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MicroRNAs DEREGULATION IN PROSTATE CANCER

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AUTHORS DECLARATION

Under the terms of the "nº 2, alínea a, do Art.º 31º do Decreto-lei nº 230/2009", is hereby declared that the author afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published articles included in this dissertation.

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Research articles

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João Ramalho-Carvalho, Inês Graça, Antonio Gomez, Jorge Oliveira, Rui Henrique, Manel Esteller, Carmen Jerónimo: *Downregulation of miR-130b~301b cluster is mediated by aberrant promoter methylation and impairs senescence in prostate cancer*. J Hematol Oncol. 2017 Feb 6; 10(1):43

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It has been a hectic journey. This work represents the combined efforts of many talented individuals. First, my mentors, Carmen Jerónimo, PhD and Rui Henrique, MD PhD, deserve much credit for guiding me through this experience. I am satisfied for their participation in my education and development. I also acknowledge the input of Manel Esteller, MD PhD, has a part in my work and I am pleased for being part of his lab during part of my PhD.

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MicroRNAs (miRNAs) are small, non-coding RNAs that function as guide molecules in RNA silencing. They target both non-coding and protein-coding transcripts, thus, being involved in virtually all homeostatic and pathological processes. The biogenesis of miRNAs is under tight temporal and spatial control, and their deregulation is associated with multiple human diseases.

Each miRNA targets hundreds of transcripts and most protein-coding genes are miRNA targets. Therefore, fine-tuning the expression of hundreds of genes is critical to cells' physiology. Indeed, miRNAs' confer precision to protein expression, thus offering an explanation for the generally observed combinatorial targeting of endogenous genes by multiple miRNAs, as well as the preferential targeting of lowly expressed genes. Although, the average level of repression induced by miRNAs is modest, the repression amongst individual cells varies considerably, which is a direct consequence of gene expression as a stochastic process.

MiRNAs have emerged as key gene regulators in diverse biological pathways involved in Prostate Cancer (PCa). PCa is the most common non-cutaneous cancer and a leading cause of cancer-related death in the Western World. Due to the clinical and molecular heterogeneity of the PCa, the role of miRNA remains elusive. Although deregulation of miRNA expression has been implicated in the development and progression of PCa, its precise role in prostate carcinogenesis is mostly unknown. Nevertheless, miRNAs have been envisaged as putatively useful for PCa management, specifically as diagnostic and prognostic biomarkers, as well as targets for miRNA-based therapeutics.

Globally, this Thesis aimed to: 1) understand the extension of miRNA deregulation in PCa, 2) investigate whether there is specific miRNAs' methylation-repression patterns, 3) understand the role of specific miRNAs in PCa biology and 4) identify potential PCa-specific biomarkers based on miRNAs methylation aberrations.

The results obtained in this Thesis proved that miRNAs are globally down-regulated in PCa compared to morphologically normal prostate tissues, due to: (i) decreased expression (and, arguably, activity) of the core genes involved in miRNA biogenesis (*DROSHA*, *DGCR8*, *DICER*); (ii) aberrations in the DNA methylation landscape of miRNA promoters; (iii) altered chromatin conformation in the loci of certain miRNAs.

Moreover, *in vitro* studies showed that restoration of miRNAs expression caused attenuation of the malignant phenotype of PCa cell lines, indicating that miR-130a, miR-130b~301b cluster and miR-152 as new PCa tumor-suppressor miRNAs. Furthermore, this Thesis showed that miR-130a and miR-152 seems to act by fine-tuning target genes' expression, whereas the cluster miR-130b~301b controls specifically cellular senescence signaling

pathways. Interestingly, miR-130a and miR-130b~301b cluster are part of the same miRNA family. The phenotype obtained by their *in vitro* manipulation was rather similar, suggesting that they might act as cooperative functional units. These results support the functional cooperation and specialization of the members of this family, as exemplified by the co-regulation of DNA Damage and Senescence-related genes.

Taking advantage of the DNA methylation dataset and a miRNA profile, an integrative approach was conceived. Thus, miR-152 was found to be downregulated and hypermethylated in PCa. Restoration of miR-152 reverted the malignant phenotype of PCa cell lines and, importantly, promoted cell cycle arrest at S and G2/M. *TMEM97* and *NOL4* were identified as targets of miR-152.

Finally, the value of miRNAs promoter methylation as PCa biomarkers was also evaluated. Using several patients' cohorts, miR-193b's hypermethylation was unveiled as a promising biomarker for non-invasive detection of PCa in urine samples. In parallel, higher miR-34b/c or miR-129-2 methylation levels independently predicted worse outcome.

