Instituto de Ciências Biomédicas Abel Salazar

Faculdade de Engenharia

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EXPLOITING THE INTERPLAY BETWEEN c-Myc AND NOVEL MICRORNAS IN PROSTATE CARCINOGENESIS

Henrique Matos Fernandes de Oliveira Duarte

Dissertação de mestrado apresentada ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto – Mestrado Integrado em Bioengenharia, ramo de Biotecnologia Molecular

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“There are no endings. Only beginings. Here is another one.”

Hilary Mantel

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Resumo

Introdução: O cancro da próstata constitui um dos tipos de cancro com maior incidência e mortalidade. A vasta maioria dos intervenientes-chave envolvidos em vias moleculares implicadas na carcinogénese prostática permanece, ainda, desconhecida. Os microRNAs (miRNAs), uma classe de pequenas moléculas de RNA não codificante, são essenciais no desenvolvimento e progressão tumoral, através da regulação pós-transcricional de genes envolvidos no processo de tumorigénese. Atualmente, têm sido feitos esforços no sentido de clarificar a interação biológica que parece existir entre o *MYC*, um reconhecido oncogene, e vários miRNAs, bem como o consequente impacto que esta interação possa ter no desenvolvimento de cancro.

Objetivos: O principal objetivo da presente Tese foi a identificação de novos miRNAs implicados na carcinogénese da próstata e a sua possível associação à sinalização pelo gene *MYC*.

Materiais e Métodos: Um vetor lentiviral contendo uma sequência de RNA de interferência foi utilizado para silenciar o gene *MYC* na linha celular de cancro de próstata PC-3. Nestas células, as alterações nos níveis de transcrito de quatro miRNAs previamente selecionados (miR-27a*, miR-126*, miR-570 e miR-1292), decorrentes do silenciamento do *MYC* [(confirmado ao nível do transcrito (RT-qPCR) e da proteína (Western Blot)], foram avaliadas por RT-qPCR. Adicionalmente, a expressão dos miRNAs 27a* e 126* foi validada, por RT-qPCR, numa série de amostras clínicas de próstata. Os níveis proteicos do fator de transcrição c-Myc foram avaliados na mesma série, por imunohistoquímica, e comparados com os níveis de expressão destes miRNAs. A análise *in silico* permitiu avaliar a existência de uma possível interação biológica entre o c-Myc e os miRNAs validados. O impacto biológico da desregulação de miRNAs, decorrente do silenciamento do *MYC*, foi determinado com base nos níveis proteicos, analisados por Western Blot, de alvos previamente validados do miR-27a*.

Resultados: Os níveis da proteína c-Myc mostraram ser significativamente mais elevados nas lesões de PIN e nos tumores primários, em comparação com tecidos prostáticos morfologicamente normais. Adicionalmente, foi obtida uma associação estatisticamente significativa entre a expressão proteína de c-Myc e os níveis de PSA sérico e Gleason score (GS) dos doentes. Em células PC-3, o silenciamento do *MYC* levou a uma redução significativa dos níveis

de expressão dos quatro miRNAs selecionados. Em amostras clínicas de próstata, os níveis de expressão dos miRNAs 27a* e 126* seguiram uma tendência oposta aos níveis da proteína c-Myc, sendo significativamente menores em lesões de PIN e ainda mais reduzidos em tumores primários, quando comparados com tecidos normais de próstata. Foi encontrada uma associação estatisticamente significativa entre os níveis de miR-27a* e o GS dos doentes. Os níveis proteicos de c-Myc associaram-se inversamente aos níveis do miR-126*, isto é, a baixa expressão de c-Myc (< 10%) associou-se significativamente a níveis elevados de miR-126*, sendo que os tumores com elevada expressão de c-Myc (> 50%) apresentaram níveis reduzidos de miR-126*. A análise realizada *in silico* revelou locais de ligação da proteína c-Myc na região promotora dos dois miRNAs validados. Esta análise identificou ainda o gene *MYC* e genes codificadores de proteínas ativadoras deste fator de transcrição como alvos putativos do miR-126*. As células PC-3 silenciadas para o gene *MYC* apresentaram um aumento dos níveis proteicos do *EGFR* e *MTOR*, dois alvos previamente validados do miR-27a*.

Discussão: O padrão de expressão obtido para miRNAs 27a* e 126* indica um papel supressor tumoral de ambos os miRNAs validados. A associação entre níveis elevados do miR-27a* e valores mais elevados de GS sugere o valor prognóstico deste miRNA. A redução da expressão dos miRNAs 27a* e 126* em células PC-3, após o silenciamento do *MYC*, indica um possível papel desta proteína na ativação transcricional destas moléculas. Adicionalmente, a existência de locais de ligação previstos para o c-Myc na região promotora de ambos os miRNAs corrobora a possível existência de uma interação entre eles. Por outro lado, padrões de expressão antagónicos observados para ambos os miRNAs validados e a proteína c-Myc na série de amostras clínicas sugerem um mecanismo distinto, no qual o c-Myc inibe a transcrição destes miRNAs supressores tumorais, contribuindo assim para a tumorigénese. Finalmente, o miR-27a* parece regular a expressão de genes envolvidos na carcinogénese prostática, de forma semelhante ao que foi reportado para outros modelos tumorais.

Conclusões e Perspetivas Futuras: Os resultados obtidos sugerem que a regulação pelos miRNAs 27a* e 126* poderá constituir um mecanismo adicional da complexa rede regulatória responsável pelo controlo da expressão e atividade do fator de transcrição c-Myc, em cancro da próstata. No entanto, estudos adicionais são necessários para confirmar a existência de tal interação e para avaliar a relevância biológica destes miRNAs na carcinogénese prostática.

Summary

Introduction: Prostate cancer (PCa) represents one of the most incident and deadliest types of cancer affecting men worldwide. Regarding PCa progression, many key players involved in the molecular pathways contributing to prostate carcinogenesis remain unveiled. MicroRNAs (miRNAs), a class of small non-coding RNA molecules, are crucial in cancer development and progression, through the post-transcriptional regulation of genes involved in the tumorigenic process. Currently, research efforts have been focused in characterizing the biologic interplay between c-Myc transcription factor, a well-recognized oncogene, and several miRNAs, and its consequent impact in cancer development.

Aims: The main goal of this Master Thesis was to identify novel miRNAs implicated in prostate carcinogenesis and to further characterize their possible link to *MYC* signaling.

Material and Methods: A lentiviral vector containing a short hairpin RNA sequence was used for *MYC* silencing in the PC-3 cell line. Alterations in the expression levels of four selected microRNA candidates (miR-27a*, miR-126*, miR-570 and miR-1292) were assessed in PC-3 cells, by RT-qPCR, upon *MYC* knockdown confirmation at both transcript (RT-qPCR) and protein (Western Blot) level. Further validation of miR-27a* and miR-126* was performed by RT-qPCR in a large set of prostate clinical samples. Protein levels of c-Myc were assessed in the same series by immunohistochemistry, and compared with the expression levels of these miRNAs. *In silico* analysis was performed to assess the possible interaction between c-Myc and both validated miRNAs. To assess the biological impact of miRNA deregulation upon *MYC* silencing, the expression levels of validated molecular targets of miR-27a*were also evaluated by Western Blot.

Results: Protein levels of c-Myc showed to be significantly higher in PIN lesion and PCa samples, when compared with morphologically normal prostate tissues (MNPTs). Additionally, there was a statistically significant association between c-Myc protein levels and patients' PSA levels and Gleason score (GS). In PC-3 cells, *MYC* silencing led to a significant decrease in the expression levels of the four selected miRNAs. Moreover, in clinical samples, we were only able to assess expression levels of miR-27a* and miR-126*. MiRNAs levels followed the opposite trend observed for c-Myc, being significantly lower in PIN lesions and, at more extent, in PCa, when compared

with MNPTs. A statistically significant association between miR-27a* expression levels and patients GS was also disclosed. Protein levels of c-Myc inversely associated with miR-126* transcript levels, i.e., < 10% c-Myc group significantly associated with high miR-126* transcript levels and the > 50% c-Myc group significantly associated with low miR-126* transcript levels. *In silico* analysis revealed c-Myc binding sites for both validated miRNAs' promoter region and indicated c-Myc and c-Myc activator proteins as putative targets of miR-126*. In PC-3 cells, protein levels of epidermal growth factor receptor (*EGFR*) and mechanistic target of rapamycin (*MTOR*), two previously validated miR-27a*-targets, increased upon *MYC* gene silencing.

Discussion: The obtained expression pattern for miR-27a* and miR-126* suggests their role as tumor suppressor miRNAs, which is in agreement with other previously published studies. Significantly higher miR-27a* expression levels were in found in the subgroup of patients with higher GS, indicating this miRNA as a promising prognostic tool. Interestingly, the decreased expression levels of miR-27a* and miR-126* observed in PC-3 cells, upon *MYC* knockdown, indicates c-Myc as a transcriptional activator of these molecules. Moreover, the existence of predicted c-Myc binding sites in both these miRNAs promoter regions additionally strengthens the possibility of such interaction to occur. On the other hand, opposite expression patterns observed for both validated miRNAs and c-Myc across a large series of prostate clinical samples suggest a different mechanism, in which c-Myc inhibits the transcription of tumor suppressor miRNAs, this way contributing to tumor progression. Additionally, miR-27a* seems to regulate the expression levels of genes implicated in prostate carcinogenesis, similarly to what was previously reported for other cancer models.

Conclusion and Future Perspectives: Taken together, the obtained results suggest that miR-27a* and miR-126* may comprise an additional layer of the complex regulatory network responsible for controlling *MYC* expression and activity in PCa. However, further functional studies are necessary to confirm the existence of a true interplay between these miRNAs and c-Myc transcription factor, and to evaluate the biological relevance of miR-27a* and miR-126* in prostate carcinogenesis.

Table of Contents

Figures Index

Tables Index

List of Abbreviations

ACTB	Beta-actin
AR	Androgen receptor
ARE	Androgen-responsive element
BAK1	BCL2-antagonist/killer 1
BCA	Bicinchoninic acid
BCL2	B-cell CLL/lymphoma 2
bp	Base pair
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
CCND1	Cyclin D1
CD44	Cluster of differentiation 44
CDH1	E-cadherin
CDK	Cyclin-dependent kinase
CDK1	Cyclin-dependent kinase 1
CDK2	Cyclin-dependent kinase 2
CDKN1B	Cyclin-dependent kinase inhibitor 1B
CDKN1C	Cyclin-dependent kinase inhibitor 1C
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CSC	Cancer stem cell
DAB	3,3’-Diaminobenzidine
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DRE	Digital rectal exam
dsRNAs	Double-stranded RNAs
dTTP	2'-deoxythymidine 5'-triphosphate

E2F1	E2F transcription factor 1
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
EZH2	Enhancer of the zeste homolog 2
FBS	Fetal bovine serum
FFPE	Formalin-fixed paraffin embedded
FISH	Fluorescence *in situ* hybridization
FN1	Fibronectin
GS	Gleason score
GUSβ	Beta-glucuronidase
KW	Kruskal-Wallis test
Let-7	Lethal-7
HGPIN	High-grade prostatic intraepithelial neoplasia
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IHC	Immunohistochemistry
MGB	Minor groove binder
miRNA	MicroRNA
MMP	Matrix metalloproteinase
MNPT	Morphologically normal prostate tissue
mRNA	Messenger RNA
MTOR	Mechanistic target of rapamycin
MW	Mann-Whitney U test
MYC	v-Myc avian myelocytomatosis viral oncogene homolog
MYCBP	Myc-binding protein
MYCBP2	Myc-binding protein 2
Oncomir	Oncogenic microRNA
PBS	Phosphate-buffered saline

PCa	Prostate cancer
PDCD4	Programmed cell death 4
PIA	Proliferative inflammatory atrophy
PIC	Protease inhibitor cocktail
PIN	Prostatic intraepithelial neoplasia
PMSF	Phenylmethylsulfonyl fluoride
pre-miRNAs	Precursor microRNAs
pri-miRNAs	Primary microRNAs
PSA	Prostate-specific antigen
PTEN	Phosphatase and tensin homolog
P-S	Penicillin-streptomycin
RAS	Rat sarcoma
RIPA	Radioimmuno precipitation assay
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROCK1	Rho-associated coiled-coil containing protein kinase 1
RT-qPCR	Quantitative real-time polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC23A	Sec23 homolog A
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SIRT1	Sirtuin 1
SLC45A3	Prostate cancer-associated protein 6
TBS-T	Tris buffered saline with tween® 20
TGFBR2	Transforming growth factor β receptor II
TNM	Tumor - Lymph Node - Metastasis
TP53	Tumor protein 53
TRBP	Transactivating response RNA-binding protein
TRUS	Transrectal ultrasound

UNG	Uracil-DNA glycosylase
UTR	Untranslated region
VIM	Vimentin
XPO5	Exportin 5
ZEB	Zinc finger E-box binding homeobox

1. Introduction

1.1. Epigenetics

The term “Epigenetics” was first used in the early 1940s, when Conrad Waddington attached the prefix *epi*, which literally means “over” or “upon”, to the word genetics (1). All cells composing a complex multicellular mammal organism share the same genetic heritage. However, the vast diversity and complexity of such organisms clearly show that, despite having the same set of genetic instructions, encoded in their DNA molecules, cells can significantly diverge from each other in what their regulatory state is concerned (2). These differences allow cells with the same genome to maintain all sorts of phenotypes. There is a series of mechanisms that allow cells to create a non-genetic memory, with which they record and integrate information arising from their own development process and their environmental context (3). Besides, the way some cellular features are propagated and maintained cannot be explained by genetic mechanisms alone. Epigenetics can then be defined as the set of modifications in the DNA or its associated proteins that carry the information content regarding gene expression during cell division, and that are not related with changes in the DNA sequence itself (4).

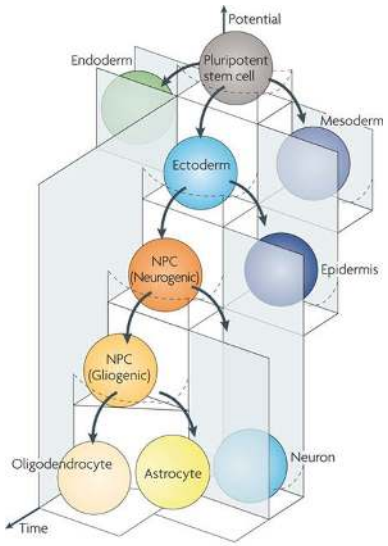

Figure 1. Differentiation potential of stem cells in each developmental stage [Adapted from: (6)].

The epigenome of a cell consists in all the chemical alterations to DNA and histones that can be transmitted from one cell generation to the next, through either mitosis or meiosis. The epigenetic mechanisms work as powerful on and off switches that determine which proteins will be expressed inside the cell (5), being partially responsible, together with other mechanisms, for a considerable cell diversity within the same multicellular organism - neurons, myocytes, hepatocytes, blood cells and many others – due to differential gene expression (Figure 1) (6). Such mechanisms also play an important role in the inactivation of one of the X chromosomes in mammal females. Currently, the set of known epigenetic mechanisms includes DNA methylation, chromatin remodeling associated with histone modifications and regulation by non-coding ribonucleic acid (RNAs) molecules (Figure 2) (7).

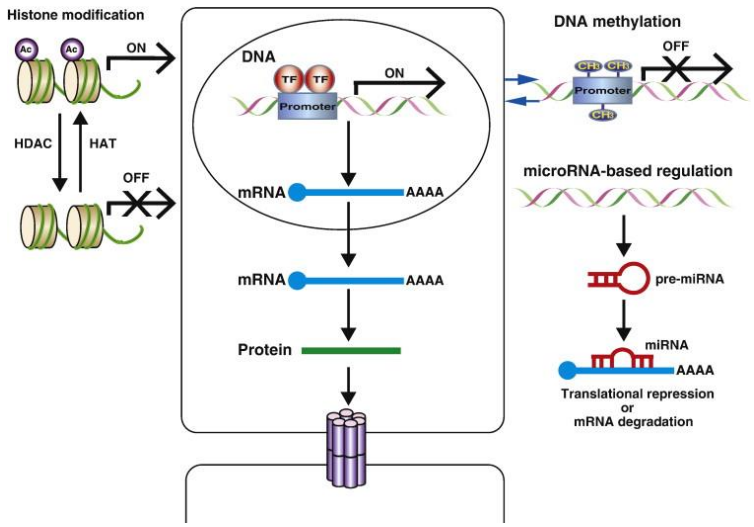

Figure 2. Epigenetic mechanisms include DNA methylation, histone modification and microRNA-based regulation [Adapted from: (7)].

Deregulation of epigenetic mechanisms is often implicated in pathological processes, since it leads to the abnormal activation or inactivation of given genes (5). Among their involvement in disease-driven processes, the role of epigenetic mechanisms in tumorigenesis is one of the major issues nowadays. Herein, the focus of this Thesis will be on epigenetic mechanisms involving microRNA regulation.

1.2. MicroRNA Regulation

Non-coding RNAs act by modifying the structure or expression of messenger RNA (mRNA) molecules, leading to alterations in expression levels of the proteins they encode (2). MicroRNAs (miRNAs) represent a set of endogenous small non-coding RNA molecules, with a length between 18 and 25 nucleotides, currently acknowledged as key players in gene regulation (8). MiRNAs were first discovered in 1993 in *Caenorhabditis elegans*, and, currently, they are known to play important roles in almost all cellular pathways, including cell cycle progress and regulation, immune response to pathogens, stem cell differentiation, stress response, apoptosis and pathological processes (Figure 3) (9).

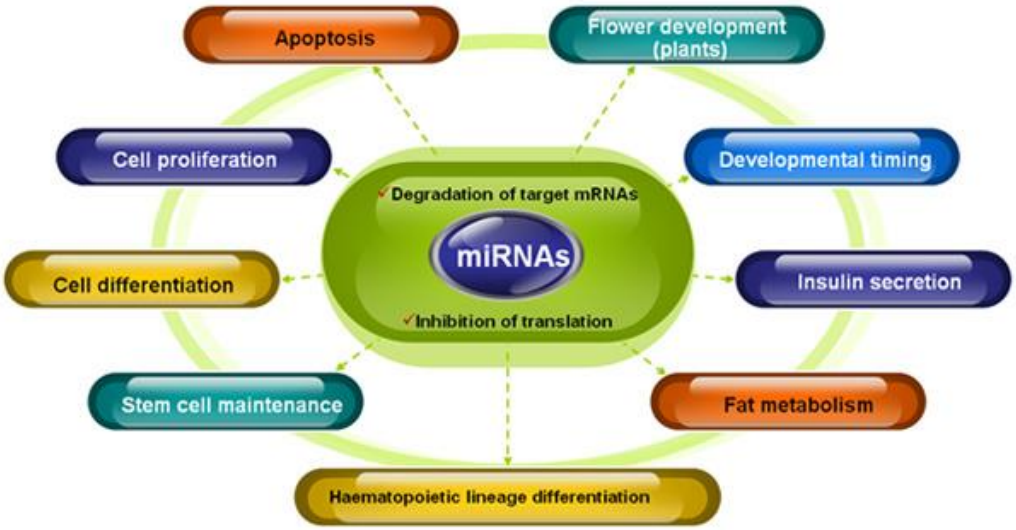

Figure 3. MiRNA-regulated cellular, biological and physiological processes [Adapted from: (9)].

MiRNA biogenesis is a relatively complex process (Figure 4) (10). The majority of these small RNA molecules derive from longer intramolecularly double-stranded RNAs (dsRNAs), also called primary miRNAs or pri-miRNAs (11). Its transcription, carried out by RNA polymerase II, may occur from independent genomic transcription units or from introns of protein-coding genes (8). After being transcribed, pri-miRNA molecules are further cleaved by specialized ribonuclease III enzymes, such as RNA polymerase III Drosha (*DROSHA*) in the case of mammals, which are located in the cell nucleus. The resulting intermediate molecules, miRNAs precursors or pre-miRNAs, have 60 to 70 nucleotides in length and, typically, display a stem-loop structure with self-complementarity. One molecule of pri-miRNAs may originate several pre-miRNAs, after cleavage

by Drosha (polycistronic miRNAs), which, by its turn, is part of a larger protein complex with about 650 kDa, known as the microprocessor complex (10). Within the pre-miRNA molecule, there is self-complementarity, which leads to the formation of a double-stranded hairpin. Pre-miRNAs are then exported to the cytoplasm through nuclear pores by the nuclear transport receptor Exportin 5 (*XPO5*). In the cytoplasm, another ribonuclease III enzyme, known as Dicer (*DICER1*), which acts in a complex with the transactivating response RNA-binding protein (*TRBP*), further cleaves the pre-miRNA molecules, originating small dsRNA molecules of approximately 22 bp. Each of these molecules is composed by an active or guide strand, which is then released and associated to the RNA-induced silencing complex (RISC), originating the mature RISC complex, and an inactive or passenger strand, which is removed and directed to degradation (12). The selection between the guide and passenger strands is made according to their thermodynamic properties (10).

At this point, mature miRNAs associated with the RISC complex, which also includes the Argonaute proteins, among others, are able to target specific mRNA molecules and interfere with their translation, through sequence-specific interactions (8). In the case of animals, this ribonucleoprotein complex typically positions itself at the 3' untranslated region (UTR) of the target mRNA molecule, by recognition of complementary sequences (Figure 4) (10). Depending on the level of complementarity between the miRNA sequence and its mRNA target sequence, post-transcriptional regulation may occur through inhibition of translation or degradation of the mRNA molecule (10). The latter may additionally be divided in site-specific cleavage, an extremely rare event in mammals that takes place when there is a perfect or almost perfect match between the miRNA and mRNA molecules, and enhanced mRNA degradation, which, together with the translational inhibition process, is associated with a mismatch between the sequences of the miRNA molecule and its target mRNA. Recently, it is thought that the degree of translation repression or mRNA degradation depends not only on the sequence complementarity between the miRNA and its target mRNA, but also on the expression levels of both (13).

