathology and genetics of tumours of the urinary system and male genital organs*. Vol World Health Organization and International Agency of Research on Cancer IARC, Lyon.
14. Ferlay, J., Shin, H., Bray, F., Forman, D., Mathers, C., and Parkin, D. (2010) *GLOBOCAN 2008: Cancer Incidence and Mortality Worldwide v1.2*.
15. Damber, J.E. and Aus, G. (2008) Prostate cancer. *Lancet*, **371**, 1710-21.
16. Kvale, R., Auvinen, A., Adami, H.O., Klint, A., Hernes, E., Moller, B., Pukkala, E., Storm, H.H., Tryggvadottir, L., Tretli, S., Wahlqvist, R., Weiderpass, E., and Bray, F. (2007) Interpreting trends in prostate cancer incidence and mortality in the five Nordic countries. *J Natl Cancer Inst*, **99**, 1881-7.
17. Baade, P.D., Youlden, D.R., and Krnjacki, L.J. (2009) International epidemiology of prostate cancer: geographical distribution and secular trends. *Mol Nutr Food Res*, **53**, 171-84.
18. Farazi, T.A., Spitzer, J.I., Morozov, P., and Tuschl, T. (2011) miRNAs in human cancer. *J Pathol*, **223**, 102-15.
19. Leitzmann, M.F. and Rohrmann, S. (2012) Risk factors for the onset of prostatic cancer: age, location, and behavioral correlates. *Clin Epidemiol*, **4**, 1-11.
20. Gronberg, H. (2003) Prostate cancer epidemiology. *Lancet*, **361**, 859-64.
21. Ramon, J. and Denis, L. (2007) *Prostate Cancer*. Springer, Berlin.
22. Heidenreich, A., Bellmunt, J., Bolla, M., Joniau, S., Mason, M., Matveev, V., Mottet, N., Schmid, H.P., van der Kwast, T., Wiegel, T., and Zattoni, F. (2011) EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and treatment of clinically localised disease. *Eur Urol*, **59**, 61-71.
23. Gomella, L.G., Liu, X.S., Trabulsi, E.J., Kelly, W.K., Myers, R., Showalter, T., Dicker, A., and Wender, R. (2011) Screening for prostate cancer: the current evidence and guidelines controversy. *Can J Urol*, **18**, 5875-83.
24. Lilja, H., Ulmert, D., and Vickers, A.J. (2008) Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. *Nat Rev Cancer*, **8**, 268-78.
25. Duffy, M.J. (2011) Prostate-specific antigen: does the current evidence support its use in prostate cancer screening? *Ann Clin Biochem*, **48**, 310-6.
26. Wolf, A.M., Wender, R.C., Etzioni, R.B., Thompson, I.M., D'Amico, A.V., Volk, R.J., Brooks, D.D., Dash, C., Guessous, I., Andrews, K., DeSantis, C., and Smith, R.A. (2010) American Cancer Society guideline for the early detection of prostate cancer: update 2010. *CA Cancer J Clin*, **60**, 70-98.
27. Epstein, J.I. (2010) An Update of the Gleason Grading System. *Journal of Urology*, **183**, 433-440.
28. Edge, S.B. (2010) *AJCC cancer staging manual*. 7th edn Springer, New York.