Overall, the work presented in this Thesis provides evidence that miRNAs are dynamically regulated in PCa carcinogenesis, and may serve as biomarkers for non-invasive or minimally invasive diagnosis and prognostication of PCa patients. Furthermore, the multiple pathways regulated by miRNA restoration described in this Thesis not only proved the functional relevance of specific miRNAs in PCa but also set the stage for a future miRNA-based therapeutic strategy. Finally, the datasets generated will help to understand the complex biological functions of miRNAs in PCa.

Keywords: microRNA, Non-coding RNAs, Prostate Cancer, Senescence, DNA Damage, Cell Cycle, Senescence-Associated Secretory Phenotype, Epigenetics, DNA Methylation.

RESUMO

Os microRNAs (miRNAs) são uma classe de RNAs não codificantes, de cadeia simples com cerca de 22 nucleótidos de tamanho que actuam como "guias" no silenciamento de genes através da degradação de moléculas de RNA mensageiro. Os miRNAs têm como alvo transcritos com capacidade de codificar proteína e transcritos não codificantes. Desta forma, os miRNAs estão envolvidos – virtualmente – em todos os processos homeostáticos e patológicos. O processo de maturação dos miRNAs é regulada de forma muito complexa e, quando desregulados, estão associados com diferentes patologias.

Cada miRNA tem a capacidade de regular um grande número de transcritos, sendo que a maioria dos genes que codificam proteína são alvos de miRNAs. Funcionalmente, estes controlam a expressão génica através de pequenos ajustes nos transcritos alvo, sendo o seu papel fundamental para a fisiologia celular. Os miRNAs conferem precisão à expressão proteica, oferecendo assim uma explicação para a abordagem combinatória comummente observada de genes regulados por múltiplos miRNAs, bem como uma acção preferencial sobre genes pouco expressos. Todavia, o nível de repressão induzido por miRNAs é modesto, sendo que a repressão inter-celular varia consideravelmente, o que é uma consequência direta da expressão génica como um processo estocástico.

Os miRNAs posicionam-se como reguladores genéticos fundamentais em diversas vias de sinalização envolvidas no cancro da próstata (CaP). O CaP é o cancro não cutâneo mais comum em homens e uma das principais causas de morte por cancro no mundo ocidental. Devido à sua heterogeneidade clínica e molecular, o papel dos miRNAs permanece impreciso. Embora a desregulação da expressão dos miRNAs esteja implicada no desenvolvimento e progressão do CaP, o seu papel preciso na carcinogénese da próstata é em grande parte desconhecida. No entanto, os miRNAs parecem apresentar valor na prática clínica em doentes com CaP, especificamente como biomarcadores de diagnóstico e prognóstico, bem como podem ser potenciais alvos para terapêutica.

Globalmente, os objectivos desta Tese foram: 1) compreender a extensão da desregulação dos miRNAs no CaP; 2) investigar se a metilação do DNA é uma das principais causas da alteração de expressão dos miRNAs em CaP; 3) caracterizar a função de alguns miRNAs na biologia das células de CaP; e 4) identificar potenciais biomarcadores específicos para o CaP baseados nos padrões de metilação nos promotores de alguns miRNAs.

Os resultados obtidos nesta Tese demonstraram que os níveis de miRNAs estão globalmente diminuídos em CaP em comparação com tecidos morfologicamente normais da próstata, devido a: (i) diminuição da expressão (e, discutivelmente, actividade) dos genes envolvidos no processamento dos miRNAs (*DROSHA*, *DGCR8*, *DICER*); (ii) aberrações ao

nível da metilação do DNA nos promotores dos miRNAs; (iii) alteração da conformação da cromatina nos loci de miRNAs.

Adicionalmente, estudos *in vitro* mostraram que a restauração da expressão de miRNAs causou atenuação do fenótipo maligno das linhas celulares representativas de CaP, indicando o miR-130a, o cluster miR-130b~301b e o miR-152 como novos miRNAs com função onco-supressora em CaP. Além disso, os resultados indicam que o miR-130a e miR-152 actuam como moduladores da expressão de genes-alvo, enquanto o cluster miR-130b~301b controla especificamente as diferentes vias de sinalização envolvidas na senescência celular.

Curiosamente, o miR-130a e o cluster miR-130b~301b fazem parte da mesma família de miRNAs. O fenótipo obtido com a sua manipulação *in vitro* foi bastante semelhante, sugerindo que eles podem actuar como unidades funcionais cooperativas. Estes resultados suportam a cooperação funcional e a especialização dos membros desta família, como exemplificado pela co-regulação dos genes relacionados com a reparação danos no DNA e senescência.