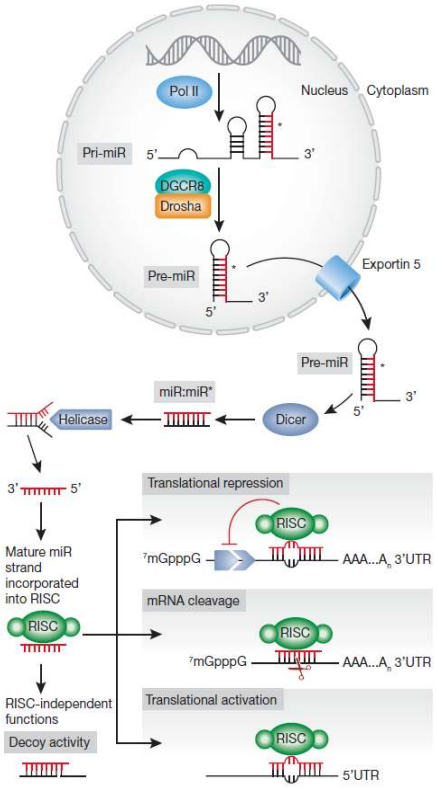

Figure 4. **Biogenesis and mechanism of action of miRNAs [Adapted from: (10)].**

Despite miRNA regulation being mainly associated to downregulation of its target mRNA molecule and, consequently, of the respective protein, these small non-coding RNAs may be responsible, in certain cellular contexts, for translation upregulation, when they happen to bind the 5'-UTR of the target mRNA (14). MiRNA-mediated regulation is highly promiscuous, since each miRNA can potentially regulate several mRNA target molecules. In fact, recent computational analysis and transcriptome profiling suggest that each miRNA molecule may have as many as 200 mRNA targets (13). On the other hand, several miRNAs can act together in the regulation of a single gene. Currently, there are over 2500 human mature miRNA molecules listed at miRBase (15). There are several computational tools and databases that can be used to predict miRNA targets (16). However, the role that many of them play in biology of the cell still remains largely unveiled. Deregulation of miRNA-mediated mechanisms and pathways has been associated with several different pathologies, including age-related, cardiac and neurological diseases, as well as immune disorders and, most importantly, cancer (17).

1.3. MicroRNA Deregulation in Cancer

1.3.1. The Role of MicroRNAs in Tumorigenesis: Oncogenic vs. Tumor Suppressive

Table 1. **MiRNAs associated with human cancers [Adapted from: (19)].**

MiRNA	Cancer Association	Function
miR-15a and miR-16-1	Frequently deleted or downregulated in B-cell chronic lymphocytic leukemia; negatively regulate the anti-apoptotic gene B-cell CLL/lymphoma 2 (*BCL2*)	Tumor suppressor
miR-21	Anti-apoptotic factor; upregulated in glioblastomas and breast cancer	Oncogene
let-7 family	Negatively regulates the rat sarcoma (*RAS*) oncogenes; directs cell proliferation and differentiation; decreased expression in lung cancer	Tumor suppressor
miR -17-19b cluster	Upregulated by v-Myc avian myelocytomatosis viral oncogene homolog (*MYC*); negatively modulates the E2F transcription factor 1 (*E2F1*) oncogene; loss of heterozygosity of this cluster is found in hepatocellular carcinoma; overexpressed in B-cell lymphomas	Tumor suppressor Oncogene

As previously mentioned, deregulation of epigenetic mechanisms plays a crucial role in many pathological processes, of which cancer is one of the best studied (1). Specifically, several miRNAs have been reported as deregulated in different human cancers (12) and studies involving miRNA expression profiling showed significant differences between normal and tumor tissues (10). Indeed, miRNAs deregulation has been associated with critical steps of tumorigenesis, including tumor growth, progression, invasion and metastasis formation, as well as with patients' response to therapy. It has been reported that about 50% of the miRNAs encoding genes are inserted in cancer-associated genomic regions (18). Thus, it becomes clear that numeric or structural chromosomal abnormalities, affecting the regions where those genes are mapped in, may lead to altered miRNAs levels, ultimately promoting tumorigenesis. Aberrant expression or deregulation of miRNA biogenesis machinery may itself be one of the causes underlying the genetic instability that fosters tumor formation (19), as illustrated by Dicer downregulation in lung cancer (20).

Since miRNAs act by regulating gene expression at a post-transcriptional level, it is clear that these molecules may play an important role in cancer development, depending on their cellular context, their target genes and their involvement in the pathological process. Hence, miRNAs may either have oncogenic functions, if their deregulation leads to inhibition of tumor suppressor genes expression, or tumor suppressor functions, if they are responsible for regulating the expression of oncogenes (21) – Figure 5 and Table 1 (19).

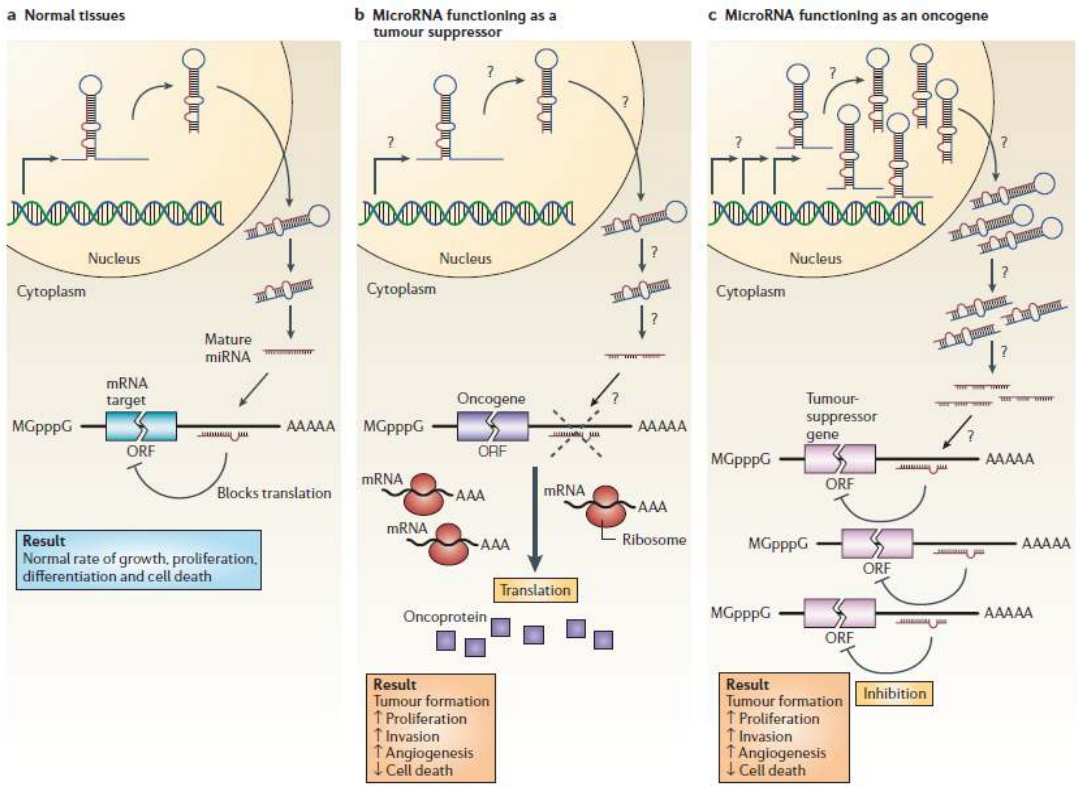

Figure 5. MiRNAs can act either as oncogenic or tumor suppressor elements [Adapted from: (19)].

The oncogenic miRNAs, also called "oncomirs", characteristically have their expression increased in tumors, and they contribute for cancer development through negative regulation of tumor suppressor genes or genes that control vital cellular processes such as apoptosis, cell cycle control and checkpoints mechanisms, cell growth and differentiation (19). For example, miR-372 and miR-373, overexpressed in human testicular germ cell tumors, promote cell proliferation and tumorigenesis by preventing tumor protein p53-mediated (*TP53*) cyclin-dependent kinase (CDK) inhibition (22).

Tumor suppressor miRNAs, which show decreased expression in cancer tissues, regulate the expression of proto-oncogenes, preventing their overexpression, and their downregulation contributes to neoplastic transformation (23). The lethal-7 (*let-7*) miRNA family was the first to be recognized as having tumor suppressor functions (24). The miRNAs included in this family target several important genes that are critically involved in cell growth and differentiation, such as rat sarcoma (*RAS*) oncogene family, which is mutated in 15-30% of human cancers (23).

MiRNAs have specific signatures, according to the type of tissue and of cancer. Several studies have been conducted to build miRNA expression profiles in normal tissues, cancer cell lines and tumors with different origins (25). Actually, miRNA profiling is becoming more and more attractive, and can be put to use together with gene profiling. That happens mostly because of miRNA's increased stability and short length (25). Such studies have revealed that a large number of miRNAs display specific altered expression levels according to tumor type and stage, allowing for miRNA expression patterns to classify tumors (26). Thus, miRNA profiling may be extremely informative, helping in the classification of poorly differentiated tumors, when mRNA profiling fails to do so (27). The development and improvement of refined and accurate miRNA detection methodologies permits the use of these small molecules in diagnosis, so that specific miRNAs may become cancer biomarkers and ancillary tools for diagnosis.

1.3.2. MicroRNA Regulation by c-Myc

The v-Myc avian myelocytomatosis viral oncogene homolog (*MYC*) gene encodes for a transcription factor, c-Myc, that plays an essential role in almost all cellular processes, many of which are important for tumorigenesis, such as cell cycle progression, proliferation, growth, metabolism, angiogenesis, differentiation, adhesion and motility, being also deregulated in several human cancers (28). Several studies have shown that c-Myc is also involved in regulating miRNA cellular levels, inducing the expression of oncogenic miRNAs, such as miR-17-92 cluster (29-33). In other cases, it represses the expression of tumor suppressor miRNAs (34-36), as it is represented in Figure 6 (28). This transcription factor has also been shown to regulate miRNA processing through transcriptional regulation of Drosha (37). Thus, clarifying the role of c-Myc-regulated miRNAs in tumourigenesis may be of great interest for developing novel cancer therapies.

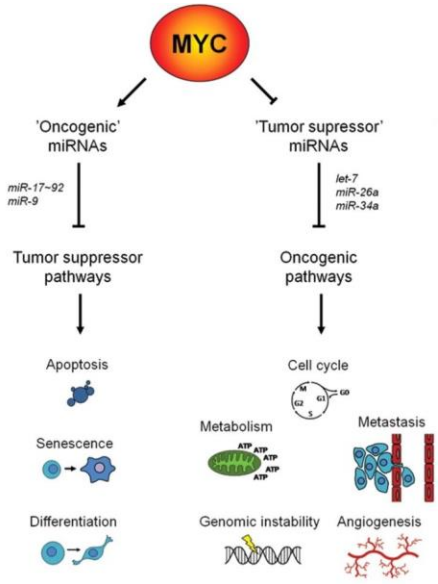

Figure 6. **MiRNA Regulation by c-Myc transcription factor [Adapted from: (28)].**

1.4. Prostate Cancer

1.4.1. Prostate Anatomy, Histology and Physiology

The prostate gland is part of the human male reproductive and urinary systems, playing a major role in secretion, ejaculation and urination (38). It lies underneath the bladder, in front of the rectum, being separated from the latter by a thin layer of connective tissue (Figure 7) (39). The human prostate weights about 20 g and has approximately 3 cm long, 4 cm wide and 2 cm thick, in young individuals. This organ completely surrounds the urethra. The apex of this walnut-shaped organ contacts with membranous urethra and is directed downward, while the basis contacts the bladder and is directed upward (38). The gland is internally divided by the prostatic urethra into distinct inner and outer regions. The ejaculatory ducts penetrate the prostate and join the prostatic urethra at the verumontanum.

Although over the years several investigators sustained a lobular structure for the human prostate, MacNeal, based on anatomic observations in different animal models, proposed the division of this gland into zones rather than lobes (Figure 7) (39), a proposition that is widely accepted among the scientific and medical community (40).

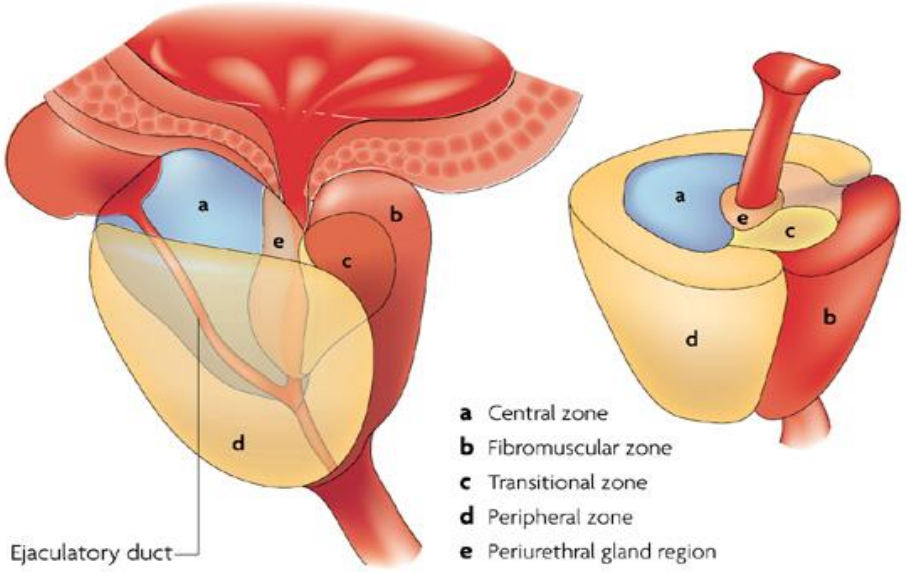

Figure 7. Anatomic position of human prostate and prostate zones [Adapted from: (39)].

The prostate is a well innervated gland by both the sympathetic and parasympathetic divisions, the former being important to mediate smooth muscle contraction, through activation of α-1 receptors, and the latter being involved in stimulation of glandular activity.

Histologically, each glandular zone is composed by acini and ducts, lined with two main cell layers: a luminal columnar cell layer with secretory functions and an underlying basal cell layer. However, there may be several intermediate cell types separating these two layers (41). Progression from basal (stem) to secretory luminal (mature) cells represents a continuous differentiation process.

1.4.2. Prostate Pathology

Several pathological processes may affect the prostate (Figure 8) (39). Benign prostatic hyperplasia (BPH), a benign prostatic enlargement, is a common condition among men over 70 years old (42) and it is defined as microscopic or macroscopic nodules with hyperplasia of glandular and stromal cells, mostly occurring at the gland's transition zone (39).

Among neoplastic lesions, prostate cancer (PCa) is the most common and it may be preceded by prostatic intraepithelial neoplasia (PIN) (43). Although most men display PIN lesions by the age of 50, only high-grade PIN (HGPIN) lesions are considered precursors of PCa, as they are

able to form multiple foci and present significant changes in cell phenotype. In PIN, the two-cell layer is preserved although normal luminal cells are replaced by neoplastic cells closely resembling PCa cells, and those have been shown to share multiple chromosomal abnormalities with PCa (44). These HGPIN lesions occur mostly in the peripheral zone.

Several factors may be responsible for inflammatory processes in the prostatic tissue, such as infectious agents, hormonal changes, dietary habits, physical trauma and urinary reflux (39). Inflammation of the prostate, which becomes more common with aging, is frequently associated with glandular atrophy and mostly located in the peripheral zone. Interestingly, a fraction of epithelial cells localized in these focal atrophy lesions shows the ability to proliferate, giving rise to the so-called proliferative inflammatory atrophy (PIA). PIA lesions are considered to potentially evolve either to HGPINs lesions or directly to PCa. Although benign, PIA lesions can share altered signaling pathways with PCa, namely downregulation of several tumor suppressor and caretaker genes (39).

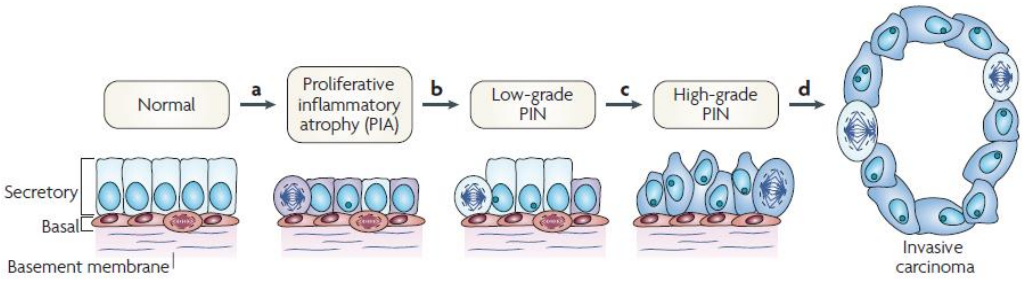

Figure 8. Cellular and molecular model of early prostate neoplasia progression [Adapted from: (39)].

Among all types of PCa, adenocarcinoma accounts for over 95% of cases, being originated from glandular cells (40). Prostate adenocarcinoma is mostly acinic, but may also be of other types, such as small-cell neuroendocrine, adenoid cystic and basal, squamous cell, urothelial and sarcomatoid (45). PCa and BPH share certain traits, such as hormone-dependent growth and response to anti-androgen therapy, but this neoplasm has often a silent/asymptomatic behavior. It is not uncommon for patients to be diagnosed when metastatic lesions have already developed (e.g. bone, brain, lymph nodes, lung and liver). Hence, the development of early diagnosis tools is of great importance for PCa patient management.

1.4.3. Prostate Cancer Epidemiology

PCa is the second most frequent type of cancer and the sixth leading cause of cancer death among men, worldwide (Figure 9) (46, 47). By the year of 2030, the number of PCa diagnosed cases is expected to reach an astonishing number of 1.7 million. In Portugal, in 2012, PCa ranked first in incidence and second in mortality, in men (48).

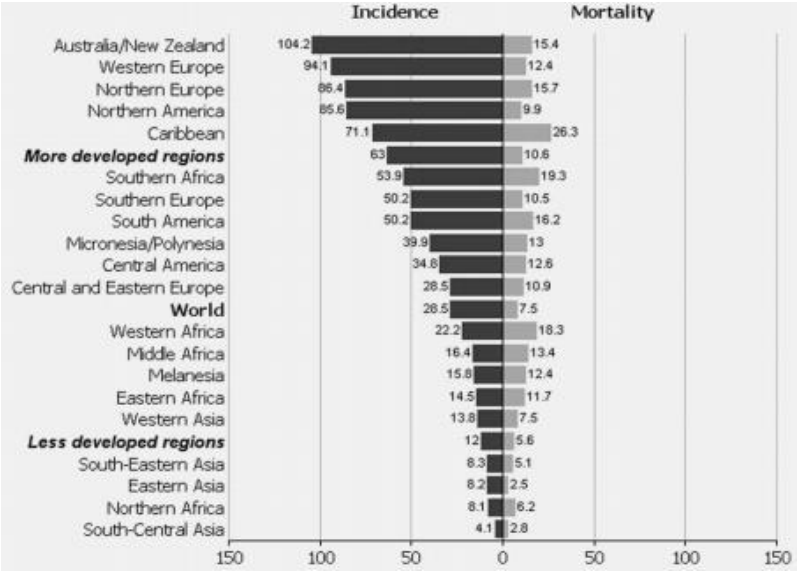

Figure 9. Estimated age-standardized incidence and mortality rates for prostate cancer [Adapted from: (46)].

1.4.4. Prostate Cancer Risk Factors

Although several factors are thought to contribute or increase the risk of PCa development, only three of them are well-established and widely accepted (49).

Family History: Familial PCa accounts for about 10-15% of all cases (50). Men whose family members have developed PCa are considered to be at high risk of developing PCa themselves, although the clinical and pathological features of the familial PCa are similar to those of non-familial cases (49). If affected family members are close relatives (e.g. father or brothers), the risk may be 2 to 8 times higher (51). The risk becomes even higher when multiple family members have been affected.

Age: PCa incidence strongly increases with age, probably due to the accumulation of oxidative stress (49). It increases nearly one hundred times from men aged 40-44 years to those between

70-74 years (52). Since PCa is characterized by a silent and slow-growing behavior, the development of pre-neoplastic lesions, that can forego PCa, may occur for many years or even decades before the malignant lesions are diagnosed. Indeed, theoretically, almost every men would develop PCa if they could reach 100 years old (53).

Ethnicity: Different ethnic groups have different risks of developing PCa (49). African-American men are those at higher risk, developing PCa 60% more frequently than Caucasian American men (52). In Europe, northern and central countries have lower incidence rates compared to southern and eastern countries. Those differences may be due to several factors: differences in exposure, dietary habits and genetic heritage, but also differences in the decision-making process, access to medical care and preferred diagnostic tools (54).

Although not firmly established, other factors have been associated with PCa, including smoking, obesity and diabetes (55-57).