29. Jani, A.B. and Hellman, S. (2003) Early prostate cancer: clinical decision-making. *Lancet*, **361**, 1045-53.
30. Felici, A., Pino, M.S., and Carlini, P. (2012) A Changing Landscape in Castration-Resistant Prostate Cancer Treatment. *Front Endocrinol (Lausanne)*, **3**, 85.
31. Mottet, N., Bellmunt, J., Bolla, M., Joniau, S., Mason, M., Matveev, V., Schmid, H.P., Van der Kwast, T., Wiegel, T., Zattoni, F., and Heidenreich, A. (2011) EAU guidelines on prostate cancer. Part II: Treatment of advanced, relapsing, and castration-resistant prostate cancer. *Eur Urol*, **59**, 572-83.
32. Beltran, H., Beer, T.M., Carducci, M.A., de Bono, J., Gleave, M., Hussain, M., Kelly, W.K., Saad, F., Sternberg, C., Tagawa, S.T., and Tannock, I.F. (2011) New therapies for castration-resistant prostate cancer: efficacy and safety. *Eur Urol*, **60**, 279-90.
33. Rodriguez-Paredes, M. and Esteller, M. (2011) Cancer epigenetics reaches mainstream oncology. *Nat Med*, **17**, 330-9.
34. Jablonka, E. and Lamb, M.J. (2002) The changing concept of epigenetics. *Ann N Y Acad Sci*, **981**, 82-96.
35. Sharma, S., Kelly, T.K., and Jones, P.A. (2009) Epigenetics in cancer. *Carcinogenesis*, **31**, 27-36.
36. Hirst, M. and Marra, M.A. (2009) Epigenetics and human disease. *The International Journal of Biochemistry & Cell Biology*, **41**, 136-146.
37. Bird, A. (2007) Perceptions of epigenetics. *Nature*, **447**, 396-8.
38. Guil, S. and Esteller, M. (2009) DNA methylomes, histone codes and miRNAs: Tying it all together. *The International Journal of Biochemistry & Cell Biology*, **41**, 87-95.
39. Taby, R. and Issa, J.P. (2010) Cancer epigenetics. *CA Cancer J Clin*, **60**, 376-92.
40. Bird, A. (2002) DNA methylation patterns and epigenetic memory. *Genes Dev*, **16**, 6-21.
41. Scarano, M.I., Strazzullo, M., Matarazzo, M.R., and D'Esposito, M. (2005) DNA methylation 40 years later: Its role in human health and disease. *J Cell Physiol*, **204**, 21-35.
42. Turek-Plewa, J. and Jagodzinski, P.P. (2005) The role of mammalian DNA methyltransferases in the regulation of gene expression. *Cell Mol Biol Lett*, **10**, 631-47.
43. Lopez-Serra, L. and Esteller, M. (2008) Proteins that bind methylated DNA and human cancer: reading the wrong words. *Br J Cancer*, **98**, 1881-5.
44. Klose, R.J. and Bird, A.P. (2006) Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci*, **31**, 89-97.

45. Bogdanovic, O. and Veenstra, G.J. (2009) DNA methylation and methyl-CpG binding proteins: developmental requirements and function. *Chromosoma*, **118**, 549-65.
46. Weber, M., Hellmann, I., Stadler, M.B., Ramos, L., Paabo, S., Rebhan, M., and Schubeler, D. (2007) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat Genet*, **39**, 457-66.
47. Jenuwein, T. and Allis, C.D. (2001) Translating the histone code. *Science*, **293**, 1074-80.
48. Lennartsson, A. and Ekwall, K. (2009) Histone modification patterns and epigenetic codes. *Biochim Biophys Acta*, **1790**, 863-8.
49. Kouzarides, T. (2007) Chromatin modifications and their function. *Cell*, **128**, 693-705.
50. Bhaumik, S.R., Smith, E., and Shilatifard, A. (2007) Covalent modifications of histones during development and disease pathogenesis. *Nat Struct Mol Biol*, **14**, 1008-16.
51. Santos-Rosa, H. and Caldas, C. (2005) Chromatin modifier enzymes, the histone code and cancer. *Eur J Cancer*, **41**, 2381-402.
52. Barski, A., Cuddapah, S., Cui, K., Roh, T.Y., Schones, D.E., Wang, Z., Wei, G., Chepelev, I., and Zhao, K. (2007) High-resolution profiling of histone methylations in the human genome. *Cell*, **129**, 823-37.
53. Costa, F.F. (2007) Non-coding RNAs: lost in translation? *Gene*, **386**, 1-10.
54. Esteller, M. (2011) Non-coding RNAs in human disease. *Nature Reviews Genetics*, **12**, 861-874.
55. Kanhere, A. and Jenner, R.G. (2012) Noncoding RNA localisation mechanisms in chromatin regulation. *Silence*, **3**, 2.
56. Costa, F.F. (2008) Non-coding RNAs, epigenetics and complexity. *Gene*, **410**, 9-17.
57. Costa, F.F. (2005) Non-coding RNAs: new players in eukaryotic biology. *Gene*, **357**, 83-94.
58. Di Leva, G. and Croce, C.M. (2010) Roles of small RNAs in tumor formation. *Trends in Molecular Medicine*, **16**, 257-267.
59. Melo, S.A. and Esteller, M. (2011) Dysregulation of microRNAs in cancer: Playing with fire. *FEBS Letters*, **585**, 2087-2099.
60. Iorio, M.V. and Croce, C.M. (2012) MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Mol Med*, **4**, 143-59.
61. Koturbash, I., Zemp, F.J., Pogribny, I., and Kovalchuk, O. (2011) Small molecules with big effects: The role of the microRNAome in cancer and carcinogenesis.