Aproveitando o conjunto de dados gerados para a metilação do DNA e o perfil de expressão dos miRNAs, foi concebida uma abordagem integrativa desta informação. Deste modo, verificou-se que o miR-152 estava sistematicamente sub-expresso em CaP e parte deste resultado é mediado pela hipermetilação do seu promotor. A restauração de miR-152 reverteu o fenótipo maligno das células de CaP e, importantemente, promoveu a paragem do ciclo celular em S e G2/M. Os genes *TMEM97* e *NOL4* foram identificados como alvos do miR-152, sugerindo, assim, que o miR-152 é capaz de controlar o desenvolvimento e progressão de CaP em diferentes etapas da carcinogénese.

Finalmente, foi avaliado se a metilação do promotor de alguns miRNAs tem potencial para identificar novos biomarcadores de CaP. Usando várias coortes de doentes, a hipermetilação do promotor do miR-193b revelou bastante potencial como biomarcador para a detecção não invasiva de CaP em amostras de urina. Paralelamente, níveis elevados de metilação de miR-34b/c ou de miR-129-2 revelaram ser factores independentes de mau prognóstico.

Em suma, o trabalho apresentado nesta Tese fornece evidências de que os miRNAs são dinamicamente regulados na carcinogénese prostática e podem servir como biomarcadores para o diagnóstico não invasivo ou minimamente invasivo e prognóstico dos doentes de CaP. Além disso, as múltiplas vias reguladas pelos miRNAs descritos nesta Tese não só provou a relevância funcional dos miRNAs no CaP, mas também pode ajudar definir uma futura estratégia terapêutica baseada em miRNAs. Finalmente, os conjuntos de dados gerados ajudarão a um melhor entendimento das complexas funções biológicas dos miRNAs em CaP.

Palavras-chave: microRNAs, RNAs não codificantes, Cancro da Próstata, Senescência, Danos no DNA, Ciclo Celular, Epigenética, Metilação do DNA

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Chapter 7

5-Aza-CdR: 5-aza-2-deoxycytidine

5caC: 5-carboxycytosine

5fC: 5-formylcytosine

5hmC: hydroxymethylcytosine

5mC: 5- methlcytosine

A

ACTB: β-actin

AGO: Argonaute protein

ADT: androgen-deprivation therapy

APC: APC, WNT signaling pathway regulator

AR: Androgen Receptor

ATR: ATR serine/threonine kinase

AUC: Area under the curve

B

BlCa: Bladder Cancer

C

CAPRA: Cancer of the Prostate Risk Assessment

CDKN1A: Cyclin-dependent kinase inhibitor 1A

CDKN1B: Cyclin-dependent kinase inhibitor 1B

CDKN2A: Cyclin-dependent kinase inhibitor 2A

CGI: CpG islands

CI: Confidence interval

COPZ2: Coatomer protein complex subunit zeta 2

CRPC: Castration-resistant prostate cancer

D

DDIT3: DNA damage inducible transcript 3

DDIT4: DNA damage inducible transcript 4

DDR: DNA damage response

DEPDC1: DEP domain containing 1

DFS: Disease-free survival

DGCR8: DGCR8, microprocessor complex subunit (DiGeorge Syndrome Critical Region Gene 8)