1.4.5. Prostate Cancer Diagnosis

Since PCa is mostly asymptomatic at its earliest stages of development, improvement of patient's prognosis highly depends on early disease detection, while the malignancy is still confined to the gland (58). Thus, in many countries, men over 50 years, or even younger if they have familial history of PCa, are recommended to be screened for PCa yearly.

At present, PCa's detection is mostly based on digital rectal exam (DRE) and prostate-specific antigen (PSA) blood test (59). Abnormalities found in these exams, although not entirely reliable and accurate, are strong indicators for the presence of PCa. DRE has been recently falling into disuse. The PSA blood test is a widespread PCa screening methodology intended to detect PCa at early stages, in asymptomatic men, but also in men suffering from suspicious PCa symptoms (60). The PSA protein is specifically produced by prostate cells and, physiologically, is mainly found in semen. However, it also diffuses into the bloodstream where it may be detected. A high PSA concentration (i.e., above 4 ng/ml) is a strong indicator for the presence of PCa and, if the cancer does, in fact, exist, this parameter correlates with tumor extension. Although, in general, the higher the PSA concentration, the greater the chance that PCa is present, an abnormally high blood PSA concentration is not an unquestionable proof of the presence of PCa, since several other conditions, such as aging, BPH and inflammation, may also be the cause of it (61). Indeed,

PSA is specific for the prostate gland, and abnormal concentrations of this molecule can therefore inform for a several different physical conditions concerning the prostate. The inadequate use of the PSA molecule as an early diagnosis tool leads to the overdiagnosis and, consequently, to the overtreatment of PCa cases (62). These are tumors that would have never progressed to a symptomatic state or would never be clinically diagnosed, in the absence of the test (63).

The gold standard tool for PCa diagnosis is the prostate biopsy, in which a set of 12 to 16 small tissue cylinders are taken from the patient's prostate, and later analyzed by a pathologist (59). A Transrectal Ultrasound (TRUS) usually guides this procedure. The combination of both methodologies, named TRUS-guided systemic needle biopsy, represents the most reliable procedure to accurately identify the presence of PCa.

Although PCa's early detection is currently possible, the available screening and diagnostic methodologies still face several difficulties and challenges (13). Neither DRE nor PSA blood test are able to accurately identify the presence of cancer (64). Indeed, several prostatic disorders, other than PCa, may be responsible for increased production and/or diffusion of PSA into the bloodstream, including BPH, prostatitis, diet alterations, intake of medications and environmental factors (61, 65). Furthermore, these tools do not predict with accuracy the pathological stage of the disease (66). Other associated problems, such as a high rate of false positives, intensify the need for development of novel, accurate and non-invasive diagnostic tools to be used for early detection of PCa.

1.4.6. The Gleason Grading System

Following histological diagnosis of PCa, there is a need to ascertain its potential clinical aggressiveness based on tumor grading. In PCa, the Gleason grading system is the most widely used and is based on the histological appearance of neoplastic glands (67). When analyzing histologic samples of PCa, and since this type of cancer is usually heterogeneous, the two most predominantly observed Gleason patterns, each one of them graded from 1 to 5, are chosen and then summed together, giving rise to the Gleason score (GS), which ranges from 2 (1+1) to 10 (5+5) (Figure 10) (68). The higher the GS, the less differentiated and more aggressive is the tumor, and therefore, worse is the patient's prognosis. There are some limitations to the predictive accuracy of the GS, due to inter- and intraobserver variability, professional experience and

sampling issues. Accurate GS information is critical for the planning of post-radical prostatectomy management in patients with PCa, which includes evaluating the risk of recurrence and planning of additional therapy (69).

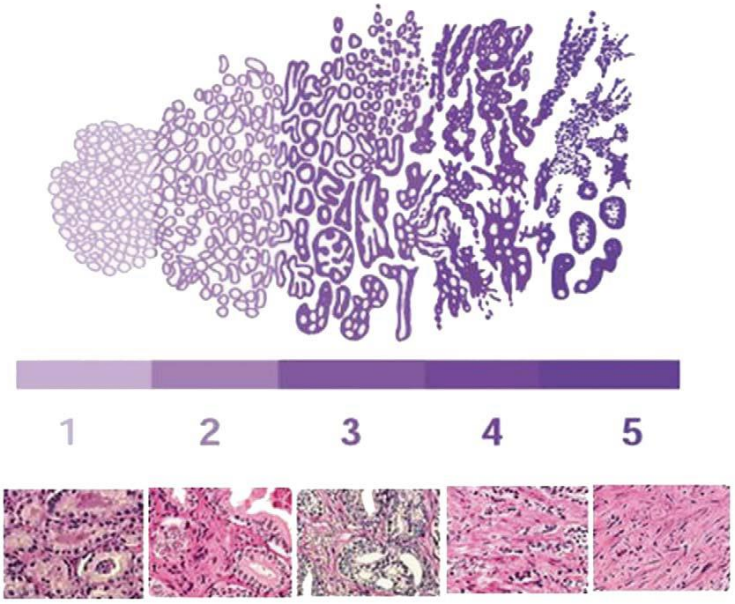

Figure 10. GS for PCa histologic grading [Adapted from: (68)].

1.4.7. Prostate Cancer Staging

Cancer staging can be divided in clinical and pathological, depending on the moment at which tumor staging is performed (70). Clinical staging is based on the collection of data prior to the first definite treatment, either by DRE, TRUS or other imaging techniques. On the other hand, pathological staging is based on the collection of histological data regarding the tumor's extent in the prostate and surrounding tissues. The staging of PCa, intimately related to the patient's prognosis, is performed according to the TNM (Tumor – Lymph Node – Metastasis) classification system, the staging tool most widely used for solid tumors (40). Tumors are staged according to the extent of the primary tumor (T category), invasion of regional lymph nodes (N category) and the absence or presence of distant metastasis (M category) (Figure 11) (71). The complete PCa staging process takes also into account information provided by the GS and PSA blood test. Altogether, each PCa case is ascribed to a specific stage category (I-IV), which becomes higher for more advanced and aggressive tumors.

Evaluation of the (primary) tumor (T)

- Clinical
  - TX: can not evaluate primary tumor
  - T0: no evidence of primary tumor
  - T1: clinically inapparent tumor neither palpable nor visible by imaging
  - T1a: tumor was incidentally found in less than 5% of prostate tissue resected
  - T1b: tumor was incidentally found in more than 5% of prostate tissue resected
  - T1c: tumor was found in a needle biopsy performed because of elevated serum PSA
  - T2: tumor confined within prostate[1]
  - T2a: the tumor is in half or less than half of one of the prostate gland's 2 lobes
  - T2b: the tumor is in more than half of one lobe, but not both
  - T2c: the tumor is in both lobes
  - T3: the tumor has spread through the prostatic capsule (if it is only part-way through, it is still T2)
  - T3a: the tumor has spread through the capsule on one or both sides
  - T3b: the tumor has invaded one or both seminal vesicles
  - T4: the tumor has invaded adjacent structures other than seminal vesicles (e.g. external sphincter, rectum, bladder, levator muscles, and/or pelvic wall)
- Pathologic (pT)[2]
  - 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 vesicles invasion
  - pT4: Invasion of rectum, levator muscles, and/or pelvic wall

Evaluation of the regional lymph nodes (N)

- (p)NX: regional lymph nodes were not assessed (sampled)
- (p)N0: there has been no spread to the regional lymph nodes
- (p)N1: there has been spread to the regional lymph nodes

Evaluation of distant metastasis (M)

- M0: there is no distant metastasis
- M1: there is distant metastasis
- M1a: the cancer has spread to lymph nodes beyond the regional ones
- M1b: the cancer has spread to bone
- M1c: the cancer has spread to other sites (regardless of bone involvement)

[1]Tumor found in one or both lobes by needle biopsy, but not palpable or reliably visible by imaging, is classified as T1c; [2]There is no pathologic T1 classification

Figure 11. The 2010 American Joint Committee on Cancer/International Union Against Cancer TNM Staging Classification for prostate cancer [Adapted from: (71)].

1.4.8. Prostate Cancer Therapy

At the early stages of PCa development (I/II), the outcome of patients is considered to be good, with over 90% of progression-free survival after 5 years (72). However, for more advanced stages (III/IV), long survival periods are less likely to occur. For patients with clinically localized tumors, standard treatment includes patient active surveillance/watchful waiting, an approach intended to spare patients with slow-growing and indolent tumors from the discomfort, pain and morbidity of unnecessary treatments (72). Radical prostatectomy and external beam radiotherapy are usually considered for more aggressive tumors, though still organ-confined, or locally advanced disease, respectively. For patients with advanced high-risk stage III or stage IV disease, androgen ablation-based therapy is implemented either by surgical or chemical castration. First-generation anti-androgen drugs include flutamide and bicalutamide. Unfortunately, mutations affecting the androgen receptor (*AR*) gene frequently occur under androgen-deprivation therapy, giving rise to a condition called castration-resistance that is associated with dismal prognosis (73). Standard treatment for patients who have developed metastatic castration-resistant PCa is based on conventional chemotherapy (e.g., docetaxel). Recently, several other therapeutic strategies became available, including new-generation anti-androgens, PCa vaccines and agents targeting bone metastasis (74-76).

1.5. Overview of the Role of MicroRNAs in Prostate Cancer

Epigenetic alterations play a major role in prostate carcinogenesis and may be identified from the earliest stages, the precursor lesions, to the most advanced forms of disease (13). Among these alterations, aberrant expression and function of miRNAs has a pivotal role in PCa's pathological pathways, being associated with critical cellular and physiological processes such as androgen signaling, apoptosis evasion, cell proliferation, migration and metabolism. Presently, over 50 miRNAs have been documented to be deregulated in PCa (4), although both function and targets still remain unveiled for most of them. Several miRNAs have been reported to be differentially expressed between normal and tumor tissue samples, arising as promising key participants in PCa carcinogenesis (77-79). Since 2007, the date of the first systematic miRNA profiling report in PCa by Porkka *et al.*, several studies have been conducted to unveil the roles

played by different miRNAs in prostate carcinogenesis (80). Currently, global miRNA profiling is performed using techniques such as microarray analysis. However, when studying a specific miRNA molecule, quantitative Real-Time polymerase chain reaction (RT-qPCR) should be used to validate the alterations that surface in the microarray analysis. Computational approaches are then used in order to identify specific target genes of the altered miRNAs (81). Frequently, different studies obtain contradictory results for the same miRNA molecule, making it difficult to conclude about its role in pathogenesis (82). This can be due to differences in study design by distinct research groups, underestimated treatment of the patients, different methods of sample collection, presence of contaminating cells and specificity and sensitivity of the used platforms. However, it can also be due to the fact that the same miRNA, depending on the type of cancer, or even in different stages of the same cancer, is expressed in different levels and plays different roles in the progress of the disease (83).

Despite the controversy in the literature concerning the role of certain miRNAs in prostate carcinogenesis, it is widely accepted that miRNAs are differentially expressed with disease progression. In other words, different miRNAs participate in different stages of PCa pathogenesis, each stage having its own characteristic miRNA profile or signature, which highlights the dynamic character of miRNA regulation, their ability to receive stimuli from the tumor microenvironment and respond accordingly, and their enormous potential for PCa diagnosis, prognosis and tumor staging (84). In a recent study, several miRNAs were found to be aberrantly expressed in the tumor stroma, instead of the tumor itself, highlighting the close and essential interaction that has to be established between neoplastic cells and their surrounding microenvironment, or stroma, so that the tumor may successfully develop, grow and invade the adjacent tissues (Figure 12) (79).

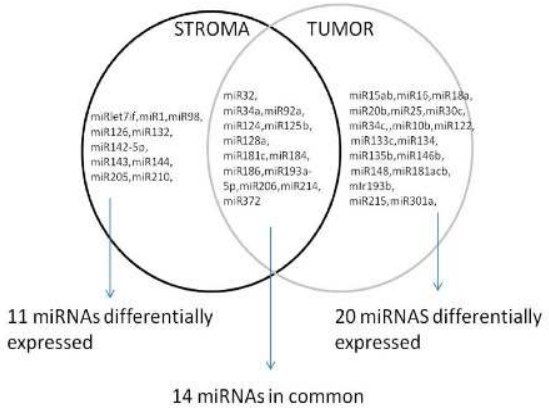

Figure 12. Differentially expressed miRNAs in PCa tumor and stroma [Adapted from: (79)].

1.5.1. Epigenetic Regulation of MicroRNAs in Prostate Cancer

MiRNAs have their levels of expression frequently regulated by other miRNAs and also other epigenetic mechanisms, such as DNA methylation and histone modification, forming complex feedback regulative loops, as depicted in Figure 13 (85). In fact, over 40% of miRNA codifying genes are located in the proximity of CpG islands, where extensive DNA methylation normally occurs. Tumor suppressor miR-145 has been previously reported to be downregulated in prostate cancer, mainly due to hypermethylation of its promoter (86, 87). Other relevant miRNAs that have been reported to be downregulated in PCa due to promoter hypermethylation include miR-21 and miR-205 (88-91). Additionally, miRNAs often act synergistically with other epigenetic mechanisms for repression of their target genes expression (13). For example, miR-101 is often downregulated in PCa, leading to an upregulation of its target gene, enhancer of zeste homolog 2 (*EZH2*), an important component of the epigenetic regulator polycomb complex. The deregulated activity of this enzymatic complex disturbs the expression levels of several other miRNA molecules, ultimately contributing to PCa progression (92).

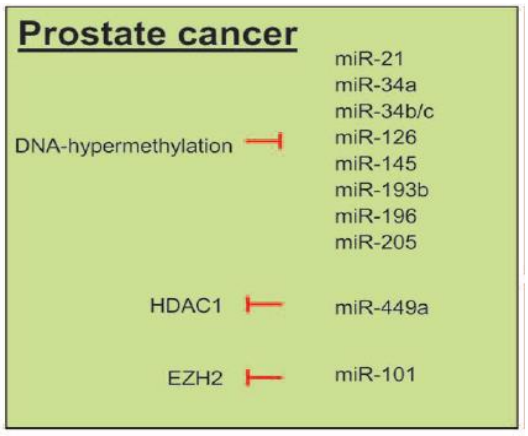

Figure 13. Epigenetics-miRNA regulatory circuit [Adapted from: (85)].

1.5.2. Involvement of MicroRNAs in Prostate Carcinogenesis

Deregulation of miRNAs expression in PCa is associated with apoptosis avoidance, as represented in Figure 14 (80), and at least 10 different miRNAs have been implicated. For example, expression of the E2F transcription factor 1 (*E2F1*) is regulated by miR-20a, which is located within the miR-17-92 cluster, along with five other miRNAs, and it is overexpressed in PCa, functioning as an oncomir (93). *E2F1* is critical for cell cycle progress and control, as well as p53 and caspase-

mediated apoptosis. MiR-25 and miR-205 also play an important role in the avoidance of apoptosis in PCa cells, targeting the same transcription factor (88, 94, 95). Oncogenic miR-21 represents one of the most important miRNAs in PCa development, attenuating the apoptotic pathways through targeting essential tumor suppressor genes, such as programmed cell death 4 (*PDCD4*) and phosphatase and tensin homolog (*PTEN*) (96, 97).

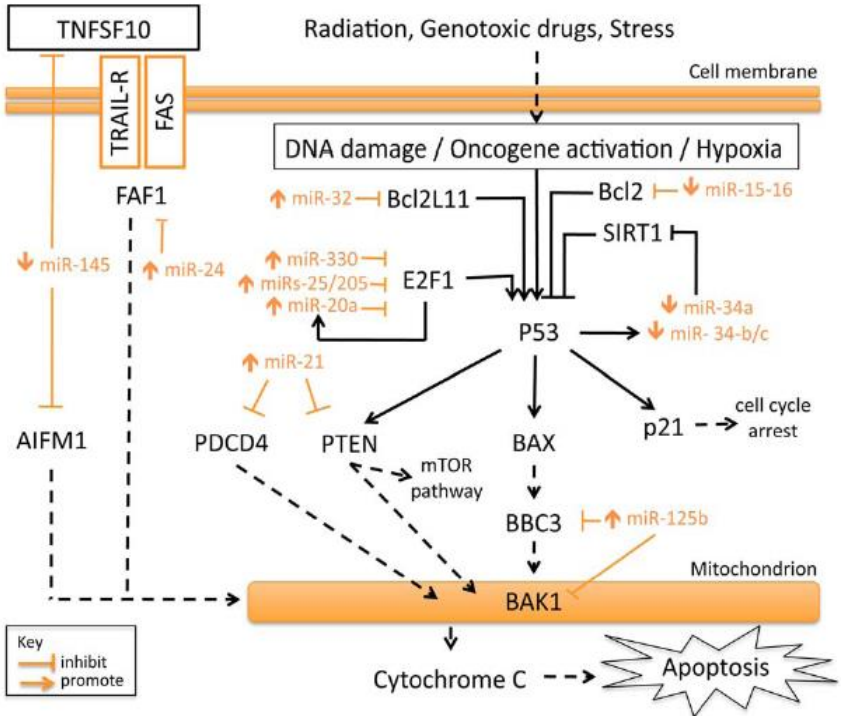

Figure 14. MiRNAs involved in the avoidance of apoptotic pathways in PCa [Adapted from: (80)].

MiRNAs can also act downstream of several transcription factors. The miR-34 cluster has tumor suppressor functions and establishes a tight regulatory loop with p53, the guardian of the genome. This loop becomes activated in cases of DNA damage or cell stress, and promotes cell cycle arrest and apoptosis through the downregulation of important genes codifying for adhesion molecules, cyclins, CDKs, sirtuin 1 (*SIRT1*) and the apoptotic factor B-cell CLL/lymphoma 2 (*BCL2*) (98-103). MiR-34 miRNA family is frequently lost or downregulated in PCa tumors and cell lines (104, 105). Other important miRNAs involved in cell cycle regulation and apoptosis are the tumor suppressor miR-15a and miR-16, frequently downregulated in PCa (106-109). Upon loss of this miRNA cluster, G1/S transition becomes facilitated and apoptosis is avoided, a consequence of the upregulation of the cyclin D1 (*CCND1*) and the anti-apoptotic factor BCL-2, respectively. This

miRNA cluster seems to target multiple oncogenic activities within the cell (80). MiR-221 and miR-222, both usually upregulated in PCa, are also crucial in cell cycle regulation and progression, acting as oncogenic miRNAs in PCa (110). Among their targets are key cell cycle regulators, including cyclin-dependent kinase inhibitors 1B and 1C (*CDKN1B* and *CDKN1C*), which inhibit the activity of CDKs, and *PTEN* (110-112).

Several miRNAs are also involved in aberrant androgen signaling, which is one of the most important pathways in PCa progression (80). In that perspective, miRNAs may either modulate androgen-related pathways, or act as androgen-responsive elements. MiR-125b belongs to the latter group, being frequently upregulated in PCa as a response to increased androgen production (113). This oncomir targets important genes that codify for proteins involved in cell cycle regulation, such as p53 and p53 target proteins, and apoptotic factors such as BCL2-antagonist/killer 1 (*BAK1*), leading to a blockage of apoptotic pathways and promoting androgen-independent tumor growth (113-115). Curiously, miR-125b has been reported to act as tumor suppressor in other cancer types, and also to be downregulated in PCa, highlighting the multifaceted character of miRNAs in different pathological contexts (77, 116, 117). MiR-21, which possesses an androgen-responsive element (ARE) in its promoter region, similarly to miR-125b, has been also reported to contribute to castration resistance, through pathways involving, among others, transforming growth factor β receptor II (*TGFBR2*) (118, 119). MiR-146a, a tumor suppressor miRNA, is also involved in promoting androgen-independent tumor growth, when downregulated in hormone refractory PCa, since its gene targets – elements of the Rho-associated coiled-coil containing protein kinase 1 (*ROCK1*) and epidermal growth factor receptor (*EGFR*) pathways - are key players in the acquisition of the castration-resistant phenotype (120, 121). When it comes to miRNAs involved in the modulation of androgen-related pathways, several candidates have already been reported, including the already mentioned miR-221/222 cluster and miR-331-3p (112, 122). The latter is frequently absent in PCa, leading to upregulation of its target gene, *ERBB-2*, which encodes a tyrosine kinase receptor. More recently, miR-19a, miR-17a and miR-133b have also been reported to be regulated by androgen receptor signaling pathways in LNCaP, an androgen-dependent PCa cell line (123).

1.5.3. MicroRNAs Deregulation in Prostate Cancer Metastasis

Since metastatic PCa remains an incurable disease, the study of the underlying molecular mechanisms ultimately responsible for tumor invasion and metastasis is of great clinical interest (78). The identification of miRNAs involved in tumor progression and metastasis, and the validation of the respective target genes, may lead to their future use as metastatic biomarkers and development of novel therapies targeting metastatic disease. Recent studies revealed a large number of miRNA molecules that may be involved in PCa metastasis, acting either as oncogenic or tumor suppressor elements (78, 103). These include miR-16, which is significantly downregulated in PCa metastatic models, suggesting a metastatic suppressive role (109, 124). Other miRNAs that may act as metastatic suppressors, and are downregulated in PCa metastatic disease, include miR-126*, 145 and 205 (88, 125, 126). MiR-34a has been suggested to be involved in the regulation of epithelial-to-mesenchymal transition (EMT) process, in which cancer cells go from a fixed epithelial phenotype to a mesenchymal phenotype, with increased motility and invasion capacity (103). During this transition, loss of typical epithelial markers, such as E-cadherin (*CDH1*) adhesion molecule, and the gain of mesenchymal markers, such as vimentin (*VIM*) and fibronectin (*FN1*), occur (127). MiR-34a is also thought to be involved in the regulation of cancer stem cells (CSCs) characteristics, through the regulation of the cluster of differentiation 44 (*CD44*) gene, which codifies for an important adhesion molecule that is one of the main biomarkers of stemness (103). CSCs comprise a small cell population within the primary tumor and are suspected to be the basis of EMT and metastatic behavior. MiR-143 and miR-145 target some EMT-related markers, thus acting in the suppression of metastasis formation (128-133). Other miRNAs with tumor suppressive functions, such as miR-29b and miR-143, whose downregulation in PCa is frequently associated with the metastatic process, are responsible for the regulation of the matrix metalloproteinases (*MMPs*) that act in the degradation of extracellular matrix components, promoting tumor invasion (134-136).