Mutation Research/Genetic Toxicology and Environmental Mutagenesis, **722**, 94-105.

62. Bratkovic, T., Glavan, G., Strukelj, B., Zivin, M., and Rogelj, B. (2012) Exploiting microRNAs for cell engineering and therapy. *Biotechnol Adv*, **30**, 753-65.
63. Garzon, R., Marcucci, G., and Croce, C.M. (2010) Targeting microRNAs in cancer: rationale, strategies and challenges. *Nat Rev Drug Discov*, **9**, 775-89.
64. Lytle, J.R., Yario, T.A., and Steitz, J.A. (2007) Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. *Proc Natl Acad Sci U S A*, **104**, 9667-72.
65. Moretti, F., Thermann, R., and Hentze, M.W. (2010) Mechanism of translational regulation by miR-2 from sites in the 5' untranslated region or the open reading frame. *RNA*, **16**, 2493-502.
66. Beitzinger, M. and Meister, G. (2010) Preview. MicroRNAs: from decay to decoy. *Cell*, **140**, 612-4.
67. Gonzalez, S., Pisano, D.G., and Serrano, M. (2008) Mechanistic principles of chromatin remodeling guided by siRNAs and miRNAs. *Cell Cycle*, **7**, 2601-8.
68. Kim, D.H., Saetrom, P., Snove, O., Jr., and Rossi, J.J. (2008) MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci U S A*, **105**, 16230-5.
69. Khraiwesh, B., Arif, M.A., Seumel, G.I., Ossowski, S., Weigel, D., Reski, R., and Frank, W. (2010) Transcriptional control of gene expression by microRNAs. *Cell*, **140**, 111-22.
70. Vasudevan, S., Tong, Y., and Steitz, J.A. (2007) Switching from repression to activation: microRNAs can up-regulate translation. *Science*, **318**, 1931-4.
71. Davis-Dusenbery, B.N. and Hata, A. (2010) MicroRNA in Cancer: The Involvement of Aberrant MicroRNA Biogenesis Regulatory Pathways. *Genes Cancer*, **1**, 1100-14.
72. Melo, S.A. and Kalluri, R. (2012) Molecular Pathways: MicroRNAs as Cancer Therapeutics. *Clin Cancer Res*.
73. Calin, G.A., Dumitru, C.D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., Rassenti, L., Kipps, T., Negrini, M., Bullrich, F., and Croce, C.M. (2002) Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A*, **99**, 15524-9.
74. Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A., Downing, J.R., Jacks, T.,

Horvitz, H.R., and Golub, T.R. (2005) MicroRNA expression profiles classify human cancers. *Nature*, **435**, 834-8.