DICER1: DICER1, ribonuclease III

DMC: DNA methylation canyons

DMV: DNA methylation valleys

DNA: Deoxyribonucleic acid

DNMT: DNA methyltransferases

DRE: Digital rectal examination

DROSHA: Drosha, ribonuclease III

DSS: Disease-specific survival

E

EFEMP1: EGF-containing fibulin-like extracellular matrix protein 1

EMT: Epithelial-to-mesenchymal transition

ENCODE: ENCyclopedia Of DNA Elements

ER: Endoplasmic reticulum

G

GADD45A: Growth arrest and DNA damage inducible alpha

GENCODE: The reference human genome annotation for The ENCODE Project

GS: Gleason score

GSTP1: GlutathioneS-transferase pi 1

H

HD: Healthy donnors

HDAC: Histone deacetylases

HDF: Human diploid fibroblasts

HR: Hazard ratio

I

IL1A: Interleukin 1 alpha

IL1B: Interleukin 1 beta

IL6: Interleukin 6

K

Kb: Kilobase

L

LNA: Locked nucleic acid

Let-7: Lethal-7

lncRNA: Long non-coding RNA

M

MBP: Methylcytosine binding-proteins

MET: Mesenchymal-to-epithelial transition

miRISC: miRNA-induced silencing complex

miRNA: microRNA

MMP1: Matrix metallopeptidase 1

MNPT: Morphological normal Prostate Tissue

MRE: miRNA response element

mRNA: Messenger RNA

MSP: Methylation-specific PCR

MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide assay

N

NBl: normal bladder

ncRNA: Non-coding RNA

NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NK: Normal kidney

NPV: Negative predictive value

O

OIS: Oncogene-induced senescence

P

PASR: Promoter-associated short RNAs

PBS: Phosphate-buffered saline

PCa: Prostate Cancer

PCA3: Prostate cancer antigen 3

PCG: Protein-coding gene

PCR: Polymerase chain reaction

PIN: Prostatic Intraepithelial Neoplasia

piRNA: Piwi-interacting RNA

PMD: Partially methylated domains

POLII: Polimerase II

PPV: Positive predictive value

pre-miRNA: Precursor miRNA

pri-miRNA: Primary miRNA

ProCam: Prostate Cancer Methylation

PSA: Prostate-specific antigen

PTM: Post-translational modifications

Q

qMSP: Quantitative real-time methylation-specific PCR

R

RARβ2: Retinoic acid receptor beta 2

RASSF1A: Ras association domain family protein 1, isoform A

RCT: Renal cell tumor

RNA: Ribonucleic acid

ROC: Receiver operator characteristics

RP: Radical prostatectomy

RT-qPCR: Reverse transcription quantitative polymerase chain reaction

S

SAHF: Senescence-associated heterochromatic foci

SASP: Senescence-associated secretory phenotype

SAM: S-adenosylmethionine

SEC23B: Sec23 homolog B, coat complex II component

siRNA: Small interfering RNAs

SNAIL1: Snail family transcriptional repressor 1

sncRNA: Small non-coding RNAs

snoRNA: Small nucleolar RNAs

SNP: Single nucleotide polymorphisms

snRNA: Small nuclear RNAs

STAT3: Signal transducer and activator of transcription 3

T

TASR: Termini-associated short RNAs

TATA Box:

TCGA: The Cancer Genome Atlas

TFIIB: Transcription Factor II B

TNM: Tumor, node and metastasis system

TP53: Tumor Protein 53

tRNA: Transfer RNAs

TSA: Trichostatin A

TSS: transcription start site

U

UMR: Unmethylated regions.

UTR: Untranslated region

UPR: Unfolded protein response

Z

ZEB1: Zinc Finger e-box binding homeobox

PART I – INTRODUCTION

CHAPTER 1 – AN OVERVIEW OF THE TRANSCRIPTIONAL DYNAMICS OF MAMMALIAN CELLS

1. Challenging the Central Dogma of Molecular Biology

In the 50 years following the discovery of DNA in the 1950s, Molecular Biology was based on a unifying principle: the central dogma of Molecular Biology. Francis Crick framed the central dogma in 1958, only 5 years after the basic structure of DNA was reported (1, 2). This model explained the relationship between the two nucleic acids and proteins. The Central Dogma postulated that once *information* has passed into protein it cannot get-out again, flowing uni-directionally. Essentially, this concept advocates that information's transfer from nucleic acid to protein, but transfer from protein to protein, or from protein to nucleic acid cannot occur. Herein, *information* means the precise sequence's determination of nucleic acid's bases or protein's aminoacid residues (1). Thus, the central dogma of biology postulates that DNA is transcribed into RNA and that RNA is translated protein.

This initial model shortly was improved. In addition to DNA and proteins, RNA, the carrier of genetic information was also added (2, 3). The Enzymatic Synthesis of Ribonucleic Acid gave the Noble Prize of Medicine to Severo Ochoa in 1959. Thus, transfer of genetic information was possible from DNA to RNA, RNA to protein, RNA to DNA, and DNA to protein (2), although Crick believed that transfer of genetic information from RNA to DNA or DNA directly to protein was unlikely (1, 2, 4).

However, the advent of high-throughput sequencing methods has assisted the exploration of transcriptomes in unprecedented depth and in an unbiased way. It is now evident that whereas less than 2% of the genome encodes proteins, at least 75% is actively transcribed into non-coding RNAs (5). Across all domains of life, a profusion of non-canonical transcripts has been detected in the transcriptome. They are clearly distinguished as they are non-coding, are not demarked by gene boundaries and are, for instance, recurrently antisense. As the occurrence of these transcripts is widespread, this phenomenon has been described as pervasive transcription. Thus, transcription is not restricted by the position of annotated protein-coding genes but can be initiated in almost any genomic context, i.e., the majority of its bases are associated with at least one primary transcript, mostly ncRNAs (6). However, it should be noticed that pervasive transcription is also subjected to skepticism: whether the detected RNAs are artifacts of the technologies used to identify them; simply inevitable byproducts of transcription from accessible, activated chromatin; and the biological significance of such transcripts (7). Nonetheless, multiple evidence from loss- and gain-of-function studies has demonstrated that ncRNAs reproducibly perturb *in vitro* physiology (8, 9). Although the functions of these transcripts, is largely unknown (10, 11), they seem to be

critical regulators of cell and developmental biology (10). RNA has been a powerful regulatory molecule, with numerous classic examples of RNA-mediated roles in splicing, translation, and genomic imprinting (10). Therefore, the pervasive transcription is unveiling a vast catalog of transcribed non-coding genes representing a wealth of different regulatory RNA elements that contribute to a physiological function primarily mediated through an RNA-based mechanism.