In conclusion, miRNAs seem to play crucial roles in every relevant pathway that contributes to the transformation of normal prostate epithelium into invasive and androgen-independent tumors (Figure 15) (82).

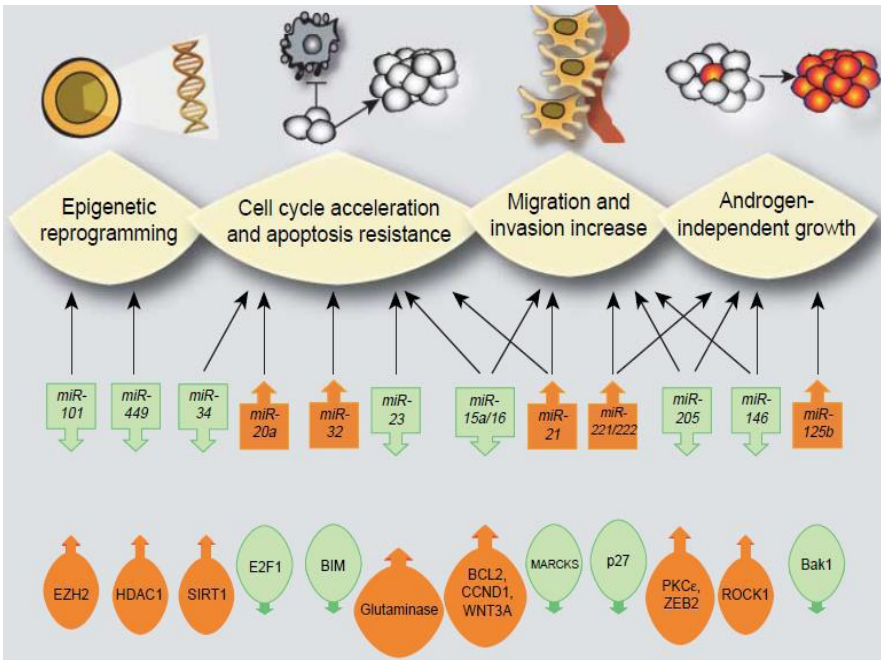

Figure 15. **MiRNAs – targets and functions with important physiological roles in PCa [Adapted from: (82)].**

1.5.4. c-Myc-Regulated MicroRNAs Are Key Players in Prostate Cancer

Recently there has been an attempt to establish a cause-consequence relation between the deregulation of several miRNAs that have been observed to be aberrantly expressed in PCa and the pathological activation of the *MYC* oncogene, a common upstream event in several human malignancies (82). This transcription factor has already been reported to bind to conserved regions in the promoter sequence of several miRNA genes, causing widespread miRNA downregulation (137). For example, the c-Myc-associated downregulation of miR-26a, miR-26b and miR-11 leads to upregulation of the gene codifying for the epigenetic regulator polycomb complex element (*EZH2*), which is known to promote PCa progression (35, 138, 139). Moreover, some miRNAs may be responsible for the regulation and suppression of c-Myc oncogenic functions in PCa, including miR-34a that was shown to suppress the activation of certain oncogenes through the inhibition of c-Myc transcriptional complexes (104). The fact that c-Myc is known to induce androgen-dependent PCa growth also supports the existence of a regulatory network established between this transcription factor and several miRNAs (140).

1.5.5. MicroRNAs Are Valuable Diagnostic, Prognostic and Predictive Tools in Prostate Cancer

As previously mentioned, aberrant miRNA expression patterns in PCa are of dynamic nature, changing along with the disease itself, thus allowing researchers to associate several altered miRNAs with different stages of PCa (Figure 16) (141). The identification and validation of miRNA signatures for each PCa stage is of great value, and may allow not only the development of novel diagnostic tools, but also help in the characterization of patients' prognosis, disease outcome and even provide information to better decide on the best therapeutic strategy. This becomes of major importance in PCa, which is characterized by heterogeneity and difficulty in patient's outcome prediction, due to slow and indolent growth and the ability of rapidly becoming lethal (142).

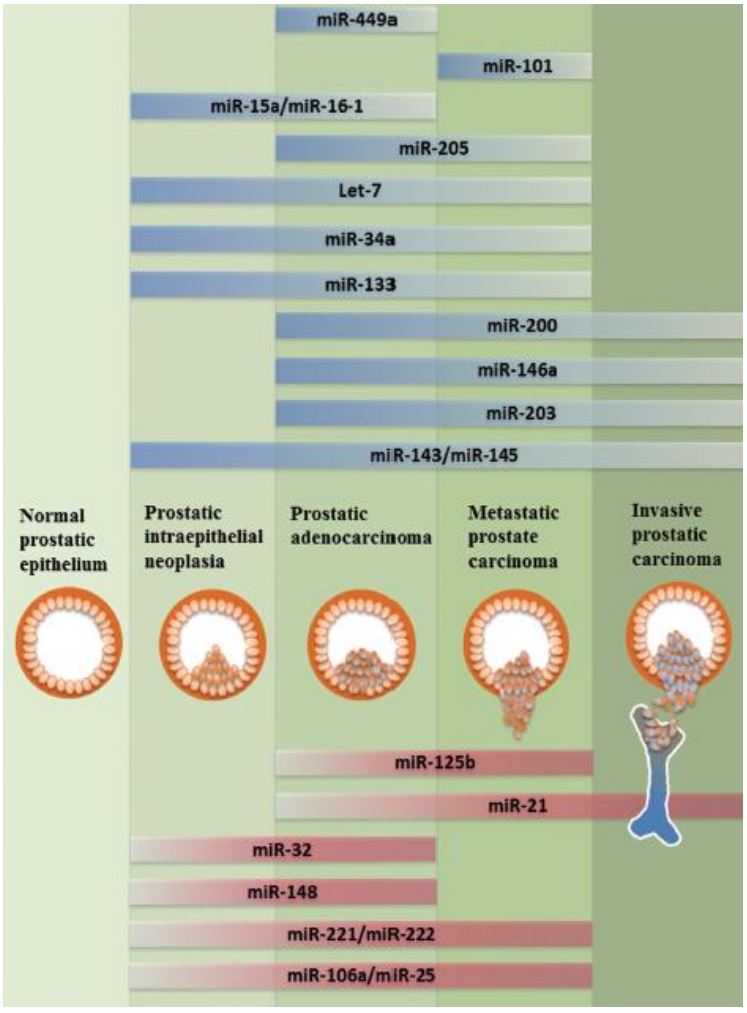

Figure 16. Key miRNAs involved in PCa pathogenesis [Adapted from: (141)].

As previously mentioned, the screening and diagnostic methodologies available for PCa still face several difficulties and challenges. In this context, miRNAs demonstrate features that are fit for PCa biomarkers (142). Their expression profiles are frequently tissue-, disease- and developmental-specific, being able to accurately discriminate tumors at different stages of development. Additionally, these molecules also show remarkable levels of stability in different clinical samples, as their small size and distinctive biochemical structure grant them higher resistance to RNase-mediated degradation. Moreover, miRNAs are easy to quantify by RT-qPCR and, because they are highly evolutionarily conserved among different species, animal models may be used in the search and validation of new biomarkers (143). Importantly, circulating miRNAs seem to be particularly resistant to extreme biochemical conditions and may be also be detected in other body fluids, such as breast milk and, most importantly for urological tumors, urine (144).

Several studies addressed the potential of miRNAs as promising PCa biomarkers. However, due to differences in miRNA array platforms, protocols for quantification, methods of statistical analyses and sample selection and size, concordance among these studies is rather low, preventing the definition of a clinical useful miRNA panel. Among all miRNAs detected in body fluids of PCa patients, miR-141 and miR-375 seem to be the most promising candidates (145-149). Other studies emphasized the diagnostic capability of miR-106a and miR-1274 in serum of patients with early stage PCa (150). MiR-21 and miR-221, which have been widely associated with PCa, have also been found to be elevated in plasma of PCa patients (147). Regarding the presence of abnormal miRNA levels in urine of PCa patients, a study revealed high levels of miR-107 and miR-574-3p (149). On the other hand, miR-205 and miR-214 were significantly downregulated in urine samples, successfully discriminating healthy individuals from PCa patients (151). Urine collection from PCa patients may be a very informative tool, as malignant cells are likely to be shed in urine before discharge (152). Another study, based on the use of tissue microarray methodology to identify miRNAs with diagnostic potential in PCa, indicated miR-205 as a promising PCa biomarker, as its downregulation is frequently associated with metastasis due to its involvement in EMT (89). Despite their potential as promising PCa diagnostic tools, circulating miRNAs may have their specificity impaired as the same miRNA may be involved in the tumorigenic process of several different cancers.

As previously mentioned, miRNAs can also be used as valuable prognostic and predictive tools, as they may be associated with different stages, histological features, aggressiveness and neoplastic behavior (153). This is of major importance, since it may influence therapeutic choices and prediction of disease outcome. Elevated blood levels of miR-141, miR-200b and miR-375 have been associated with castration-resistant metastatic PCa and high GS, discriminating these high-risk patients from those with low-risk, clinically localized PCa (146, 149). The same was reported for miR-21 and miR-221 (147). Expression of these miRNAs has also been associated with biochemical relapse, which consists in an increase of PSA blood levels in patients who have already undergone curative-intent surgery or radiation therapy (148). Other miRNAs have also been correlated with tumor aggressiveness, including miR-125b and miR-222 (64). Another study suggested a correlation between elevated tissue levels of miR-96 and high GS, as well as increased likelihood of recurrence (89).

Despite androgen deprivation therapy being the first line treatment for advanced-stage PCa, most patients will eventually develop a more aggressive form of disease, characterized by androgen-independent growth, formation of distant metastasis and very poor prognosis (154). A second line treatment, directed to patients who have been diagnosed with castration-resistant PCa, includes microtubule stabilizing agents, such as paclitaxel and docetaxel. Nonetheless, few successful therapeutic strategies are available for treating chemoresistant forms of PCa (155). Other important events and elements, in which miRNAs deregulation plays a key role in, including emergence of CSC phenotypes, EMT and metastatic capabilities, may be involved in the acquisition of chemoresistance. Since miRNAs have already been implicated in resistance to chemotherapy in several different human cancers, their role in chemoresistant PCa has been the subject of great interest, and some miRNAs have been associated with the drug resistance emerging during PCa treatment (156). A recent study uncovered the role of miR-34a and miR-200c in paclitaxel resistance in PCa cell PC-3 and DU145 cells, as both cell lines displayed low expression levels of these miRNAs in CSC populations (155). MiR-34a has also been reported to be involved in camptothecin chemoresistance in PC-3 cells, due to upregulation of its target gene, *SIRT1* (157). Elevated levels of miR-21, both in serum of PCa patients and in PCa cell lines, have also been associated with docetaxel and temozolomide resistance (158, 159). In a recent study, miR-141 showed potential to be used as a biomarker to assess patients' response to treatment and to predict clinical progression (160).

1.5.6. MicroRNAs in Prostate Cancer: A Therapeutic Perspective

When compared to more conventional drugs, miRNA-based compounds may have several advantages, including the ability to target several downstream effectors and genes, either by mimicking or inhibiting a single miRNA, and the potential to simultaneously use several miRNAs to obtain a synergistic effect (143). The small size of these molecules also prevents them from having antigenic properties. Despite the great potential of using miRNAs either as therapeutic agents or molecular targets, the development of miRNA-based therapies faces some challenges, one of the most limiting being their delivery to cancer cells, both in clinically localized disease or distant metastasis. This obstacle may be overcome through the incorporation of miRNA molecules or miRNA-based compounds in appropriate vehicles, capable of making their specific delivery to the affected organs, tissues or cell populations. Such vehicles include liposomes and other types of nanoparticles, which may have the ability to specifically target cancer cells, depending on their design (161-164). These carriers with nanometric dimensions, or the miRNAs themselves, can be associated with peptides with cell-penetrating properties or targeting elements (Figure 17) (165).

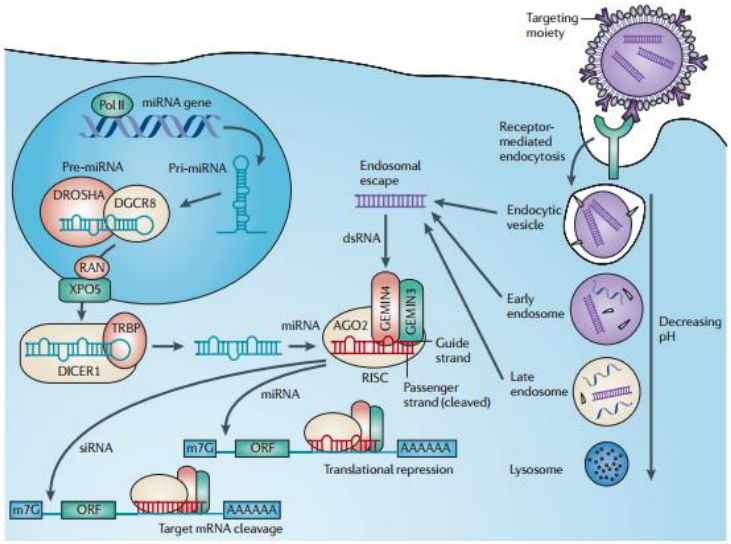

Figure 17. Intracellular fate of nanoparticles with encapsulated miRNA or small synthetic interference RNA (siRNA) following receptor-mediated endocytosis [Adapted from: (165)].

Other major challenges to the development of miRNA-based therapies include the disturbance of unintended mRNA targets, due to the fact that a single miRNA may regulate several different mRNA molecules, the inhibition of synthesis or processing of other known or yet to discover miRNAs and several aspects concerning the safety and toxicity associated with the developed therapeutic agents (166-168).

To date, no miRNA-targeted therapy has been implemented in clinical practice (143). MiR-141 and 375, whose levels are elevated both in serum and in tumor tissues of PCa patients, target important tumor suppressor genes, Sec23 homolog A (*SEC23A*) and zinc finger E-box binding homeobox family of transcription factors (*ZEB*), respectively, and are involved in preventing EMT, an essential step in the metastatic process (160, 169). Thus, those miRNAs represent promising molecular targets for development of novel therapies. In a recent study, the liposome-based delivery of miR-34a to PCa stem cells prevented these cells from replicating, through downregulation of *CD44*, a miR-34a target gene (103). In a different study, the systemic delivery of synthetic miR-16, frequently downregulated in PCa cells, showed inhibitory effects in PCa cell lines proliferation and growth of bone metastasis, in a PCa xenograft model, probably because this miRNA targets important cell cycle regulators such as cyclin-dependent kinases 1 and 2 (*CDK1* and *CDK2*) (124). Other studies evaluated the capability of certain compounds to restore normal miRNA expression in PCa. Recently, Sousa *et al.* demonstrated the ability of enoxacin, an antibiotic, to restore normal miRNA biogenesis and expression in PCa cell lines, attenuating the malignant phenotype (170).

There are several ongoing clinical trials addressing the role of miRNAs in PCa pathogenesis, but none of them is considering, so far, the potential use of miRNAs as therapeutic agents (143).

2. Aims of the Study

According to what was previously stated, it is becoming increasingly evident that miRNAs play a crucial role in many relevant cellular pathways involved in prostate carcinogenesis. However, many different aspects of their mechanisms of action, specific targets and integrated pathways, remain unveiled. Additionally, the regulatory crosstalk between the c-Myc transcription factor and several miRNAs appears to be a promising therapeutic target in prostate carcinogenesis, although many aspects of this interaction require further characterization. Understanding the precise way in which c-Myc controls the expression of different miRNA molecules, and also how some of them may affect the expression of this transcription factor, in PCa, represents an important step towards a better understanding of the biology of this complex disease.

Therefore, the main goal of this Master Thesis is to further explore the importance of the regulatory network that is established between c-Myc and different miRNAs, and its impact in prostate carcinogenesis. Prior to the start of this dissertation within the Cancer Biology and Epigenetics Group (CBEG), c-Myc transcript levels had been previously analyzed in a series of primary PCa, PIN lesions and morphologically normal prostate-tissues (MNPTs). Following these results, an expression array was made using total RNA from tumor samples containing high and low levels of *MYC* transcript levels to assess which candidate miRNAs could have their expression regulated by this transcription factor in PCa. Additionally, the *MYC* gene was silenced in a PCa cell line (PC-3) to validate the results obtained in the microarray array. The work of this Master Thesis consisted in accomplishing the following set of tasks:

1. Confirm and validate the silencing of the *MYC* gene in PC-3 cell line;
2. Assess the content of c-Myc transcription factor in the series of primary PCa cases, PIN lesions and normal tissues, at protein level, and associate it with the corresponding transcript levels;
3. Validate the importance of a group of miRNAs, identified in the expression array, in PCa carcinogenesis and investigate their potential regulation by c-Myc, through the assessment of their expression levels in a series of primary PCa, PIN lesions

and normal prostate tissues, as well as in the PCa cell line in which *MYC* gene was silenced;

4. Verify the association between the expression of different candidate miRNAs with clinical and pathological features of PCa;
5. Predict molecular targets of those miRNA and assess their relevance in prostate carcinogenesis.

3. Preliminary Data

The work comprised in this Master Thesis is integrated in a broader project of the CBEG (CI-IPOP-4/2012), whose main aim is to explore and understand the regulatory network established between c-Myc and miRNAs, as well as its importance in prostate carcinogenesis.

Firstly, *MYC* expression was assessed by quantitative RT-qPCR in a series of 198 primary PCa cases, 43 high-grade PIN lesions (from now on only referred to as "PINs"), and 13 morphologically normal prostate tissue samples (MNPTs) (Figure 18). PIN lesions showed the highest levels of *MYC* transcript, and both PIN lesions and primary PCa cases depicted, in a statistically significant manner, higher *MYC* levels compared to MNPTs. *MYC* expression was also assessed, at chromosome [fluorescence *in situ* hybridization (FISH)], transcript (RT-qPCR) and protein (Western blot) level, in six different PCa cell lines, which are *in vitro* models representative of PCa heterogeneity, three of which are hormone-dependent (LNCaP, 22Rv1 and VCaP) and the remaining are hormone-resistant (DU145, MDA-PCa-2b and PC-3). Taking together the transcript and protein levels, LNCaP and PC-3 displayed the highest expression levels of c-Myc.

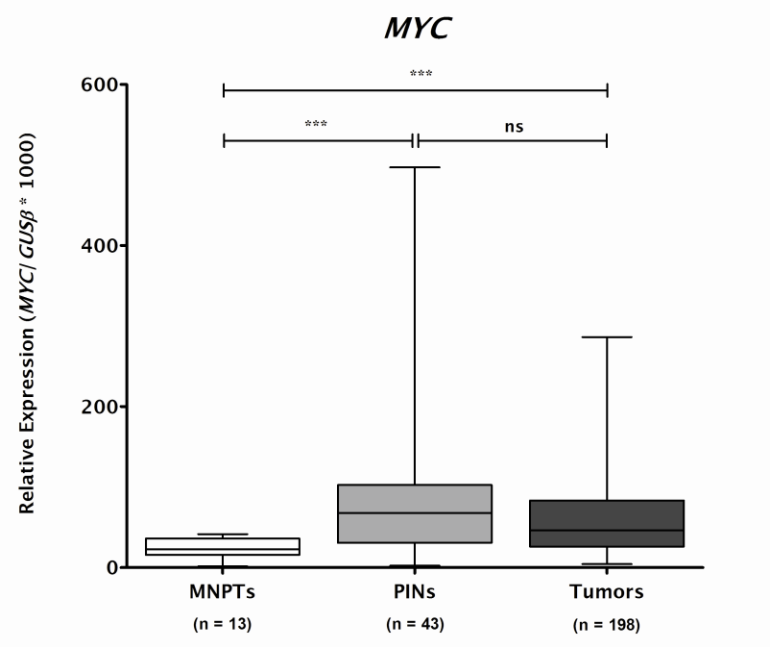

Figure 18. *MYC* transcript levels, assessed by RT-qPCR and normalized to *GUSβ*, in a series of primary PCa, PIN and MNPTs (* p < 0.001; ** p < 0.01; * p < 0.017; ns – non-significant).**

Three PCa cases with low *MYC* expression and four with high *MYC* expression were chosen for subsequent microarray analysis. Total RNA was then extracted, using the miRNeasy Mini Kit (Qiagen, Limburg, Netherlands) and used to perform a microRNA microarray, using the SurePrint G3 Human v16 miRNA 8x60K Microarray Kit with SurePrint Technology (Agilent Technologies Inc., Santa Clara, CA, USA). MicroRNA Spike In Kit (Agilent Technologies Inc.) was used to efficiently monitor microarray workflow for linearity, sensitivity and accuracy. The statistical analysis of the microarray was performed using R language (The R Project for Statistical Computing). Student's T-Test, with Bonferroni correction, was the chosen statistical method, with p-value < 0.01 and Fold Change > 1.3. The resulting heatmap, represented in Figure 19, shows only miRNAs that achieved statistical significance, and revealed 78 miRNAs that were overexpressed in samples with high *MYC* transcript content, representing possible targets of c-Myc regulation. From that list of candidates, a panel of 3 miRNAs (miR-27a*, miR-570 and miR-1292) was chosen for further validation of its abnormal expression and potential c-Myc regulation in PCa. Selection of miRNA candidates was based on a critical review of published studies so that miRNAs without prior documented implication in PCa or other cancer models were not considered for further analysis. Although it was not one of the top overexpressed miRNAs, miRNA-1292 was also included after a Rank Product non-parametric test was performed, with the aim of shortening the list of candidate miRNAs. Expression levels of miRNA-126*, although not listed in the miRNA microarray, were also assessed, since this miRNA has been described in the literature as a regulator of *MYC* gene expression, in multiple myeloma cells (171).