75. Calin, G.A. and Croce, C.M. (2006) MicroRNA signatures in human cancers. *Nature Reviews Cancer*, **6**, 857-866.
76. Blenkiron, C., Goldstein, L.D., Thorne, N.P., Spiteri, I., Chin, S.F., Dunning, M.J., Barbosa-Morais, N.L., Teschendorff, A.E., Green, A.R., Ellis, I.O., Tavare, S., Caldas, C., and Miska, E.A. (2007) MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. *Genome Biol*, **8**, R214.
77. Sempere, L.F., Christensen, M., Silahtaroglu, A., Bak, M., Heath, C.V., Schwartz, G., Wells, W., Kauppinen, S., and Cole, C.N. (2007) Altered MicroRNA expression confined to specific epithelial cell subpopulations in breast cancer. *Cancer Res*, **67**, 11612-20.
78. Lebanony, D., Benjamin, H., Gilad, S., Ezagouri, M., Dov, A., Ashkenazi, K., Gefen, N., Izraeli, S., Rechavi, G., Pass, H., Nonaka, D., Li, J., Spector, Y., Rosenfeld, N., Chajut, A., Cohen, D., Aharonov, R., and Mansukhani, M. (2009) Diagnostic assay based on hsa-miR-205 expression distinguishes squamous from nonsquamous non-small-cell lung carcinoma. *J Clin Oncol*, **27**, 2030-7.
79. Lowery, A.J., Miller, N., Devaney, A., McNeill, R.E., Davoren, P.A., Lemetre, C., Benes, V., Schmidt, S., Blake, J., Ball, G., and Kerin, M.J. (2009) MicroRNA signatures predict oestrogen receptor, progesterone receptor and HER2/neu receptor status in breast cancer. *Breast Cancer Res*, **11**, R27.
80. Iorio, M.V. and Croce, C.M. (2009) MicroRNAs in Cancer: Small Molecules With a Huge Impact. *Journal of Clinical Oncology*, **27**, 5848-5856.
81. Zhang, L., Huang, J., Yang, N., Greshock, J., Megraw, M.S., Giannakakis, A., Liang, S., Naylor, T.L., Barchetti, A., Ward, M.R., Yao, G., Medina, A., O'Brien-Jenkins, A., Katsaros, D., Hatzigeorgiou, A., Gimotty, P.A., Weber, B.L., and Coukos, G. (2006) microRNAs exhibit high frequency genomic alterations in human cancer. *Proc Natl Acad Sci U S A*, **103**, 9136-41.
82. Lamy, P., Andersen, C.L., Dyrskjot, L., Torring, N., Orntoft, T., and Wiuf, C. (2006) Are microRNAs located in genomic regions associated with cancer? *Br J Cancer*, **95**, 1415-8.
83. Calin, G.A., Ferracin, M., Cimmino, A., Di Leva, G., Shimizu, M., Wojcik, S.E., Iorio, M.V., Visone, R., Sever, N.I., Fabbri, M., Iuliano, R., Palumbo, T., Pichiorri, F., Roldo, C., Garzon, R., Sevignani, C., Rassenti, L., Alder, H., Volinia, S., Liu, C.G., Kipps, T.J., Negrini, M., and Croce, C.M. (2005) A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med*, **353**, 1793-801.

84. Jazdzewski, K., Murray, E.L., Franssila, K., Jarzab, B., Schoenberg, D.R., and de la Chapelle, A. (2008) Common SNP in pre-miR-146a decreases mature miR expression and predisposes to papillary thyroid carcinoma. *Proc Natl Acad Sci U S A*, **105**, 7269-74.

85. Hu, Z., Chen, J., Tian, T., Zhou, X., Gu, H., Xu, L., Zeng, Y., Miao, R., Jin, G., Ma, H., Chen, Y., and Shen, H. (2008) Genetic variants of miRNA sequences and non-small cell lung cancer survival. *J Clin Invest*, **118**, 2600-8.

86. Chang, T.C., Wentzel, E.A., Kent, O.A., Ramachandran, K., Mullendore, M., Lee, K.H., Feldmann, G., Yamakuchi, M., Ferlito, M., Lowenstein, C.J., Arking, D.E., Beer, M.A., Maitra, A., and Mendell, J.T. (2007) Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell*, **26**, 745-52.