2. Non-coding RNAs

The biggest surprise of the postgenomic era has been the massive number and diversity of transcriptional products arising from the previously presumed *'dark matter'* portion of the genome. This pervasive impact of genome structure is now emerging. It includes an overabundance of small regulatory RNAs and a plethora of polyadenylated and nonpolyadenylated long non-coding RNAs (lncRNAs) that are antisense, intronic, intergenic and overlapping with respect to protein-coding loci (12). The ncRNAs are classified by an *ad hoc* size threshold cutoff. The distinction of two separate classes is somewhat arbitrary and might reflect technical bias rather than a true biological bimodal length distribution. LncRNA refers to a transcript >200 nucleotides in length that does not appear to contain a protein-coding sequence, while a small RNA is a transcript of 20 to 200 nucleotides. The abundance of lncRNA genes outnumbers protein-coding gene (13), and more than 90% have no significant peptide products (14). However, a tiny percentage of transcripts originally annotated as lncRNAs, are able to encode cryptic micropeptides (15, 16). The catalog of non-coding transcripts has grown enormously over the past few years, in large part due to the identification of extensive lncRNA genes (17). These lncRNAs elicit functional outcomes through interactions with DNA, chromatin, signaling and regulatory proteins, and a variety of cellular RNA species. Mechanistically, lncRNAs can act at neighboring intrachromosomal genes in *cis* (18) or targeting of genes in *trans* (19) on different chromosomes.

Several dozen classes of small ncRNAs have been proposed (20). These include well-characterized housekeeping ncRNAs (e.g. transfer RNA and some ribosomal RNA) essential for fundamental aspects of cell biology or splicing RNAs (21). Moreover, other small infrastructural and regulatory RNAs are now being characterized. A significant proportion of coding and non-coding transcripts are processed into steady-state stable RNAs shorter than 200 nucleotides (5). These precursors include transfer RNA, microRNA, small nuclear RNA and small nucleolar RNA (tRNA, miRNA, snRNA and snoRNA, respectively).

2.1 Small RNAs

Currently, more than 7,000 small RNAs are annotated by GENCODE, 85% of which correspond to four major classes: small nuclear (sn)RNAs, small nucleolar (sno)RNAs, micro (mi)RNAs and transfer (t)RNAs (5). The distribution of annotated small RNAs varies markedly between cytosolic and nuclear compartments (22). The small RNA classes enriched in each compartment depend on their functions. Thus, whereas miRNAs and tRNAs are in the cytosol, snoRNAs are in the nucleus. Interestingly, snRNAs are equally abundant in both the nucleus and the cytosol (4). Apart from this abundant small ncRNAs, the diversity of small ncRNAs has, perhaps, grown the most. Several dozen classes of small ncRNAs have been proposed, including splicing RNAs (small nuclear RNA (snRNAs)), and a variety of recently-observed RNAs associated with protein-coding gene transcription. It includes tiny transcription-initiation RNAs, promoter-associated short RNAs, termini-associated short RNAs, 3'UTR-derived RNAs, and antisense termini-associated short RNAs (5, 21).

The use of small RNA sequencing allowed the identification of previously unannotated small RNAs (5). These newly, low copy numbers, small RNAs are associated with promoter and terminator regions of annotated genes (promoter-associated short RNAs (PASRs) and termini-associated short RNAs (TASRs)), and their position relative to transcription start sites (TSS) and transcription termination sites.

The transcriptome resolution obtained from RNA-sequencing detected an accumulation of reads at the start of snoRNAs and at the guide and passenger sequences of annotated miRNAs, both in the nucleus and cytoplasm (23).