The *MYC* gene was permanently silenced in PCa cell lines using short hairpin RNA (shRNA) lentiviral particles. Although the initial intent was to silence the *MYC* gene in two distinct PCa cell lines, hormone-dependent LNCaP and hormone-resistant PC-3 (the ones that showed the highest *MYC* expression), this was only successfully accomplished, so far, for PC-3, probably due to LNCaP strong dependence on paracrine signaling that impaired the clonal propagation of single cells (172).

The work comprised in this Master Thesis provides continuity to the tasks already accomplished and described in this subsection.

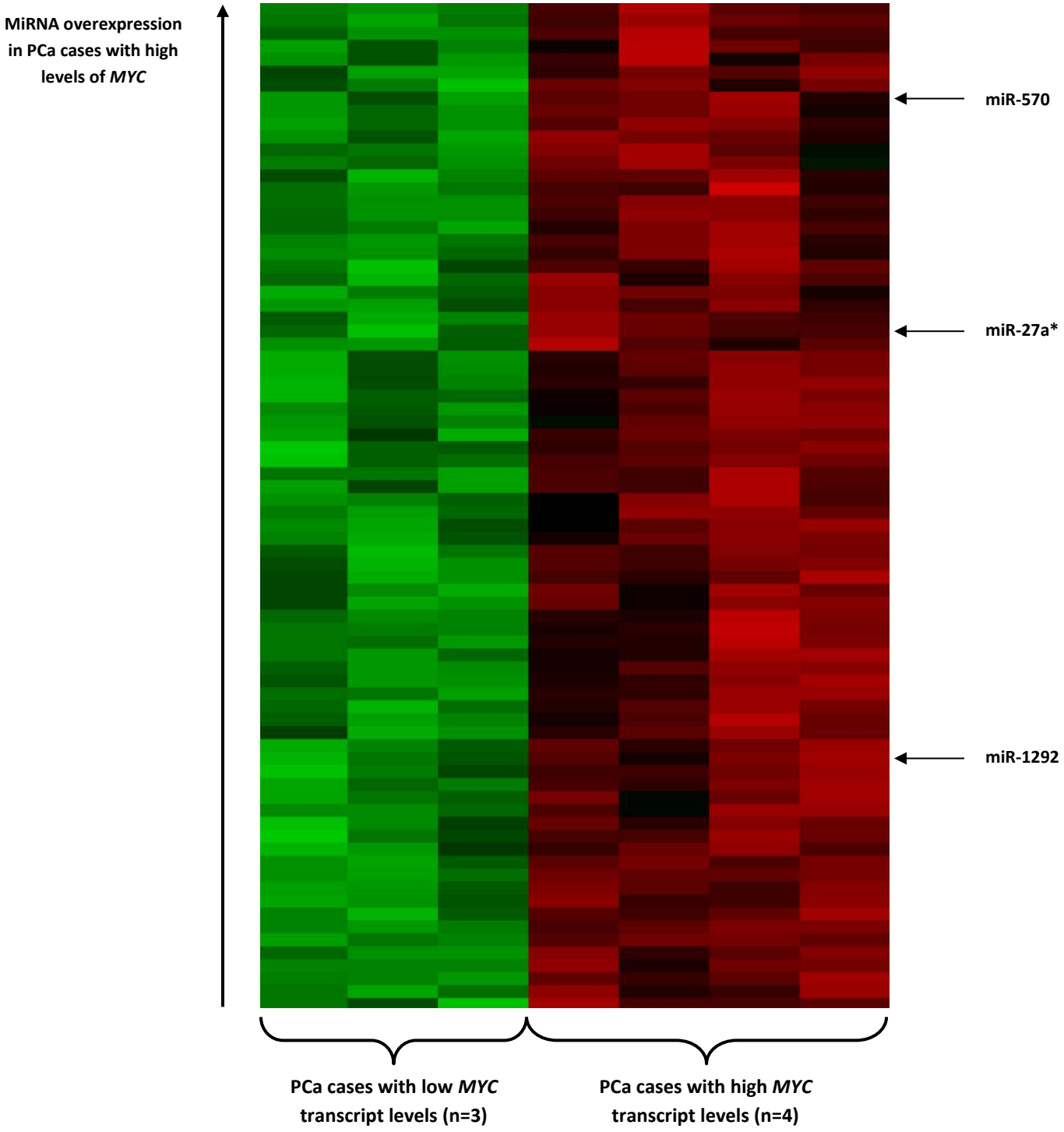

Figure 19. **Heatmap resulting from the miRNA microarray performed with total RNA extracted from PCa cases with high and low levels of *MYC* transcript. Selected miRNA candidates are indicated by the black arrows.**

4. Materials and Methods

4.1. Clinical Samples

4.1.1. Patients and Sample Collection

In the present study, samples of 198 prostate adenocarcinomas and 43 PIN lesions were prospectively collected from patients consecutively diagnosed with clinically localized disease and submitted to radical prostatectomy at the Portuguese Institute of Oncology – Porto, Portugal, between 2001 and 2006. As control samples, 13 MNPTs were collected from the prostatic peripheral zone of patients diagnosed with bladder cancer and submitted to cystoprostatectomy. All specimens were immediately frozen after surgical procedure and stored at -80^{o}C for further analysis. After histological confirmation of the presence of tumor and normal tissue within a specimen, fresh-frozen tissue fragments were trimmed to augment the yield of target cells (> 70%). Histological slides from formalin-fixed paraffin-embedded (FFPE) tissue fragments were also obtained from the same surgical specimens and assessed for Gleason grade and TNM stage. Relevant clinical data was collected from the clinical records, and these studies were approved by the institutional review board [Comissão de Ética para a Saúde-(IRB-CES-IPOFG-EPE 198/2012)] of Portuguese Institute of Oncology - Porto, Portugal.

4.1.2. Immunohistochemistry

Histological slides from FFPE tissue fragments were obtained from the same surgical specimens referred in the previous subsection, and samples were sectioned at a thickness of 4 µm. Slides were deparaffinized through two consecutive passages in xylene (Sigma-Aldrich®, St. Louis, MO, USA) and then hydrated in a series of ethanol solutions (Merck, Darmstadt, Germany) with decreasing ethanol content (100% - 90% - 70% - 50%). Epitope retrieval was performed with pre-heated ethylenediaminetetraacetic acid (EDTA) buffer (Thermo Scientific, Waltham, MA, USA) for 20 minutes, in a microwave oven, at a potency of approximately 700W. Endogenous peroxidase activity was neutralized for 20 minutes with 0.6% hydrogen peroxide (Merck). Protein detection was performed using the Novolink™ Max Polymer Detection System (Leica Biosystems, Nussloch, Germany), according to manufacturer instructions. Slides were incubated overnight with a rabbit

monoclonal antibody specific for c-Myc (Abcam®, Cambrige, United Kingdom) in a 1:100 dilution at 4°C inside a humid chamber. All washing steps were performed with tris buffered saline with Tween® 20 (TBS-T) (Sigma-Aldrich®). Antigen-antibody binding reaction was unveiled as the slides were incubated for 7 minutes, in the dark, in a 0.05% (m/v) 3,3'-diaminobenzidine (DAB) solution (Sigma-Aldrich®) in phosphate-buffered saline (PBS) (Biochrom Ltd., Cambridge, United Kingdom) activated with a 30% hydrogen peroxide solution, in a volume corresponding to 0.1% of the final DAB solution volume. Slides were counterstained with hematoxylin (Merck) for less than 5 seconds and washed for 1 minute in a 0.25% ammonium solution (Merck). Slides were dehydrated through consecutive passages in a series of ethanol solutions with increasing ethanol content (50% - 75% - 90% - 100%) and diaphanized through two consecutive passages in xylene. After the coverslip was mounted, slides were dried. As a positive control for the immunohistochemistry (IHC) reaction, FFPE tissue from a Burkitt's lymphoma was included.

Slides were observed at the optical microscope by an experienced pathologist for c-Myc immunoexpression evaluation. The used scoring criteria was previously reported by our research group (173) (0 – no observed expression; +1 – weak expression in ≤ 10% of the epithelial (in the case of MNPTs) and neoplastic cells; +2 – weak expression in > 10% of the neoplastic cells or moderate expression in ≤ 50% of the neoplastic cells; +3 – moderate/intense expression in > 50% of the neoplastic cells). Moreover, cases with a +1 score were considered negative for c-Myc expression, and cases with a +2 or +3 score were considered as positive for c-Myc expression.

4.1.3. RNA Extraction – TRIzol® Reagent Method

Total RNA was extracted from clinical samples with PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA, USA), according to manufacturer instructions. Briefly, 750 µL of TRIzol® Reagent (Invitrogen) were added to a tube containing a tissue sample. Each tissue sample was grinded and homogenized in 750 µL of TRIzol® with a homogenizing device (VWR International, PA, USA). Samples were incubated for 10 minutes at room temperature, after which, 300 µL of chloroform (Merck) were added. Then, tubes were briefly vortexed and incubated for three minutes at room temperature, before being centrifuged for 15 minutes at 12,000 g and 4°C. Afterwards, approximately 600 µL of the upper transparent phase, containing RNA, were transferred to a new tube and 600 µL of 70% ethanol were added, followed by agitation. Next, 700 µL of the previous mixture were transferred to a Spin Cartridge placed inside a collection tube, and centrifuged at

12,000 g for 15 seconds, at room temperature. The flow-through was subsequently discarded, and the last two steps were repeated until all the previously prepared mixture containing RNA was passed through the Spin Cartridge. At this point, 700 µL of Wash Buffer I were added and another centrifugation was performed. After this, Spin Cartridge was moved to a new collection tube. Next, 500 µL of Wash Buffer II were added to the Spin Cartridge and another centrifugation was performed. The previous step was repeated. Finally, a one minute centrifugation was performed to ensure maximum cleaning. Lastly, Spin Cartridge was placed in a new RNase-free tube to collect RNA by elution with 50 µL of RNase-free water, after an incubation of 3 minutes at room temperature and a 2 minutes centrifugation at 12,000 g. This step was repeated, so that a total of 100 µL of RNA solution was obtained. RNA concentrations and purity ratios were ascertained using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was verified by electrophoresis and RNA samples were stored at -80^{o}C.

4.1.4. MicroRNA cDNA Synthesis

MicroRNA complementary DNA (cDNA) synthesis was accomplished using the TaqMan® MicroRNA Reverse Transcription Kit and Megaplex™ Primer Human Pools A v2.1. and B v3.0. (Applied Biosystems®, Life Technologies™, Foster City, CA, USA). The latter contain two distinct sets (A and B) of stem-looped reverse transcription primers that allow the simultaneous synthesis, into cDNA, of over 750 different human miRNAs (Figure 20).

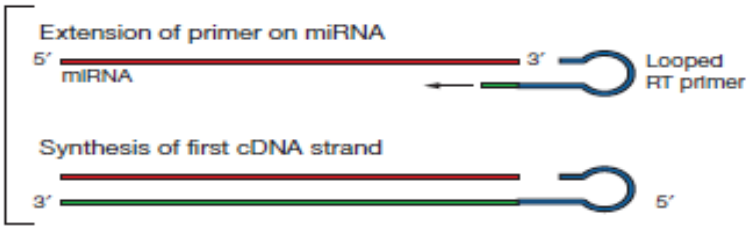

Figure 20. Specific miRNA cDNA synthesis [Adapted from: *TaqMan® MicroRNA Assays*, provided by Applied Biosystems®, Life Technologies™].

Briefly, distilled water (B. Braun Medical Inc., Melsungen, Germany) was added to each sample of extracted RNA to a final volume of 3 µL, containing 350 ng of RNA, in a nuclease-free PCR tube. Additionally, 1.6 µL of the Megaplex™ RT Primers, 0.4 µL of deoxynucleotide

triphosphates (dNTPs) with 2'-deoxythymidine 5'-triphosphate (dTTP) (100 mM), 3 µL of MultiScribe™ Reverse Transcriptase (50 U/µL), 1.6 µL of 10X RT Buffer, 1.8 µL of $MgCl_2$ solution (25 mM), 0.2 µL of RNase Inhibitor (20 U/µL) and 0.4 µL of nuclease-free water were also added to each tube. The tubes were gently vortexed, briefly centrifuged and incubated for 5 minutes on ice. Reverse transcription was performed in a Veriti® Thermal Cycler (Applied Biosystems®). Thermal-cycling conditions consisted in: 40 cycles constituted by 2 minutes at 16°C, 1 minute at 42°C, 1 second at 50°C and one holding stage of 5 minutes at 85°C. Samples were finally stored at -20°C.

4.1.5. MicroRNAs Expression: Individual Assays

RT-qPCR was performed using TaqMan® Small RNA Assays (Applied Biosystems®), in a 7500 Real-Time PCR system (Applied Biosystems®), according to the manufacturer instructions. A TaqMan® MGB probe contains a reporter dye (FAM™ Dye) linked to its 5'-end, and a minor groove binder (MGB) at the 3'-end. Additionally, there is a non-fluorescent quencher (NFQ) at the 3'-end of the probe. During the PCR reaction, the TaqMan® MGB probe specifically anneals to a complementary sequence between the forward and reverse primer sites (Figure 21). When the probe is intact, the proximity between the reporter dye and the quencher dye suppresses the fluorescence of the first one, primarily by Förster-type energy transfer. The DNA polymerase cleaves only probes that are hybridized to the target. Once the probe is cleaved, the reporter dye and quencher dye become separated, resulting in increased fluorescence by the reporter. However, the increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is amplified during PCR, which avoids non-specific amplification. Polymerization of the strand continues, but because the 3' end of the probe is blocked, there is no extension of the probe during PCR (174).

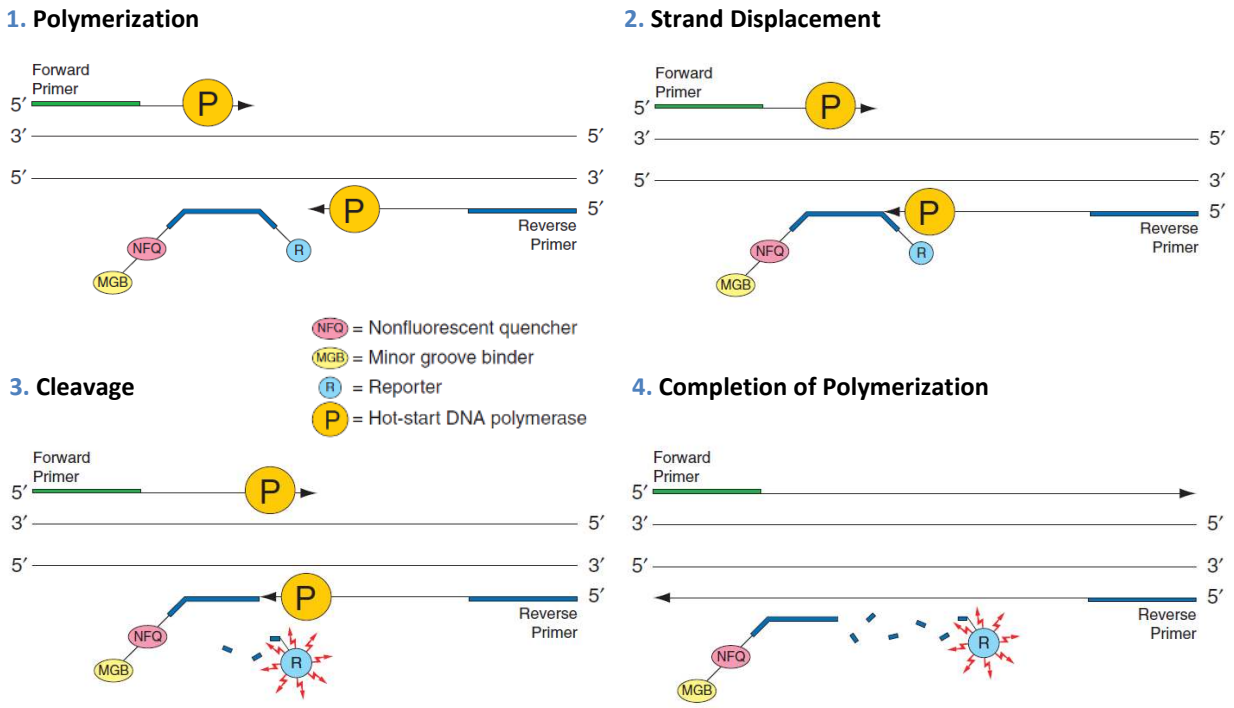

Figure 21. **Nuclease assay process [Adapted from: *TaqMan® MicroRNA Assays*, provided by Applied Biosystems®, Life Technologies™].**

The Megaplex™ RT products were thawed on ice. Then, each well of a 96-well plate received: 5 µL of TaqMan® Universal Master Mix II, with no uracil-DNA glycosylase (UNG) (Applied Biosystems®, Life Technologies™), 0.5 µL of TaqMan® Small RNA Assay (20X) (Applied Biosystems®, Life Technologies™), 0.75 µL of Megaplex™ RT product and 3.75 µL of nuclease-free water. Each sample was run in triplicate, and, in every plate, two negative template controls (reaction mix) were included. Synthetized similarly to all samples, cDNA from total human RNA (Agilent Technologies Inc.) was included, in each plate, in five consecutive 1:10 dilutions. These serial dilutions allowed the generation of a standard curve for relative quantification and also, to ascertain PCR efficiency. According to the manufacturer instructions, the running method consisted in: a holding stage of 2 minutes at 50°C, followed by another holding stage at 95°C for 10 minutes, during which enzyme activation occurs, and 40 cycles composed of a denaturation stage at 95°C that lasts for 15 seconds and an annealing/extending stage at 60°C during 60 seconds.

RNU6B was used as a reference gene for normalization, since it is known to be stably expressed in PCa and other cancer models and, therefore, the most suitable for this analysis (175).

Relative expression of targets tested in each sample was obtained using the following formula:

$$\textbf{\textit{Relative Expression}} = \frac{miRNA\ Candidate\ Mean\ Quantity}{RNU6B\ Mean\ Quantity}$$

The obtained ratio was then multiplied by 1000 for easier tabulation. In Table 2 can be found a list of all the individual assays that were used, the specific sequence that each one targets and the Megaplex™ RT Primers Pool containing the specific primers for the reverse transcription reaction.

Table 2. Specific target sequences and Megaplex™ RT Primers Pools of the studied miRNAs.

MiRNA	Target Sequence	Megaplex™ RT Primers Pool
RNU6B	5'-CGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTTT-3'	A v2.1.
miR-27a*	5'- AGGGCUUAGCUGCUUGUGAGCA-3'	B v3.0.
miR-126*	5'- CATTATTACTTTTGGTACGCG-3'	B v3.0.
miR-570	5'- CGAAAACAGCAAUUACCUUUGC-3'	A v2.1.
miR-1292	5'- TGGGAACGGGTTCCGGCAGACGCTG-3'	B v3.0.

4.2. *In Vitro* Studies

4.2.1. Cell Culture

In the present study, two PCa cell lines were chosen for *MYC* gene silencing: PC-3 and LNCaP. However, as previously explained, successful silencing was only achieved in PC-3 cell line. PCa cell lines are valuable *in vitro* tools to study PCa, possessing different features and behaviors that can be representative of distinct stages of the disease. However, it is important to keep in mind that they differ from prostate adenocarcinoma in several aspects, and comparisons with

primary tumors must be made with caution. These two cell lines have significant different features, namely their sensitivity to hormonal stimuli (172). LNCaP is considered to be androgen-sensitive and PC-3 manifests a castration-resistant phenotype (androgen-independent). Furthermore, their origin also differs: LNCaP cell line is originated from lymph node metastasis and PC-3 is originated from lumbar metastasis in Caucasian males. Both cell lines used in this study were kindly provided by Prof. Ragnhild A. Lothe, from the Department of Cancer Prevention at The Institute for Cancer Research at Oslo, Norway.

All PCa cell lines were grown using the recommended medium and correspondent supplements, which can be consulted in Table 3, as well as 1% of penicillin-streptomycin (P-S) (GIBCO®, Life Technologies™, Carlsbad, CA, USA). Cell culture flasks were kept at 37°C and 5% CO_2, inside a humidifying chamber. The use of a dissociation reagent, TrypLE™ Express (GIBCO®), allowed cells harvesting and culture in the appropriate number of 75 cm^3 cell culture flasks (Sarstedt, Nümbrecht, Germany). All cell lines were routinely tested by a specific multiplex PCR for contamination by *Mycoplasma spp*.

Table 3. PCa cell lines growth conditions: used mediums and respective supplements.