87. Choudhry, H. and Catto, J.W. (2011) Epigenetic regulation of microRNA expression in cancer. *Methods Mol Biol*, **676**, 165-84.

88. Nakamura, T., Canaani, E., and Croce, C.M. (2007) Oncogenic All1 fusion proteins target Drosha-mediated microRNA processing. *Proc Natl Acad Sci U S A*, **104**, 10980-5.

89. Melo, S.A., Moutinho, C., Ropero, S., Calin, G.A., Rossi, S., Spizzo, R., Fernandez, A.F., Davalos, V., Villanueva, A., Montoya, G., Yamamoto, H., Schwartz, S., Jr., and Esteller, M. (2010) A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells. *Cancer Cell*, **18**, 303-15.

90. Hill, D.A., Ivanovich, J., Priest, J.R., Gurnett, C.A., Dehner, L.P., Desruisseau, D., Jarzembowski, J.A., Wikenheiser-Brokamp, K.A., Suarez, B.K., Whelan, A.J., Williams, G., Bracamontes, D., Messinger, Y., and Goodfellow, P.J. (2009) DICER1 mutations in familial pleuropulmonary blastoma. *Science*, **325**, 965.

91. Melo, S.A., Ropero, S., Moutinho, C., Aaltonen, L.A., Yamamoto, H., Calin, G.A., Rossi, S., Fernandez, A.F., Carneiro, F., Oliveira, C., Ferreira, B., Liu, C.G., Villanueva, A., Capella, G., Schwartz, S., Jr., Shiekhattar, R., and Esteller, M. (2009) A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. *Nat Genet*, **41**, 365-70.

92. Zhang, B., Pan, X., Cobb, G.P., and Anderson, T.A. (2007) microRNAs as oncogenes and tumor suppressors. *Dev Biol*, **302**, 1-12.

93. Porkka, K.P., Pfeiffer, M.J., Waltering, K.K., Vessella, R.L., Tammela, T.L.J., and Visakorpi, T. (2007) MicroRNA Expression Profiling in Prostate Cancer. *Cancer Research*, **67**, 6130-6135.

94. Li, X., Zhang, Y., Ding, J., Wu, K., and Fan, D. (2010) Survival prediction of gastric cancer by a seven-microRNA signature. *Gut*, **59**, 579-85.

95. Caramuta, S., Egyhazi, S., Rodolfo, M., Witten, D., Hansson, J., Larsson, C., and Lui, W.O. (2010) MicroRNA expression profiles associated with mutational status and survival in malignant melanoma. *J Invest Dermatol*, **130**, 2062-70.

96. Ji, J., Shi, J., Budhu, A., Yu, Z., Forgues, M., Roessler, S., Ambs, S., Chen, Y., Meltzer, P.S., Croce, C.M., Qin, L.X., Man, K., Lo, C.M., Lee, J., Ng, I.O., Fan, J., Tang, Z.Y., Sun, H.C., and Wang, X.W. (2009) MicroRNA expression, survival, and response to interferon in liver cancer. *N Engl J Med*, **361**, 1437-47.

97. Schetter, A.J., Leung, S.Y., Sohn, J.J., Zanetti, K.A., Bowman, E.D., Yanaihara, N., Yuen, S.T., Chan, T.L., Kwong, D.L., Au, G.K., Liu, C.G., Calin, G.A., Croce, C.M., and Harris, C.C. (2008) MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA*, **299**, 425-36.

98. Liu, X., Liu, L., Xu, Q., Wu, P., Zuo, X., and Ji, A. (2012) MicroRNA as a novel drug target for cancer therapy. *Expert Opin Biol Ther*, **12**, 573-80.

99. Corsini, L.R., Bronte, G., Terrasi, M., Amodeo, V., Fanale, D., Fiorentino, E., Cicero, G., Bazan, V., and Russo, A. (2012) The role of microRNAs in cancer: diagnostic and prognostic biomarkers and targets of therapies. *Expert Opin Ther Targets*, **16 Suppl 2**, S103-9.