The ENCODE project was also able to establish short RNAs' genealogy. Globally, 27% of annotated small RNAs are mapped within 8% of protein-coding and 5% within 3% of lncRNA genes (23). Overall, about 6% of all annotated long transcripts overlap with small RNAs and are probably precursors to these small RNAs (23). Although most of these small RNAs reside within introns, exons from lncRNAs are comparatively enriched as hosts for snoRNAs (23). Additionally, 8.4% of GENCODE annotated small RNAs map are within novel intergenic transcripts derived from non-polyadenylated RNAs, with most overlapping annotated tRNAs (23). As expected, several long RNAs, both novel and annotated, seem to have dual roles, as functional (protein coding) RNAs, and as precursors for many important classes of small RNAs (23). To date, the most extensively studied small RNAs in cancer are microRNAs.

2.1.1 microRNAs

Numerous forms of small non-coding RNAs (sncRNAs) have evolved in eukaryotes to suppress undesirable genetic elements and transcripts, in order to maintain cell and genome integrity (24). SncRNAs are RNA sequences from 20-30 nucleotides in length that are intimately associated with Argonaute family proteins (AGO), acting as guides of AGO

proteins to RNA targets (24). Although the number of AGO proteins varies across species, four paralogues thought to have overlapping activities are known in human and mice. The current model, classify these sncRNAs into three different classes: miRNA, small interfering RNAs (siRNA) and PIWI-interacting RNA (piRNA). MiRNAs were discovered by Victor Ambros in 1993 (25). The first to be discovered were the lin-4 and lethal-7 (let-7), which are components of the gene regulatory network that controls the timing of *Caenorhabditis elegans* larval development (25-27). Both lin-4 and let-7 were found to bind the 3' Untranslated Region (3'UTR) of their respective targets, *lin-14* and *lin-41*, respectively. Furthermore, several studies demonstrated that the recruitment of the miRNA-induced silencing complex (miRISC) to messenger RNA (mRNA) targets reduced the output of the target proteins, thus impacting in cell homeostasis. The discovery of *let-7* and its conservation from worms, flies and humans, emphasized the functional significance of miRNA-dependent gene regulation.

It is now estimated that miRNA genes comprise from 0.5-2% of all genes in worms, flies, and mammals (28). MiRNAs function as guide molecules in RNA silencing, targeting both protein-coding transcripts and lncRNAs (24). MiRNAs repress gene expression by binding (mostly) to complementary sequences in the mRNAs 3´UTR to target them for degradation and thereby prevent their translation (24).

The human genome comprises more than 2000 hairpin structures that generate detectable small RNAs, although the genuineness and functional importance of several miRNAs candidates remains to be proved (29). Selective pressure during evolution not only selected miRNAs with a critical role in sculpting gene regulatory networks, but apparently have maintained the paring between miRNAs and the vast majority of all human transcripts (30). Considering that more than 500 *bona fide* individual miRNA genes have been identified (31), that an individual miRNA can target hundreds or thousands of different mRNAs and that an individual mRNA can be simultaneously suppressed by multiple different miRNAs, support that biological processes are subject to miRNA dependent regulation (32).

The last years have witnessed the acknowledgment that miRNAs are cancer genes (33). The biogenesis of miRNAs is under tight cellular, temporal and spatial control, and their deregulation was associated with several human diseases, particularly cancer (24, 32). They often are classified as oncomiRNA (an over-expressed miRNA with oncogenic activities) or tumor-suppressor miRNAs (when a miRNA expression is down-regulated and unable to repress a oncogene) (33). Using disease models, both loss-of-function and gain-of-function experiments in human cancer cells, mouse xenografts, transgenic mouse models and knockout mouse models demonstrated that miRNAs have key roles in cancer initiation, progression and metastasis (32, 33), including in Prostate Cancer (PCa).

The data available supports a model in which miRNA expression is globally suppressed in cancer compared with normal tissue, suggesting that when miRNA biogenesis is disrupted it accelerates neoplastic transformation (32, 34). Indeed, miRNAs may control cell proliferation, differentiation, apoptosis, metabolism, genome stability, inflammation, invasion and angiogenesis to affect tumor development (28, 32, 33). These pathways might also represent putative therapeutic strategies for different types of cancer.

Comparative genomics studies indicate that a miRNA binds to hundreds of miRNA Response Elements (MRE) across the transcriptome (as well a given RNA is targeted by multiple miRNAs) (35). However, when the expression of the miRNA is perturbed most predicted miRNA targets undergo small changes at mRNA and protein levels (36, 37). Taking together these observations generated the idea that rather than acting as genetic switches – where strong repression of one or few targets results in a clear phenotypic outcome – most miRNA act as rheostats, fine tuning the expression of hundreds of genes (36, 37) to reinforce cell fate decisions brought about through other mechanisms (28).