PCa Cell Line	Growth Medium	Supplements
LNCaP	RPMI-1640	10 % Fetal bovine serum (FBS)
PC-3	F-12 + RPMI-1640	

4.2.2. *MYC* Gene Silencing

MYC gene silencing in PC-3 cell line was achieved through the use of particles carrying the pGIPZ lentiviral vector containing a shRNA sequence targeting *MYC* (Thermo Scientific) that functions as a small interfering RNA (siRNA) (Figure 22). Three different shRNAs were used, although their specific sequence was not revealed by the Thermo Scientific Company. As a negative control, one scrambled siRNA (sh-scramble RNA) sequence was used, with the same nucleotide composition as the three shRNAs randomly ordered. This sequence was designed so it would have no known target in the PC-3 cell line's genome. The use of a negative control is

important, so that sequence-specific silencing can be distinguished from non-specific effects that the particles may have on the cells.

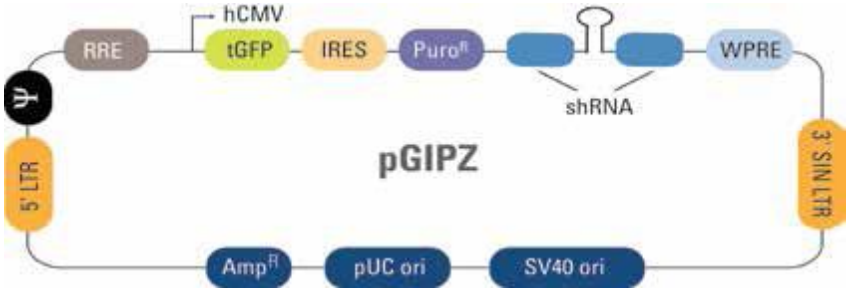

Figure 22. pGIPZ lentiviral vector [Adapted from: *Thermo Scientific GIPZ Lentiviral sh-MYC*, provided by Thermo Scientific].

Briefly, cells were cultured on a 24-well plate at a density of 5 x 10^4 cells/ml (1 ml per well), in complete growth medium, supplemented with fetal bovine serum (FBS) and P-S. Plates were incubated overnight at 37°C and 5% CO_2 in a humidifying chamber. At day 1, growth medium was removed and 15 µL of the lentiviral particles were added, together with 250 µL of serum-free media supplemented with 5 µg/mL of polybrene, which increases the efficiency of transfection. Five hours after transfection, 1 ml of complete growth medium was added to each well. After 48 hours from the transfection, cells were incubated with growth medium supplemented with puromycin, at a concentration of 2 µg/mL for LNCaP and 4 µg/mL for PC-3. Puromycin was used as a selection agent, since the used lentiviral vectors included the gene responsible for the resistant phenotype to this antibiotic. Fresh selective medium was added to the cells every 2-3 days.

The above-described procedure did not lead to a significant reduction of the *MYC* gene expression. This was probably caused by the fact that not all cells successfully incorporated the lentiviral vector, giving rise to a cell population heterogeneous for *MYC* expression. Therefore, clonal selection was performed with the previously transfected cells. Briefly, cells were counted and cultured, in selective medium, in 96-well plates, at a density of 1 cell/well. Clonal propagation proceeded until cells were able to fill 75 cm^3 cell culture flasks. Clonal selection was not possible for LNCaP cell line since growth was not achievable from a single cell, probably due to the lack of paracrine stimuli and factors. At this moment, *MYC* silencing was confirmed in PC-3 both by RT-qPCR and Western Blot.

4.2.3. RNA Extraction – TRIzol® Reagent Method

Cell culture flasks (75 cm^3) at 100% confluence with transfected PC-3 cells originated by clonal selection and propagation were harvested with a dissociation reagent, TrypLE™ Express (GIBCO®) and centrifuged for 5 minutes at 1,200 rpm. Cell pellets were ressuspended in 1 mL of PBS (GIBCO®), and centrifuged for 5 minutes at 1,200 rpm. The supernatant was discarded and cell pellets were stored at -80^oC.

Total RNA from PC-3 cell line was extracted by TRIzol® Reagent, according to manufacturer instructions. In brief, cell pellets were defrosted on ice and ressuspended in 1 mL of TRIzol® Reagent. A 0.9 mm needle was used to homogenize the cell suspension and, after that, samples were incubated for 5 minutes at room temperature. Finally, 200 µL of chloroform (Sigma-Aldrich®) were added. Samples were briefly vortexed for about 15 seconds and incubated for 3 minutes at room temperature. Tubes were then centrifuged at 11,900 g for 15 minutes at 4^oC. The upper transparent phase containing RNA was transferred to a new tube RNase-free and 500 µL of 100% isopropanol (Merck) were added. Tubes were vigorously shaken by inversion and RNA precipitation was achieved by a 10 minutes incubation of the tubes at room temperature, followed by a 10 minutes centrifugation at 11,900 g at 4^oC. Supernatant was discarded and RNA pellets were washed with 1 mL of 75% ethanol. Samples were briefly vortexed and centrifuged for 5 minutes at 8,600 g at 4^oC. To enhance pellet washing, the ethanol addition step and the centrifugation that followed were repeated. Finally, supernatant was discarded and RNA pellets were dried during 15 to 20 minutes. Next, pellets were eluted in 40 µL of RNA storage solution (1 mM sodium citrate, pH 6.4) (Ambion®, Life Technologies™, Foster City, CA, USA). RNA concentrations and purity ratios were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA quality was assessed by electrophoresis and RNA samples were stored at -80^oC.

4.2.4. RNA Extraction – miRNeasy Mini Kit

Total RNA from cells and tissues, extracted through the TRIzol® Reagent method, is not appropriate for posterior microarray analysis. For that reason, total RNA from the PCa cases used in the preliminary microarray and from PC-3 cells was extracted according to a more appropriate protocol, using the miRNeasy Mini Kit (Qiagen, Limburg, Netherlands), according to manufacturer instructions.

Briefly, 700 µL of QIAzol Lysis Reagent were added to the samples (cell pellets, previously obtained and washed with PBS, in the case of PC-3 cells, or fresh tissue for PCa cases), followed by homogenization [by vortex, in the case of PC-3 cells, and disposable pestles attached to a cordless motor (VWR International), in the case of fresh tissues], to allow sample disruption. Samples were then incubated at room temperature for 5 minutes and 140 µL of chloroform (Sigma-Aldrich®) were added, following vigorous vortexing for 15 seconds. Samples were incubated at room temperature for 3 minutes, followed by a 15 minutes centrifugation at 12,000 g and 4°C. The upper aqueous phase was transferred to a new collection tube, to which 525 µL of 100% ethanol were added. For each sample, a total of 700 µL were loaded into the RNeasy® Mini columns in a 2 ml collection tube, which were centrifuged for 15 seconds at room temperature at 8,000 g. From this step on, flow-through in the collection tubes was immediately discarded after centrifugation. This step was repeated until the entire sample was loaded in the column. Then, 700 µL of RWT Buffer were added to the RNeasy Mini columns, followed by another centrifugation of 15 seconds at room temperature at 8,000 g (optional step, if the sample is composed by cultured cells). Then, 500 µL of Buffer RPE were loaded onto the RNeasy Mini column, followed by a centrifugation of 15 seconds at room temperature at 8,000 g. The previous step was repeated, with a centrifugation of 2 minutes at room temperature at 8,000 g. Collection tubes were discarded and each column was transferred to a new 2 ml collection tube, followed by a centrifugation of 1 minute at room temperature at maximum speed. The columns were transferred to a new 1.5 mL collection tube, and 30 µL of RNase-free water were added to each column. Columns and collection tubes were centrifuged for 1 minute at room temperature at 8,000 g. To obtain maximum RNA concentration, the previous step was repeated, with an additional 30 µL of RNase-free water. RNA concentrations and purity ratios were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA quality was verified by electrophoresis and RNA samples were stored at -80°C.

4.2.5. cDNA Synthesis

In order to evaluate *MYC* transcript expression in PC-3 cells upon silencing protocol, cDNA was synthesized from total extracted RNA. A cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®). Briefly, distilled water was added to a final volume of 10 µL to each RNA sample (1,000 ng) in a nuclease-free PCR tube. On ice, to each tube were also added: 2 µL of 10X RT Buffer, 0.8 µL of dNTP Mix (100 mM), 2 µL of 10X RT

Random Primers, 1 µL of MultiScribe™ Reverse Transcriptase, 1 µL of RNase Inhibitor and 3.2 µL of nuclease-free water. Afterwards, tubes were gently vortexed and briefly centrifuged. Reverse transcription was performed in a Veriti® Thermal Cycler (Applied Biosystems®). Thermal-cycling conditions consisted in: 10 minutes at 25°C, 120 minutes at 37°C and 5 minutes at 85°C. Samples were then stored at -20°C.

4.2.6. *MYC* Expression Assay

MYC transcript levels were quantified by RT-qPCR in PC-3 cells. Samples of cDNA were diluted 10X in distilled water and reactions were carried out in 96-well plates using a 7500 Real-Time PCR system (Applied Biosystems®). Briefly, in each well, 10 µL of TaqMan® Universal PCR Master Mix (Applied Biosystems®), 1 µL of TaqMan® Gene Expression Assay, specific for *MYC* (Applied Biosystems®), and 9 µL of previously diluted cDNA sample were added. Each sample was run in triplicate, and, in every plate, two negative template controls were included. According to the manufacturer instructions, the running method consisted in: 2 minutes at 50°C, followed by a stage of 10 minutes at 95°C, for enzyme activation, and 40 cycles composed of a denaturation stage at 95°C for 15 seconds and an annealing/extending stage at 60°C during 60 seconds.

Analysis to ascertain *MYC* silencing was performed using ΔΔCt method, where PC-3 cells treated with sh-scramble RNA particles, from now on addressed to as sh-scramble, were used to normalize results for PC-3 cells treated with shRNA particles specific for *MYC*, from now on addressed to as sh-MYC. Beta-glucuronidase (*GUSβ*), a housekeeping gene, was used as a reference gene to normalize results obtained for the *MYC* gene. Moreover, miRNAs that were chosen for further validation were also quantified in these same samples and normalized according to the same method. As previously mentioned, mean values for *RNU6B* housekeeping gene were used for normalization. In these experiments, fold variation of the target genes was obtained using the following formula:

$$\Delta\Delta Ct = \underbrace{(\Delta Ct_{\text{Target Gene}} - \Delta Ct_{\text{Reference Gene}})}_{\text{sh-MYC PC-3 Cells}} - \underbrace{(\Delta Ct_{\text{Target Gene}} - \Delta Ct_{\text{Reference Gene}})}_{\text{sh-scramble RNA PC-3 Cells}}$$

4.2.7. Protein Extraction

Protein was extracted from whole-cell lysates using the radioimmuno precipitation assay (RIPA) (Santa Cruz Biotechnology Inc., Dallas, TX, USA). Briefly, growth medium was removed from 25 cm^3 cell-culture flasks, and cells were washed with 2 mL of PBS to allow removal of residual culture medium. RIPA buffer was prepared, on ice, in an appropriate volume: 10 µL of phenylmethylsulfonyl fluoride (PMSF) protease inhibitor, 10 µL of sodium orthovanadate phosphatase inhibitor and 20 µL of protease inhibitor cocktail (PIC) per mL of 1X RIPA Lysis Buffer (Santa Cruz Biotechnology Inc.). After PBS removal from cell-culture flasks, 100 µL of 1X RIPA Lysis Buffer were added to each flask. Cells were scrapped with the help of a cell scrapper (Santa Cruz Biotechnology Inc.), to promote cell detachment and lysis. Cells were collected to a 1.5 mL tube, which was gently shaken every 5 minutes while incubating on ice for 15 minutes. After that, tubes containing cell lysates were centrifuged for 30 minutes at 13,000 rpm at 4°C. Supernatant was carefully transferred to a new 1.5 mL tube. Extracted protein samples were stored at -20°C.

4.2.8. Protein Quantification

Concentration of total protein from the previously obtained cell lysates was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific), according to manufacturer instructions. This is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion (176). This water-soluble complex exhibits a strong absorbance at 562_{nm} that is nearly linear with increasing protein concentrations over a broad working range (20-2,000 µg/mL). Briefly, a series of bovine serum albumin (BSA) dilutions were prepared, in PBS, with well-known protein concentrations and were later used as standards to create a non-linear regression, correlating absorbance at 562_{nm} and total protein concentration. Cell lysates were diluted 5X, on ice, in PBS to a final volume of 25 µL. Both BSA standards and diluted samples were incubated for 30 minutes at 37°C, inside a humidifying chamber, after receiving 200 µL of a mixture of Reagent A and Reagent B, combined in a 50:1 proportion. Upon completion of the incubation period, 200 µL of each diluted sample and BSA standard was transferred to a 96-well plate (Ratiolab®, Dreieich, Germany). Samples and BSA standards absorbance was red at 562_{nm}, in the high-performance, filter-based multimode microplate reader FLUOstar Omega (BMG Labtech, Ortenberg, Germany). A four-parameter fit

model was used for curve-fitting the samples 562_{nm} absorbance to the non-linear regression built from the BSA standards, and to determine sample total protein concentration.

4.2.9. Western Blot

MYC gene silencing and alterations in the protein levels of *EGFR* and *MTOR* were assessed by Western Blot analysis. Briefly, to 10 µg of total protein, in the case of *MYC*, and 30 µg in the case of *EGFR* and MTOR, completed with PBS up to a final volume of 20 µL, were added 5 µL of 5X loading buffer. Samples were heated for 5 minutes at 95°C to promote protein denaturation, briefly centrifuged and finally loaded in a 10% sodium dodecyl sulfate polyacrylamide gel, for further electrophoresis (SDS-PAGE). Protein separation was achieved with a voltage of 120 V at room temperature. Proteins were blotted onto 0.2 µm nitrocellulose membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA) for 1 hour at a voltage of 50 V at 4°C. After that, membranes were blocked for one hour at room temperature, while shivering, with a 5% milk solution in TBS-T. Membranes were incubated overnight at 4°C with primary antibody: rabbit monoclonal antibody for c-Myc (Abcam®), in a dilution of 1:5000, rabbit monoclonal antibody for mTOR (Cell Signaling Technology, Inc., Danvers, MA, USA), in a dilution of 1:1000, and rabbit monoclonal antibody for EGFR (Kinexus, Vancouver, Canada), in a dilution of 1:3000, in 5 mL of 5% milk in TBS-T, in a 15 mL tube with rotational agitation. Membranes went through three washing steps of 10 minutes in TBS-T, before being incubated with the secondary antibody, goat anti-rabbit and goat anti-mouse immunoglobulin G (IgG) (H+L) horseradish peroxidase (HRP) conjugate (Bio-Rad Laboratories Inc.), in a dilution of 1:300 in 5 mL of 5% milk in TBS-T, for 1 hour with rotational agitation at room temperature, in a 15 mL tube. Membranes were washed for three additional 10-minute periods in TBS-T, while shaking. After that, membrane developing was performed with the Clarity™ Western ECL Substrate (Bio-Rad Laboratories Inc.) and Amersham™ Hyperfilm ECL (GE™ Healthcare, Buckinghamshire, United Kingdom), to which membranes were exposed.

Beta-actin, or β-actin (*ACTB*), was used as a loading control, to ensure equal protein loading in the gel. Membrane stripping was achieved with a washing step of 15 minutes, with vigorous agitation, in Antibody Erasing Buffer (Komabiotech, Seoul, South Korea), followed by five additional washing steps of 5 minutes, with agitation, with distilled water. Membranes were probed with the primary antibody, mouse β-actin (Sigma-Aldrich®), in a dilution of 1:8000 in 5 mL

of 5% milk in TBS-T in a 15 mL tube, for 30 minutes at room temperature, with rotational agitation. After that, membranes went through three washing steps of 10 minutes in TBS-T, before being incubated with the secondary antibody, goat anti-mouse IgG (H+L) HRP conjugate (Bio-Rad Laboratories Inc.), in a dilution of 1:300 in 5 mL of 5% milk in TBS-T, for 15 minutes with rotational agitation at room temperature, in a 15 mL tube. Membranes were washed for three additional 10-minute periods in TBS-T, while shaking. After that, membrane developing was performed.

4.3. Regulatory Network between c-Myc and MicroRNAs – *In Silico* Analysis

In silico analysis was performed to calculate the probability of c-Myc to bind to the promoters of genomic regions where validated miRNAs are inserted, based on a recently reported c-Myc binding sequence and its respective binding matrix (177). MiRNA genomic location and promoter sequence, with a length of 5000 base pairs (bp) upstream of the gene transcription start site, were obtained from *Genome Browser* database. The number of transcription factor binding sites was retrieved with the help of *ConSite* web-based tool, after the alignment between miRNA promoter sequence and c-Myc binding sequence. Here, results are based on the integration of binding site prediction, generated with high-quality transcription factor models, and cross-species comparison filtering (phylogenetic footprinting) (178). The number of c-Myc binding sites in the miRNA gene promoter region was determined with an identity threshold of 95%.

Additionally, *in silico* analysis was performed to predict whether any of the validated miRNAs targets *MYC* gene. For such prediction, *miRWalk* database was used, which integrates information provided by eight different databases, including *miRanda*, *PICTAR* and *TargetScan*. For each predicted target of a given miRNA, *miRWalk* retrieves a score, corresponding to the number of databases in which that same target is listed. Targets mentioned in the present study were considered when listed in, at least, one of the mentioned databases.

4.4. Statistical Analysis

Non-parametric tests were used to ascertain statistical significance for the all the performed comparisons, since biological samples usually do not follow the shape of a normal Gaussian distribution.

Kruskal-Wallis test (KW) was used for comparisons between more than two non-related groups, and Mann-Whitney U test (MW) was used in pair-wise comparisons. These tests were used both in clinical samples and *in vitro* studies. When comparing multiple groups, Bonferroni's correction was applied in subsequent paired comparisons, dividing the P-value by the number of comparisons performed (0.017, in the case of the present study).

Sign test, another non-parametric test, was used for comparisons between matched samples from the same individual in study.

Fisher's exact test was used in the analysis of contingency tables, i.e., to assess whether the proportion of a certain variable was statistically significant between different groups. The P-value calculated in this type of test is exactly correct, being preferable than the one calculated in the chi-square test, since it can be applied to sample groups of any size (179).

Additionally, Somer's D ordinal measure of association was used to assess the existence of a statistical relationship between two ordinal variables. The value of this coefficient, which can vary from -1 to 1, indicates if the compared variables move to the same (when the coefficient is positive) or opposite (when the coefficient is negative) directions.

P-values were considered statistically significant when inferior to 0.05. Statistical analyses were performed using SPSS software, version 22.0 (IBM-SPSS Inc., Chicago, IL, USA). Graphs were built using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA).

5. Results

5.1. Assessment of c-Myc Protein Levels in Clinical Samples

IHC for assessment of c-Myc protein levels was performed in a total of 254 tissue samples (198 PCa primary tumors, 42 PINs and 13 MNPTs). Table 4 summarizes the score distribution obtained for each group of samples, and illustrative examples of different immunostaining scores are shown in Figure 23. A significant increase of c-Myc protein levels was apparent from MNPTs to PIN lesions, and from the latter to primary PCa. The score distribution of c-Myc immunostaining across the three groups of clinical samples is illustrated in Figure 24 - A.

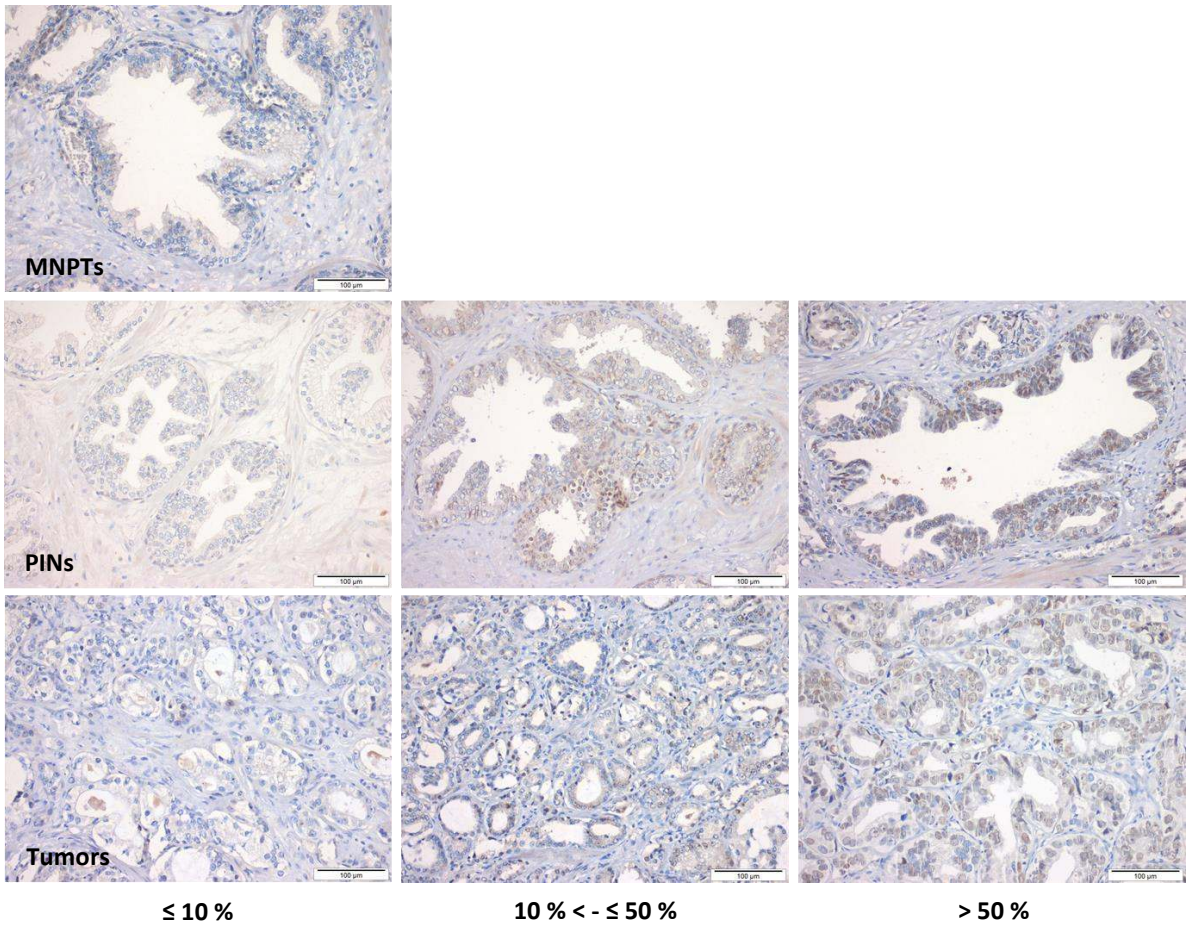

Figure 23. Representative images of c-Myc immunostaining in PCa, PIN and MNPT clinical samples.