100. McDermott, A.M., Heneghan, H.M., Miller, N., and Kerin, M.J. (2011) The Therapeutic Potential of MicroRNAs: Disease Modulators and Drug Targets. *Pharmaceutical Research*, **28**, 3016-3029.

101. Li, C., Feng, Y., Coukos, G., and Zhang, L. (2009) Therapeutic microRNA strategies in human cancer. *AAPS J*, **11**, 747-57.

102. Bhanot, S.K., Singh, M., and Chatterjee, N.R. (2001) The chemical and biological aspects of fluoroquinolones: reality and dreams. *Curr Pharm Des*, **7**, 311-35.

103. Schaeffer, A.J. (2002) The expanding role of fluoroquinolones. *Am J Med*, **113 Suppl 1A**, 45S-54S.

104. Espinosa-Mansilla, A., Pena, A.M., Gomez, D.G., and Salinas, F. (2005) HPLC determination of enoxacin, ciprofloxacin, norfloxacin and ofloxacin with photoinduced fluorimetric (PIF) detection and multiemission scanning: application to urine and serum. *J Chromatogr B Analyt Technol Biomed Life Sci*, **822**, 185-93.

105. Shan, G., Li, Y., Zhang, J., Li, W., Szulwach, K.E., Duan, R., Faghihi, M.A., Khalil, A.M., Lu, L., Paroo, Z., Chan, A.W., Shi, Z., Liu, Q., Wahlestedt, C., He, C., and Jin, P. (2008) A small molecule enhances RNA interference and promotes microRNA processing. *Nat Biotechnol*, **26**, 933-40.

106. Melo, S., Villanueva, A., Moutinho, C., Davalos, V., Spizzo, R., Ivan, C., Rossi, S., Setien, F., Casanovas, O., Simo-Riudalbas, L., Carmona, J., Carrere, J., Vidal, A., Aytes, A., Puertas, S., Ropero, S., Kalluri, R., Croce, C.M., Calin, G.A., and

Esteller, M. (2011) Small molecule enoxacin is a cancer-specific growth inhibitor that acts by enhancing TAR RNA-binding protein 2-mediated microRNA processing. *Proc Natl Acad Sci U S A*, **108**, 4394-9.

107. Catto, J.W.F., Alcaraz, A., Bjartell, A.S., De Vere White, R., Evans, C.P., Fussel, S., Hamdy, F.C., Kallioniemi, O., Mengual, L., Schlomm, T., and Visakorpi, T. (2011) MicroRNA in Prostate, Bladder, and Kidney Cancer: A Systematic Review. *European Urology*, **59**, 671-681.
108. O'Kelly, F., Marignol, L., Meunier, A., Lynch, T.H., Perry, A.S., and Hollywood, D. (2012) MicroRNAs as putative mediators of treatment response in prostate cancer. *Nat Rev Urol*, **9**, 397-407.
109. Ozen, M., Creighton, C.J., Ozdemir, M., and Ittmann, M. (2008) Widespread deregulation of microRNA expression in human prostate cancer. *Oncogene*, **27**, 1788-93.
110. Coppola, V., De Maria, R., and Bonci, D. (2010) MicroRNAs and prostate cancer. *Endocr Relat Cancer*, **17**, F1-17.
111. Todorova, K., Mincheff, M., Hayrabedyan, S., Mincheva, J., Zasheva, D., Kuzmanov, A., and Fernandez, N. (2012) Fundamental Role of microRNAs in Androgen-Dependent Male Reproductive Biology and Prostate Cancerogenesis. *Am J Reprod Immunol*.
112. Lin, S.L., Chiang, A., Chang, D., and Ying, S.Y. (2008) Loss of mir-146a function in hormone-refractory prostate cancer. *RNA*, **14**, 417-24.
113. Bourguignon, L.Y., Singleton, P.A., Zhu, H., and Diedrich, F. (2003) Hyaluronan-mediated CD44 interaction with RhoGEF and Rho kinase promotes Grb2-associated binder-1 phosphorylation and phosphatidylinositol 3-kinase signaling leading to cytokine (macrophage-colony stimulating factor) production and breast tumor progression. *J Biol Chem*, **278**, 29420-34.
114. Waltering, K.K., Porkka, K.P., Jalava, S.E., Urbanucci, A., Kohonen, P.J., Latonen, L.M., Kallioniemi, O.P., Jenster, G., and Visakorpi, T. (2011) Androgen regulation of micro-RNAs in prostate cancer. *Prostate*, **71**, 604-14.
115. Brase, J.C., Johannes, M., Schlomm, T., Falth, M., Haese, A., Steuber, T., Beissbarth, T., Kuner, R., and Sultmann, H. (2011) Circulating miRNAs are correlated with tumor progression in prostate cancer. *Int J Cancer*, **128**, 608-16.
116. Gonzales, J.C., Fink, L.M., Goodman, O.B., Jr., Symanowski, J.T., Vogelzang, N.J., and Ward, D.C. (2011) Comparison of circulating MicroRNA 141 to circulating tumor cells, lactate dehydrogenase, and prostate-specific antigen for determining treatment response in patients with metastatic prostate cancer. *Clin Genitourin Cancer*, **9**, 39-45.