Most of the miRNA targets show evidence that they are regulated by depletion of mRNA levels. Thus, mRNA destabilization is the major effect of miRNAs by the time that substantial target gene repression arises (30). Interestingly, functionally critical genes that are spatially or temporally expressed are stringently regulated by miRNAs. Longer 3'UTRs are preferred as they have a higher average density of MRE, suggesting that their evolutionary selection. In its turn, housekeeping genes, however conserved, are selected to have shorter 3'UTRs and therefore less subjected to miRNA-mediated regulation (28).

Globally, miRNAs shape the dynamics of target gene expression in an intricate, tight process, involving multistep processing and requiring multi-protein complexes responsible for accurate miRNA function. Any disruption in miRNA biogenesis will impair cell homeostasis and leading to disease (28, 32).

Moreover, cancer-associated changes in miRNA's expression patterns are evolving as promising diagnostic markers that often correlate with clinical and pathological parameters used in clinical practice (32, 33).

2.1.2 microRNAs as fine tuners of gene expression

Gene expression is a stochastic process. Predictions indicate that each miRNA targets hundreds of genes and the majority of protein-coding genes are miRNA targets. Some miRNAs are expressed as high as 50,000 copies per cell; and by their sequence conservation, with some miRNAs conserved from sea urchins to humans (31). Therefore, miRNAs can regulate a large variety of cellular processes, from differentiation and proliferation to apoptosis. MiRNAs also confer robustness to systems by stabilizing gene expression during stress and in developmental transitions (31, 37). MiRNAs can generate

thresholds in target gene expression and mediate feedforward and feedback loops in gene networks (38).

However, miRNAs only marginally decrease the mean expression of most targeted proteins, leading to speculation about their role in the variability, or noise, of protein expression. It is now accepted that miRNAs decrease protein expression noise for lowly expressed genes but increase noise for highly expressed genes (37). Therefore, genes that are regulated by multiple miRNAs show more marked noise reduction. These observations suggest that miRNAs confer precision to protein expression and thus offer plausible explanations for the commonly observed combinatorial and overlapping targeting of endogenous genes by multiple miRNAs, as well as the preferential targeting of lowly expressed genes (37).

It is accepted that inactivation of a miRNA results in very modest de-repression of its direct targets, typically less than two fold even for highly abundant miRNAs (36). These differences are well within the range that could be attributed to fluctuations of gene expression between two genetically identical cells or between individuals (39). For most genes, such modest changes in expression can be well tolerated by the organism, which might explain why genetic inactivation of miRNAs often does not have obvious phenotypic consequences (28). Globally, these observations suggest that rather than acting as genetic switches - i.e. robust repression of one or few targets results in a strong phenotypic outcome (40, 41) – most miRNA act as rheostats, fine-tuning the expression of hundreds of genes to reinforce cell fate decisions caused through other mechanisms (39, 42, 43). Altered miRNA levels might lead to widespread de-repression of target genes and an unbuffering of gene expression. In cancer biology, such deregulation might be associated in increased genomic noise and an associated increase in the clonal heterogeneity of cancer cells.

2.1.3 Functional redundancy among family members

One notable aspect of miRNA genes is that a large number display paralogs in the genome. Paralog miRNAs arise both from tandem and nonlocal gene duplication events, which give rise to either duplication of sequences in the same transcript – thus originating miRNA clusters – or on distant loci, typically on different chromosomes (31, 44). These miRNAs not only retain a high degree of sequence homology, but also share the same seed sequence. Hence, and by convention, they are grouped into 'miRNA seed families' (45). Notably, roughly one third of human miRNA genes are categorized into families based on sequence similarity (46). As paralog miRNAs share the same seed sequence, they are likely to have similar correspondences to target genes. When expressed in the same cells, these associated miRNAs can co-regulate targets, leading to higher levels of repression than those that could be achieved by each miRNA individually (28). Thus, the existence of miRNAs with

redundant functions suggests that, in particular conditions, numerous members of the family need to be deleted to obtain a clear phenotypic alteration (28).

Polycistronic miRNA clusters are a common feature of vertebrate genomes. Up to two-thirds of the genome are encoded in polycistroninc clusters, i.e., they are co-transcribed with their cluster partners. Such co-regulated miRNAs seem to have a propensity to target the same gene or different genes in the same pathways. Overall, this emphasizes the pathway-regulating roles of these miRNAs (e.g. miR-200 family targeting of actin cytoskeleton). Moreover, in a recent study, it was exposed evidence that strong functional specialization and cooperation can coexist among members of the same polycistronic cluster (47).

2.1.4. The transcriptional landscape aberrations of prostate cancer

The complex eukaryotic transcriptome is dynamically transcribed [16]. Indeed, there are many different RNA species generated by pervasive transcription, including mRNAs, lncRNAs and sncRNAs [16]. The coding potential defines a mRNA as able to translate the DNA sequence information into an aminoacid sequence, forming a protein. This is the boundary used to distinguish between a protein-coding gene (PCG) and ncRNAs.