Table 4. **Immunohistochemical expression of c-Myc in a series of of primary PCa, PIN lesions and MNPTs.**

Clinical Sample Group	Negative	Positive	
	≤ 10 % (+1) N (%)	10 % < - ≤ 50 % (+2) N (%)	> 50 % (+3) N (%)
MNPTs	13 (100 %)	-	-
PIN lesions	21 (50 %)	16 (38.1 %)	5 (11.9 %)
PCa primary tumors	74 (37.4 %)	83 (41.9 %)	41 (20.7 %)

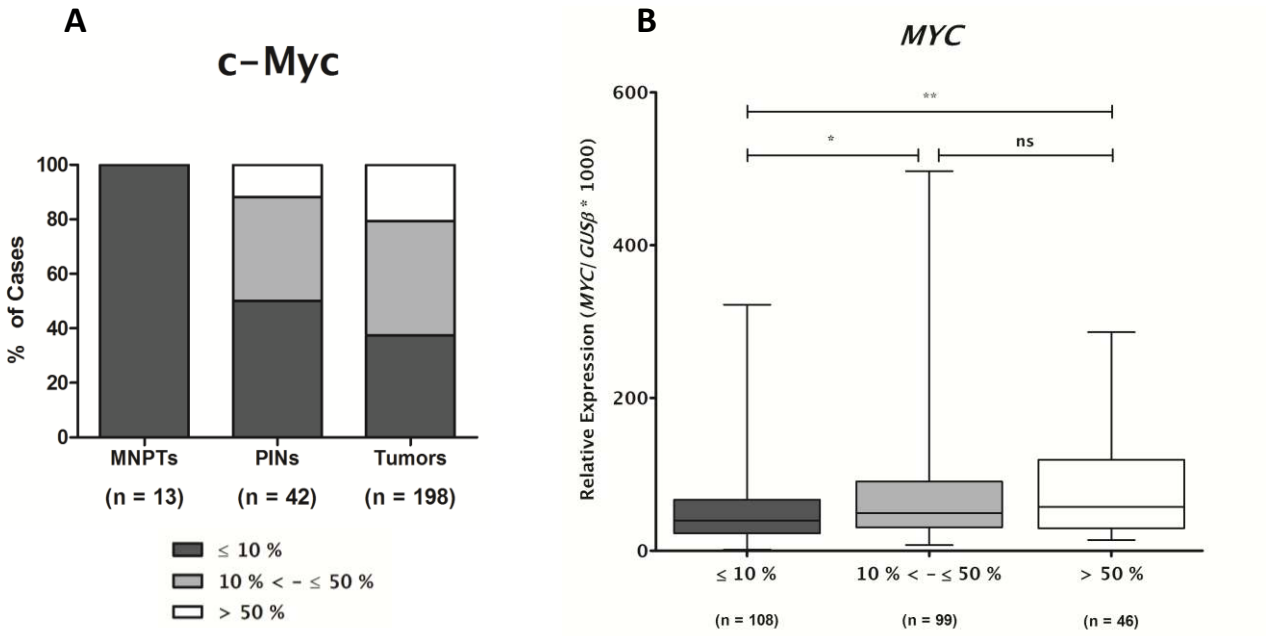

Figure 24. **Distribution of c-Myc protein (A), by immunohistochemistry, and of *MYC* transcript (B), by RT-qPCR, levels in a series of PCa, PIN lesions and MNPTs, grouped according to c-Myc protein immunostaining. (*** p < 0.001; ** p < 0.01; * p < 0.017; ns – non-significant).**

Statistically significant differences were observed concerning *MYC* transcript and respective protein levels across the three groups of immunostaining scores, indicating that, in general, c-Myc protein levels follow the same trend of the transcript (Figure 24 – B). In pairwise comparisons, however, statistical significance was only observed between ≤ 10% / 10% < - ≤ 50% and ≤ 10% / > 50% score groups ($p < 0.017$ and $p < 0.01$, respectively).

5.2. Confirming *MYC* Silencing

MYC silencing in PC-3 cells was confirmed at the transcript level by RT-qPCR and at the protein level by Western Blot, in two distinct biological replicates. A reduction of 82.90% in *MYC* transcript levels was achieved in sh-MYC PC-3 cells (Figure 25 – A), and a clearly visible reduction was also observed at protein level (Figure 25 – B).

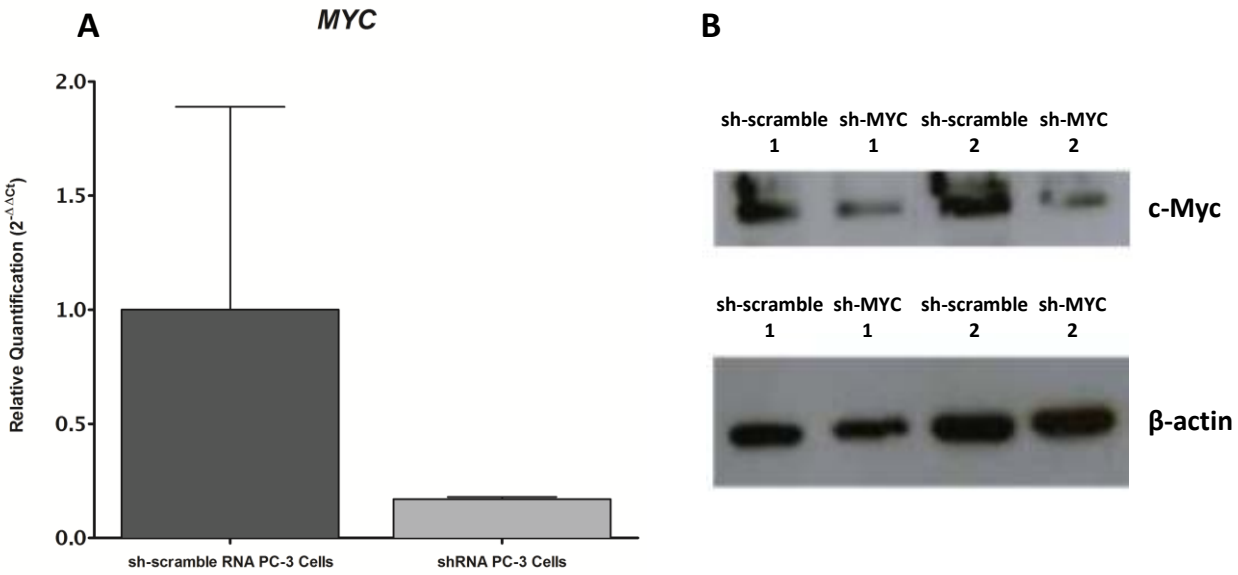

Figure 25. Confirmation of *MYC* gene silencing in PC-3 cell line. A – Relative expression of the *MYC* transcript (RT-qPCR, normalized to *GUSβ*); B – c-Myc protein expression (Western blot, immediate exposure).

5.3. Expression Levels of Selected MicroRNA Candidates in PC-3 Cells with Permanent *MYC* Silencing

To further explore the regulatory network established between *MYC* and the selected miRNA candidates, alterations in expression levels of these molecules were assessed in sh-MYC PC-3, by comparison to sh-scramble RNA PC-3 cells (Figure 26). Overall, a significant reduction in expression levels was observed for all candidate miRNAs, although this was more dramatic for miR-126*, almost reaching 100%. Thus, permanent *MYC* silencing led to a significant decrease of the expression levels of the selected miRNA candidates. Subsequently, expression levels of the selected miRNAS were determined in the series of PCa, PIN lesions and MNPTs.

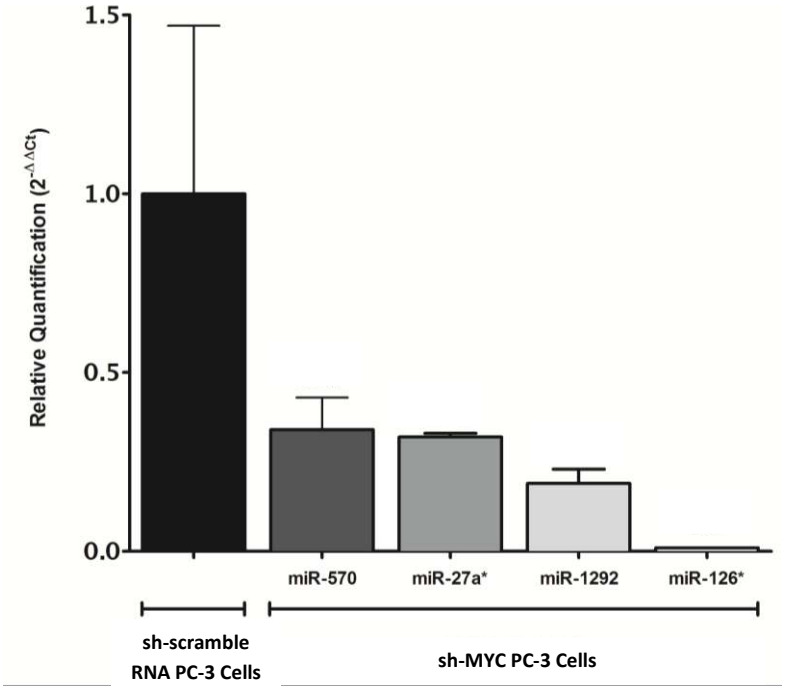

Figure 26. Expression levels of selected miRNA candidates in PC-3 cells with permanent *MYC* silencing (RT-qPCR, normalized to *RNU6B*).

5.4. Validation of Selected MicroRNA Candidates

Further validation was only accomplished for miR-27a* and miR-126*, since very low expression levels of miR-570 and miR-1292 in the clinical samples impaired the amplification reaction. Both miR-27a* (Figure 27 - A) and miR-126* (Figure 27 - B) were significantly underexpressed in PIN lesions ($p < 0.001$ and $p < 0.017$, respectively) and PCa ($p < 0.001$, for both miRNAs), compared to MNPTs, as well as in PCa compared to PIN lesions ($p = 0.01$ and $p < 0.001$, respectively).

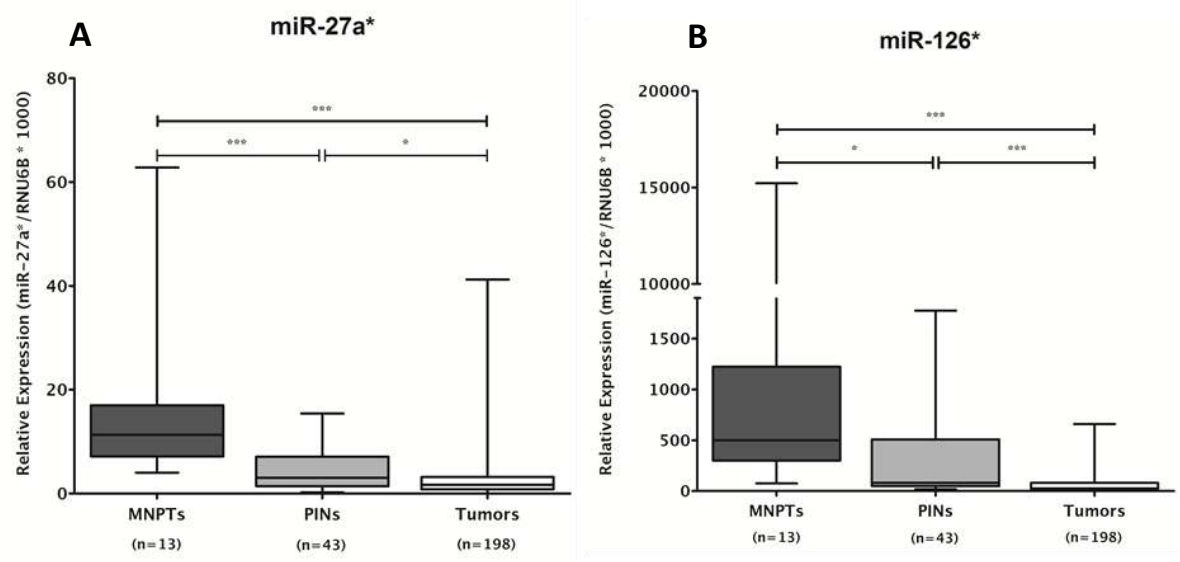

Figure 27. Expression levels of selected miRNA candidates, after validation, in a series of PCa, PIN lesions and MNPTs. A – miR-27a*; B – miR-126* (RT-qPCR, normalized to *RNU6B*). (* $p < 0.001$; ** $p < 0.01$; * $p < 0.017$; ns – non-significant).**

5.5. MicroRNA Status of Matched PINs and Primary Tumors

From the total number of PIN lesion samples, 37 of them matched with a primary tumor, i.e., both samples derived from the same patient, although this does not imply a relation of causality between them, but rather reflects a common origin from the same prostate tissue. A non-parametric signal test was then performed to assess differences in expression levels of miRNA candidates between matched PINs and primary tumors. For miR-27a* and miR-126*, expression levels were significantly higher in the PIN lesions, compared to the respective PCa ($p < 0.05$ and $p < 0.001$, respectively). These results are graphically represented in Figure 28, where the ratio between miR-27a* (Figure 28 – A) or miR-126* (Figure 28 – B) expression levels in primary tumors and in the respective PIN lesions is depicted.

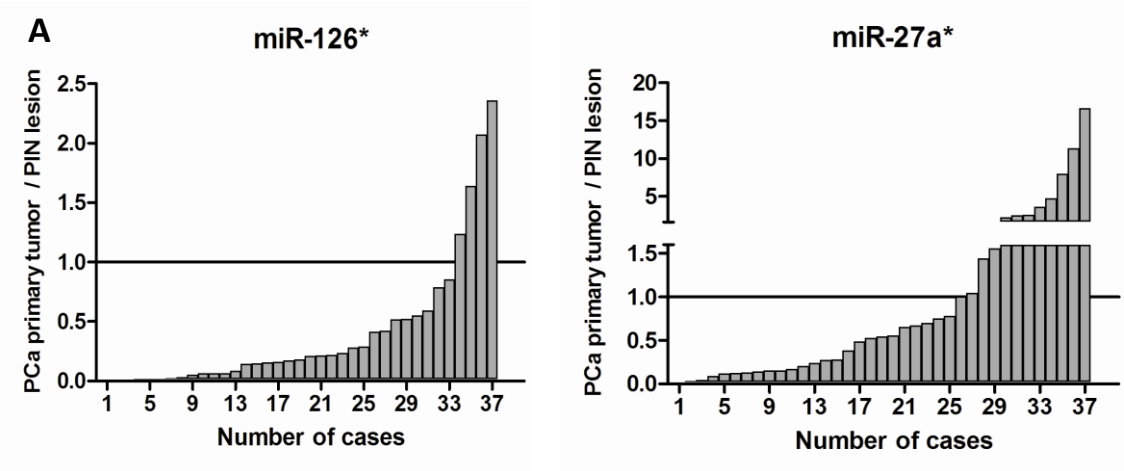

Figure 28. Relative expression levels of miR-27a* (A) and miR-126* (B) in paired PIN lesions and PCa samples (miRNA expression in tumors normalized to the expression in the matched PINs, assessed by RT-qPCR and normalized to *RNU6B*).

5.6. Association Between c-Myc and Validated MicroRNA Expression Levels and Clinicopathological Parameters

The data of different clinicopathological parameters from all clinical samples tested in this study are depicted in Table 5.

Table 5. Clinical and pathological data of the patients included in this study.

Clinicopathological Parameter	PCa (n = 198)	PIN (n = 43; 37 matched with a PCa)	MNPT (n = 13)
Age (years), median (range)	**64 (49 - 75)**	**65 (51 - 75)**	**64 (49 - 80)**
PSA (ng/mL), median (range)	**8.10 (2.66 - 35.50)**	**n.a.**	**n.a.**
Pathological Stage, N (%)			
pT2	**110 (55.6 %)**	**n.a.**	**n.a.**
pT3a	**65 (32.8 %)**	**n.a.**	**n.a.**
pT3b	**23 (11.6 %)**	**n.a.**	**n.a.**
Gleason score, N (%)			
< 7	**67 (33.8 %)**	**n.a.**	**n.a.**
= 7	**115 (58.1 %)**	**n.a.**	**n.a.**
> 7	**16 (8.1 %)**	**n.a.**	**n.a.**

n.a., not applicable / not available

Concerning age, no statistically significant difference between PCa and MNPT samples was verified. Furthermore, a statistically significant association was disclosed between c-Myc protein levels and both PSA blood levels and GS ($p < 0.05$, for both clinicopathological parameters). The calculated Somer's D coefficients revealed that higher c-Myc protein levels were associated with higher serum PSA and GS (0.157 and 0.131, respectively). Considering miRNA-27a* expression levels, a statistically significant difference was observed between GS < 7 and GS ≥ 7 groups ($p < 0.01$), revealing lower expression of this miRNA in less aggressive tumors, with GS < 7 (Figure 29). On the other hand, no statistically significant association was found between miR-126* expression levels and any of the clinicopathological parameters.

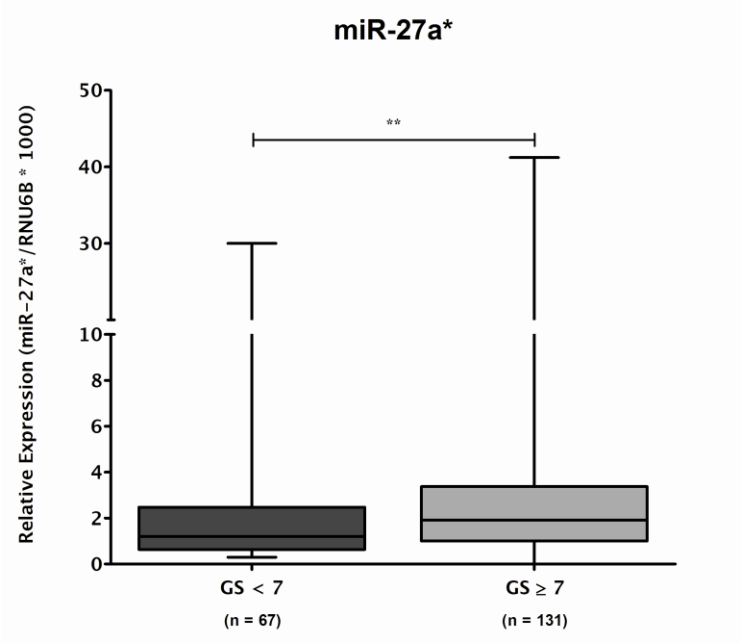

Figure 29. Association between miR-27a* expression levels and the GS of the PCa primary tumor samples (RT-qPCR, normalized to *RNU6B*). (* p < 0.001; ** p < 0.01; * p < 0.05; ns – non-significant).**

5.7. Association Between Validated MicroRNAs Expression and c-Myc Protein Levels

Differential expression of c-Myc protein was observed among the three groups of clinical samples ($p < 0.001$). Moreover, when expression levels of candidate miRNAs were compared among c-Myc immunoscore groups, a statistically significant difference was only observed for miR-126*. Although a trend for decreased expression is apparent across groups of increasing immunoscore, statistical significance was only achieved for the differences between the < 10% and the > 50% c-Myc immunoscore groups ($p < 0.01$) (Figure 30).