117. Sun, T., Wang, Q., Balk, S., Brown, M., Lee, G.S., and Kantoff, P. (2009) The role of microRNA-221 and microRNA-222 in androgen-independent prostate cancer cell lines. *Cancer Res*, **69**, 3356-63.

118. Epis, M.R., Giles, K.M., Barker, A., Kendrick, T.S., and Leedman, P.J. (2009) miR-331-3p regulates ERBB-2 expression and androgen receptor signaling in prostate cancer. *J Biol Chem*, **284**, 24696-704.

119. Bommer, G.T., Gerin, I., Feng, Y., Kaczorowski, A.J., Kuick, R., Love, R.E., Zhai, Y., Giordano, T.J., Qin, Z.S., Moore, B.B., MacDougald, O.A., Cho, K.R., and Fearon, E.R. (2007) p53-mediated activation of miRNA34 candidate tumor-suppressor genes. *Curr Biol*, **17**, 1298-307.

120. He, L., He, X., Lim, L.P., de Stanchina, E., Xuan, Z., Liang, Y., Xue, W., Zender, L., Magnus, J., Ridzon, D., Jackson, A.L., Linsley, P.S., Chen, C., Lowe, S.W., Cleary, M.A., and Hannon, G.J. (2007) A microRNA component of the p53 tumour suppressor network. *Nature*, **447**, 1130-4.

121. Fujita, Y., Kojima, K., Hamada, N., Ohhashi, R., Akao, Y., Nozawa, Y., Deguchi, T., and Ito, M. (2008) Effects of miR-34a on cell growth and chemoresistance in prostate cancer PC3 cells. *Biochem Biophys Res Commun*, **377**, 114-9.

122. Yamakuchi, M., Ferlito, M., and Lowenstein, C.J. (2008) miR-34a repression of SIRT1 regulates apoptosis. *Proc Natl Acad Sci U S A*, **105**, 13421-6.

123. Kojima, K., Fujita, Y., Nozawa, Y., Deguchi, T., and Ito, M. (2010) MiR-34a attenuates paclitaxel-resistance of hormone-refractory prostate cancer PC3 cells through direct and indirect mechanisms. *Prostate*, **70**, 1501-12.

124. Hagman, Z., Larne, O., Edsjo, A., Bjartell, A., Ehrnstrom, R.A., Ulmert, D., Lilja, H., and Ceder, Y. (2010) miR-34c is downregulated in prostate cancer and exerts tumor suppressive functions. *Int J Cancer*, **127**, 2768-76.

125. Noonan, E.J., Place, R.F., Pookot, D., Basak, S., Whitson, J.M., Hirata, H., Giardina, C., and Dahiya, R. (2009) miR-449a targets HDAC-1 and induces growth arrest in prostate cancer. *Oncogene*, **28**, 1714-24.