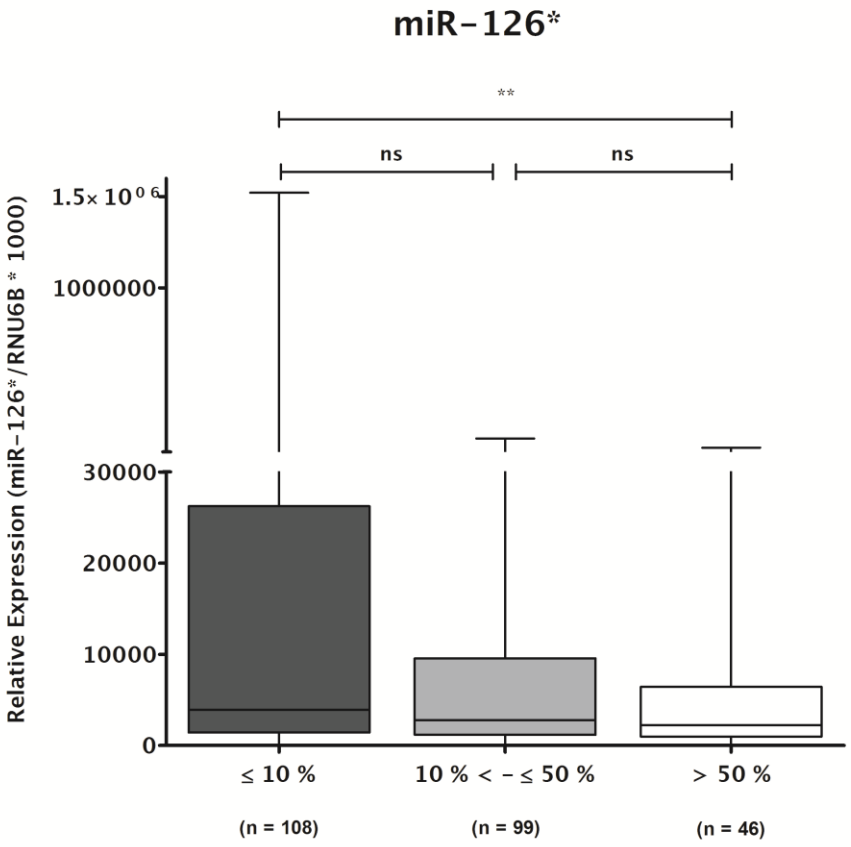

Figure 30. MiR-126* expression levels, assessed by RT-qPCR and normalized to *RNU6B*, in a series of PCa, PIN lesions and MNPTs, grouped according to c-Myc protein expression, assessed by immunohistochemistry. (* p < 0.001; ** p < 0.01; * p < 0.017; ns – non-significant).**

5.8. Regulatory Network Between c-Myc and Validated MicroRNAs – *In Silico* Analysis

In a previously published study, c-Myc was reported as a transcriptional activator of the miR-23a/24-2/27a cluster, in breast cancer cell lines (180). We performed *in silico* analysis that revealed the presence of two c-Myc binding sites at the promoter region of this cluster. As for the promoter region of the gene allocating miR-126*, no information concerning c-Myc binding has, so far, been published. Nevertheless, the same *in silico* analysis revealed one c-Myc binding site at this promoter region.

Regarding previously reported targets of the validated miRNAs, a study performed in a different cancer model identified miR-126* as a translational regulator of *MYC* (171). *In silico* analysis revealed that *MYC* is a putative target for miR-126* regulation. Interestingly, two additional genes, *MYCBP* and *MYCBP2*, codifying for Myc-binding proteins, which are closely associated with c-Myc activity, were identified as putative targets of miR-126* regulation. *MYCBP* codifies for a protein that activates c-Myc-mediated transcription (181). This protein is able to bind to the N-terminal region of c-Myc and stimulate the activation of E box-dependent transcription. On the other hand, no information was found in the literature concerning *MYC* as a possible target of miR-27a* and the *in silico* analysis did not indicate *MYC* as a putative target of miR-27a*.

5.9. Alterations in Expression Levels of MiR-27a* Known Targets

Upon *MYC* silencing in PC-3 cells, an impressive reduction in the expression levels of the four candidate miRNAs was observed. To further explore the biological impact of this reduction in PCa and in signaling pathways reported to be involved in prostate carcinogenesis, the expression levels of some of the known targets of those four miRNAs were assessed at protein level, through western blot analysis. The mechanistic target of rapamycin (*MTOR*) and the epidermal growth factor receptor (*EGFR*) and have been described as targets of miR-27a* (182), in head and neck squamous cell carcinoma cell lines, participating in a signaling axis that is frequently deregulated in different types of cancer (183, 184). Both mTOR and EGFR proteins displayed increased expression in sh-MYC PC-3 cell total protein extracts, compared to sh-scramble RNA PC-3 cells (Figure 31).

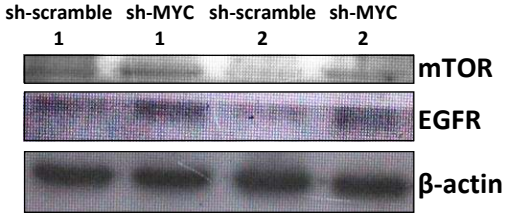

Figure 31. Western blot analysis of miR-27a* protein targets mTOR and EGFR in total protein extracts from sh-scramble and sh-MYC PC-3 cells (5 minutes exposure).

6. Discussion

Occupying the second position in terms of incidence and being in the top-ten when it comes to mortality among men worldwide, PCa represents one of the most prominent types of cancer (47). Additionally, the lack of sensitivity and specificity of widely used serum PSA levels as a biomarkers for PCa screening, leads to the overdiagnosis and overtreatment of the disease (185). Thus, it is critical to ascertain the level of threat that a diagnosed PCa poses to that particular patient. The identification of biomarkers associated with disease progression could be helpful for that purpose. However, many key players involved in the molecular pathways contributing to PCa initiation and progression remain unveiled. Therefore, the study of the basic biology of PCa is critical, since the identification of novel promising molecular targets may improve current diagnostic and therapeutic approaches, as well as to allow for the development of novel and more effective targeted therapies.

In the recent years, Epigenetics became a major field of research when it comes to understanding the biology of several diseases, including cancer (1). Since its discovery, deregulation of microRNAs biogenesis and function has been associated with progression of various tumors, including PCa (82). These small non-coding RNA molecules seem to control the expression of essential genes involved in every cellular process (77, 143) and the study of differential expression patterns of many of these molecules between tumor and normal tissue helped to clarify their role in neoplastic transformation and progression, turning miRNAs into powerful diagnostic, prognostic and therapeutic tools.

Abnormal activation of the *MYC* oncogene may occur through several distinct mechanisms, including gene rearrangement and amplification, and it is currently recognized as a major key player in many cellular pathways leading to the development of various types of neoplasia, including PCa (186-188). Indeed, the c-Myc transcription factor, which can simultaneously act as a transcription activator or repressor, greatly contributes to neoplastic transformation, by targeting genes with critical functions in cell cycle, cell differentiation, growth, metabolism, protein synthesis, adhesion, migration, angiogenesis and many others processes (189-196). More recently, efforts have been made to establish a connection between c-Myc and miRNA regulation, within the tumor progression context, in PCa as well as in other cancer models

(30, 34, 197). In fact, not only has c-Myc been reported to activate or repress the transcription of several miRNA containing genes and of genes encoding for miRNA biogenesis machinery, but also several miRNAs seem to be able to regulate c-Myc expression (29, 34, 37, 171, 198). This suggests the existence of a complex regulatory network established between *MYC* oncogene and several miRNAs, which tightly controls the expression levels of target genes in a normal cell but, once deregulated, may represent a key element for cancer development.

The main aim of this Master Thesis was to identify and validate novel miRNAs with a relevant role in prostate carcinogenesis, as well as to ascertain the possibility of such candidate miRNAs expression being regulated by c-Myc.

Firstly, *MYC* expression levels were assessed, at transcript (RT-qPCR) and protein (IHC) level, in a series of 198 PCa, 43 PIN lesions and 13 MNPTs. It should be emphasized that the normal prostate tissues used in this study as control were collected from patients not harboring PCa, whereas, in most of the studies with similar objectives, tissue localized adjacently to the tumor frequently serves as control. The use of such tissues, however, is not suitable, since both genetic and epigenetic alterations, including altered miRNA expression, have been reported to occur in morphologically normal cells adjacent to tumor foci (79). Globally, the distribution of *MYC* expression in our study is in agreement with previous publications: *MYC* increased expression is observed both in PIN lesions and prostate primary tumors, compared to normal tissue samples (188, 199). These results are also in agreement with the well-known oncogenic role of *MYC*, whose overexpression is considered an important event in prostate carcinogenesis (188, 200). Additionally, higher c-Myc protein levels were statistically associated with increased serum PSA levels and GS value, which are associated with worse disease outcome (201, 202). These results are also in line with the alleged prognostic value of c-Myc overexpression owing to its association with biochemical recurrence and patients' survival (199, 200, 203, 204).

Expression levels of *MYC*, at both transcript (RT-qPCR) and protein (Western Blot) level, were also characterized in six different PCa cell lines, which provided valuable information for future *in vitro* studies. From the analyzed cell lines, PC-3 and LNCaP were chosen for posterior *MYC* silencing, since they displayed the highest expression levels in the hormone-resistant and hormone-responding groups, respectively. However, at this point, effective silencing was only accomplished in PC-3 cells. The candidate miRNAs - miR-27a*, miR-570 and miR-1292 - were selected after careful analysis of both the results obtained in the miRNA microarray and the data already published by other researchers. Although it did not surfaced in the array, miR-126* was

also included because it has been reported to target and regulate *MYC* expression in multiple myeloma cells (171). In sh-MYC PC-3 cells, general downregulation of these four miRNA was apparent, supporting our hypothesis that they constitute potential targets for c-Myc regulation. Interestingly, c-Myc has already been reported to bind and activate transcription of the miR-23a-27a-24-2 cluster (180), although in a different cancer model. On the other hand, the observations concerning miR-126* might seem paradoxical, as in another cancer model this miRNA was shown to target *MYC* mRNA, causing a decrease in c-Myc protein expression (171). However, in PC-3 cells, *MYC* knockdown led to an almost complete depletion of miR-126* levels, implying a causal relationship. Remarkably, complex feedback regulatory loops between c-Myc and several miRNAs, namely miR-9*, has been described in Burkitt lymphoma, in which *MYC* translocation is recognized as the main event leading to tumorigenesis (28, 197). In such cases, c-Myc and specific miRNAs reciprocally control each other's expression and jointly generate differential gene expression patterns that ultimately contribute to tumor development. We are, thus, tempted to speculate whether a similar mechanism occurs in PCa cells, involving c-Myc and miR-126*. However, it should be taken into account that the observed miRNA downregulation may also represent an indirect consequence or cellular adaptation to *MYC* knockdown.

After analyzing the effects of *MYC* silencing in the selected miRNA expression levels in PC-3 cells, further miRNA candidate validation was attempted in a series of primary PCa, PIN lesions and MNPTs. However, this was only accomplished for miR-27a* and miR-126*, because no successful amplification reaction for miR-570 and miR-1292 was obtained, despite their identification in the miRNA microarray. This might be due to very low levels of those two miRNAs in prostatic tissues, highlighting the importance of microarray validation through different techniques, as microarray and RT-qPCR methodologies have different detection sensitivities (205). Concerning miR-27a*, available information about its role in cancer is scarce. This miRNA is part of the intergenic miR-23a-27a-24-2 cluster, located in the short arm of the chromosome 19, where no chromosome alterations have been reported, so far, in PCa (206). Interestingly, the miR-23a-27a-24-2 cluster has been previously reported to be androgen-regulated in PCa cell lines (207) and the same cluster was previously showed to be targeted by c-Myc regulation in breast cancer cells (180). Moreover, c-Myc-mediated transcriptional activation of the miRNAs contained in that cluster led to increased invasion and migration capacity of breast cancer cells, although the precise role of miR-27a* in tumorigenesis was not addressed. Intriguingly, this miRNA has been classified as tumor suppressor in the head and neck squamous cell carcinoma, by targeting multiple elements of the

EGFR/Akt1/mTOR signaling pathway (182). In this particular type of cancer, miR-27a* was found to be underexpressed both in cancer cell lines and clinical tumor samples. Our study is the first to identify miR-27a* as a possible key player in prostate carcinogenesis and possible target of c-Myc regulation in this particular cancer model. Expression levels of this miRNA were significantly lower in PIN lesions compared to the normal tissues and in PCa compared to PIN, which is considered a PCa precursor. This pattern of expression is very suggestive of a miRNA with tumor suppressive functions, which is in agreement with previous findings in a different cancer model (182). Moreover, samples with higher c-Myc expression display lower levels of miR-27a*, further suggesting a relationship between those two molecules, which at this time we could not substantiate statistically.

Concerning miR-126*, an intronic product of the vascular endothelial EGF-like 7 (*EGFL7*) gene, which maps to 9q, no chromosome-level alterations have been described for this particular *locus* in PCa (206). Previous studies strongly suggest a tumor suppressive role for this miRNA in prostate carcinogenesis and other cancer models, through the regulation of genes with important roles in cell proliferation, migration and invasion (208). The low expression levels of miR-126* and its complementary mature form, miR-126, have been reported to contribute to the development of PCa and other cancer types (82, 209, 210). Additionally, induced expression of this miRNA leads to translational repression of prostein or prostate cancer-associated protein 6 (*SLC45A3*), which correlates with a reduced migration and invasion capacity of LNCaP cells, in which miR-126* is naturally absent (125). As previously mentioned, miR-126* was also reported to target and repress the translation of *MYC* mRNA molecules, in a different cancer model (171). Nevertheless, our study is the first to address c-Myc regulation of miR-126* in primary PCa. Similarly to miR-27a*, expression levels of miR-126* significantly decrease from MNPTs to PINs to PCa, also suggesting a tumor suppressive role. On the other hand, a significant association between miR-126* expression levels and c-Myc protein levels was depicted, further supporting the existence of a complex regulatory interaction between *MYC* and miR-126* in PCa.

As previously pointed out, several aspects should be taken into careful consideration concerning analysis of results from miRNA microarray assays. Microarray analysis has been widely used for miRNA profiling, allowing for the simultaneous detection of multiple differentially expressed miRNAs in the same sample set (205). Results obtained in microarray analysis, however, require further validation, usually by means of RT-qPCR. Thus, data obtained from those two different methodologies may yield discordant results (211). At a first glance, the expression

patterns we observed for miR-27a* and miR-126* across the three groups of clinical samples might be surprising, given that both miRNAs were identified as overexpressed in PCa cases harboring elevated *MYC* transcript levels. However, the fact that lower expression levels of miR-27a* and miR-126* were observed in primary PCa and PIN lesions, compared to normal tissues, could not have been predicted by the microarray, since only PCa samples, but not MNPTs or PINs were included in the analysis. Additionally, microarray analysis was performed based on *MYC* transcript information, since data regarding c-Myc protein levels across the series of clinical samples was not available, at the time. Additionally, the use of a reduced number of samples in the microarray may also contribute to increase the bias associated with this technique.

Considering the limited information regarding the role of miR-27a* and miR-126* in prostate carcinogenesis, we verified whether miRNA downregulation upon *MYC* silencing in PC-3 cells might have a relevant biological impact. As previously mentioned, miR-27a* represses the translation of key players of the EGFR/Akt1/mTOR signaling axis, widely deregulated in various solid tumors, including PCa (212, 213). Also, no prior association has ever been made between the genes encoding for any of those proteins and c-Myc transcription factor. After assessing *EGFR* and *MTOR* protein levels by Western blot, an upregulation of both these protein was observed in sh-MYC PC-3 cells, in which *MYC* knockdown was previously shown to decrease miR-27a* levels. These findings further support the previously reported role of this miRNA in translational repression of both EGFR and mTOR proteins. Strikingly, it also highlights the importance of this miRNA in the regulation of signaling pathways that are relevant in prostate carcinogenesis.

Finally, it is noteworthy that both validated miRNAs constitute the passenger strand of the dsRNA precursor molecule. Until recently, only the leading strand was thought to play a relevant biological role within the cell. However, recent publications have identified several miRNA passenger strands as key regulators of many essential cellular processes and pathways (214).

Considering our results, a simple model explaining the interplay between c-Myc and miR-27a* / miR-126* in PCa seems unlikely. On the one hand, *MYC* silencing in PC-3 cells leads to miR-27a* / miR-126* downregulation, suggesting that c-Myc activates their transcription, whereas in prostate tissues, the opposite trend is observed across three distinct groups of clinical samples, in which higher c-Myc protein levels are associated with reduced expression of both miRNAs, indicating a repressive role of c-Myc. Although cancer cell lines are widely used as valuable *in vitro* cancer models, they fall short in mimicking the complex biology of primary tumors (215). These *in vitro* models, which are relatively homogenous and with well-established features and origin, are

studied in a controlled environment that lacks many aspects present in the extremely complex and heterogeneous tumor context (e.g., the tumor microenvironment does not exist in cell culture). It should be recalled, however, that the interaction of c-Myc and miR-27a* and miR-126* was predicted by our *in silico* analysis, unveiling c-Myc binding sites at miR-27a* / miR-126* promoter regions. Interestingly, two other putative targets of miR-126* regulation, *MYCBP* and *MYCBP2*, were predicted *in silico*. These proteins represent an additional regulatory mechanism of c-Myc activity, through their capacity to bind to the N-terminal of this transcription factor and, consequently, to stimulate c-Myc-dependent transcription mechanisms (181).

Thus, a hypothetic model, represented in Figure 32, may be proposed, linking our (apparently) contradictory observations. In normal cells, expression of c-Myc leads to miR-27a* / miR-126* expression, creating a negative feed-back loops that tightly tunes *MYC* activity within the cell. MiR-126* would be able to regulate c-Myc expression and activity both directly, by inhibiting its translation into a functional protein, and indirectly, by controlling the expression levels of genes codifying for proteins with an important role in c-Myc activation (*MYCBP* and *MYCBP2*). In a cancer cell in which c-Myc is overexpressed (e.g., due to gene amplification), miR-27a* and miR-126* expression might also be stimulated but their levels remain low, as generally observed for tumor suppressor miRNAs (21). It has been previously demonstrated that c-Myc overexpression leads to a widespread downregulation of miRNA levels, eventually through impairment of miRNA biogenesis (28, 34, 170). In this scenario, miR-27a* and miR-126* downregulation might further contribute to increase c-Myc expression and ultimately favor tumor progression. Additional functional studies are required to confirm this hypothetic model.

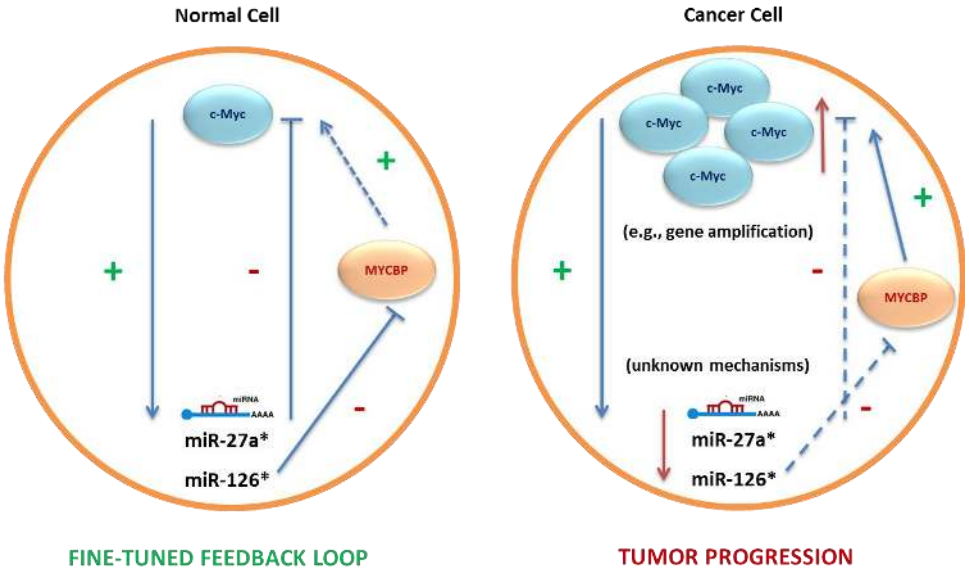

Figure 32. Biological interplay between c-Myc and miR-27a*/miR-126* in normal and tumor prostate cells - proposed hypothetic model.

7. Conclusion and Future Perspectives

In this study, expression levels of two poorly characterized miRNA molecules, putatively regulated by c-Myc, were assessed in a large series of primary PCa, PIN lesions and MNPTs. The observed expression patterns are in line with previous publications, in which both miRNAs have been considered to play tumor suppressive functions in different cancer models.

Altered miRNA expressions upon *MYC* silencing in PC-3 cell line allowed for the establishment of an association between c-Myc and the selected miRNA, concerning the existence of a regulatory network. The opposite expression patterns of c-Myc protein and both miRNAs in a large series of clinical samples, as well as the *in silico* prediction of c-Myc binding sites at these miRNAs promoter region, also suggest a mechanistic interplay between these molecules in PCa. However, additional studies are mandatory to validate the previously mentioned association and to determine its real biological importance in prostate carcinogenesis.

In the near future, and taking in consideration our findings, a new microarray analysis will be carried out to broaden previous results and to identify new candidate miRNAs, which can be further validated in the same series of clinical samples used in the present study. In this second analysis, total RNA extracted from MNPTs will also be included, together with total RNA extracted from more PCa primary tumors expressing either high or low *MYC* protein levels. Total RNA extracted from both sh-scramble RNA and sh-MYC PC-3 cells will also be included in this new analysis, and it should provide more reliable information concerning miRNA altered expression potentially due to c-Myc regulation.

Silencing of *MYC* will also be attempted in additional PCa cell lines, such as LNCaP, as initially intended. Since different cell lines mirror different features, stages and behaviors of PCa, it is important to assess the effects of *MYC* knockdown in these different *in vitro* models to better understand the complex miRNA regulation.

To confirm the existence of a real interaction between c-Myc and the selected miRNAs, functional assays, such as luciferase assay and chromatin immunoprecipitation (ChIP) analysis, will be performed in both sh-scramble and sh-MYC PC-3 cells.

Finally, to assess the biological impact of miR-27a* and miR-126* in prostate carcinogenesis, silencing and induced expression of these miRNAs will be carried out in PCa cell lines, and its impact will be evaluated by means of phenotypic assays.

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