126. Weichert, W., Roske, A., Gekeler, V., Beckers, T., Stephan, C., Jung, K., Fritzsche, F.R., Niesporek, S., Denkert, C., Dietel, M., and Kristiansen, G. (2008) Histone deacetylases 1, 2 and 3 are highly expressed in prostate cancer and HDAC2 expression is associated with shorter PSA relapse time after radical prostatectomy. *Br J Cancer*, **98**, 604-10.

127. Le, X.F., Merchant, O., Bast, R.C., and Calin, G.A. (2010) The Roles of MicroRNAs in the Cancer Invasion-Metastasis Cascade. *Cancer Microenviron*, **3**, 137-47.

128. Watahiki, A., Wang, Y., Morris, J., Dennis, K., O'Dwyer, H.M., Gleave, M., and Gout, P.W. (2011) MicroRNAs associated with metastatic prostate cancer. *PLoS One*, **6**, e24950.

129. Gandellini, P., Folini, M., Longoni, N., Pennati, M., Binda, M., Colecchia, M., Salvioni, R., Supino, R., Moretti, R., Limonta, P., Valdagni, R., Daidone, M.G., and Zaffaroni, N. (2009) miR-205 Exerts tumor-suppressive functions in human prostate through down-regulation of protein kinase Cepsilon. *Cancer Res*, **69**, 2287-95.

130. Steele, R., Mott, J.L., and Ray, R.B. (2010) MBP-1 upregulates miR-29b that represses Mcl-1, collagens, and matrix-metalloproteinase-2 in prostate cancer cells. *Genes Cancer*, **1**, 381-387.

131. Ru, P., Steele, R., Newhall, P., Phillips, N.J., Toth, K., and Ray, R.B. (2012) miRNA-29b suppresses prostate cancer metastasis by regulating epithelial-mesenchymal transition signaling. *Mol Cancer Ther*, **11**, 1166-73.

132. Pearson, H. and Stirling, D. (2003) DNA extraction from tissue. *Methods Mol Biol*, **226**, 33-4.

133. van Meerloo, J., Kaspers, G.J., and Cloos, J. (2011) Cell sensitivity assays: the MTT assay. *Methods Mol Biol*, **731**, 237-45.

134. Kupcsik, L. (2011) Estimation of cell number based on metabolic activity: the MTT reduction assay. *Methods Mol Biol*, **740**, 13-9.

135. Scarpino, S., Duranti, E., Stoppacciaro, A., Pilozzi, E., Natoli, G., Sciacchitano, S., Luciani, E., and Ruco, L. (2009) COX-2 is induced by HGF stimulation in Met-positive thyroid papillary carcinoma cells and is involved in tumour invasiveness. *J Pathol*, **218**, 487-94.

136. Mestdagh, P., Van Vlierberghe, P., De Weer, A., Muth, D., Westermann, F., Speleman, F., and Vandesompele, J. (2009) A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol*, **10**, R64.

137. Xu, Y., Fang, F., Zhang, J., Josson, S., St Clair, W.H., and St Clair, D.K. (2010) miR-17* suppresses tumorigenicity of prostate cancer by inhibiting mitochondrial antioxidant enzymes. *PLoS One*, **5**, e14356.

138. Song, Y., Shiota, M., Tamiya, S., Kuroiwa, K., Naito, S., and Tsuneyoshi, M. (2011) The significance of strong histone deacetylase 1 expression in the progression of prostate cancer. *Histopathology*, **58**, 773-80.

139. Volinia, S., Calin, G.A., Liu, C.G., Ambs, S., Cimmino, A., Petrocca, F., Visone, R., Iorio, M., Roldo, C., Ferracin, M., Prueitt, R.L., Yanaihara, N., Lanza, G., Scarpa, A., Vecchione, A., Negrini, M., Harris, C.C., and Croce, C.M. (2006) A microRNA

expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A*, **103**, 2257-61.