MiRNAs have been characterized either as tumor-suppressors or oncogenes, varying on the deregulated downstream targets. A growing frame of literature has investigated the biology of miRNA deregulation in PCa. Comparative genomics analyses and high-throughput experimental studies indicate that a miRNA binds to a multitude of sites across the transcriptome, in an intricate regulatory mechanism. Advances in experimental and computational approaches are revealing not just cancer pathways controlled by single miRNAs but also interlinked regulatory networks controlled by multiple miRNAs, which often engage in reciprocal feedback interactions with the targets that they regulate. Thus, miRNAs can establish thresholds and increase the coherence of the expression of their target genes, as well as reduce the cell- to- cell variability in target gene expression. Owing to recent developments in sequencing technologies, surveying other ncRNAs' molecular mechanisms are now providing the tools to functionally interpret these cancer-associated transcripts, making these genes attractive targets for therapeutic intervention in PCa management.

The following review, entitled "Deciphering the function of non-coding RNAs in prostate cancer", summarizes and compares the multitude of ncRNA aberrations in PCa. This is an attempt to characterize the most relevant ncRNA elements, and provide evidence that the non-coding transcriptome is functionally relevant, especially miRNAs, for PCa carcinogenesis.

2.1.5 REVIEW ARTICLE: Deciphering the function of non-coding RNAs in prostate cancer

João Ramalho-Carvalho, Bastian Fromm, Rui Henrique, Carmen Jeronimo:

Deciphering the function of non-coding RNAs in Prostate Cancer

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Abstract

The advent of next-generation sequencing methods is fuelling the discovery of multiple noncoding RNA transcripts with direct implication in cell biology and homeostasis. This new layer of biological regulation seems to be of particular importance in human pathogenesis, including cancer. The aberrant expression of ncRNAs is a feature of Prostate cancer, as they promote tumor-suppressive or oncogenic activities, controlling multicellular events leading to carcinogenesis and tumor progression. From the small RNAs involved in the RNAi pathway to the long noncoding RNAs controlling chromatin remodeling, alternative splicing and DNA repair, the noncoding transcriptome represents the significant majority of transcriptional output. As such, ncRNAs appear as exciting new diagnostic, prognostic, and therapeutic tools. However, additional work is required to characterize the RNA species, their functions, and their applicability to clinical practice in Oncology. In this review, we summarize the most important features of ncRNA biology, emphasizing its relevance in prostate carcinogenesis and its potential for clinical applications.
Key Words: ncRNA, microRNA, lncRNA, Prostate Cancer, Transcription.

1. Introduction

Following the central dogma of Molecular Biology, DNA is transcribed into messenger RNAs, which in turn serves as the guide for protein synthesis (1, 2). Although exceptions to this rule were known to occur, in the form of transfer RNAs and ribosomal RNAs for a long time (3, 4), only over the last few years' evidence emerged that RNA displayed functional roles beyond the messenger between DNA and protein. It is now widely accepted that RNA plays a key role in the regulation of genome organization and gene expression (5, 6). Multiple studies have demonstrated that the vast majority of the human genome is dynamically and differentially transcribed to produce multiple and complex non-coding RNAs (ncRNAs), i.e., RNA transcripts that do not encode for a protein but rather act as regulatory RNAs, in a phenomenon called pervasive transcription (2, 7-9).

The human genome comprises more than 3.2 billion nucleotides that unfolded correspond to more than 2 meters of linear DNA, which is packed into three-dimensional structures in the nucleus of each cell (10). However, the part of the genome that represents protein-coding genes is approximately only 2-3%, while a vast and diverse plethora of ncRNAs originate from the remaining nucleotides in the genome (5, 10). Current ENCODE predictions suggest that ~80% of the genome's DNA is transcribed into RNA, and contribute to the overall estimates of 80% of the genome being biochemically active/functional (11). Although these include some functionally well characterized small and long ncRNAs (11-15), it has been hypothesized that their abundance and sheer complexity alone is reason enough to believe that they must play major regulatory roles in complex organisms. Furthermore, because such complexity is not the result of the amount of synthesized proteins, it should represent the extent and nature of genome regulation (12, 16). However, caution should be taken when considering those transcribed DNA elements as functional players. Multiple lines of evidence indicate that this genome wide transcription is a stochastic process rather than a sign of function p*er se* (17, 18). Indeed, any given DNA element can be transcribed when it is associated with specific histone marks, binds to transcription factors and is located in an open chromatin area (11). Transcription is certainly a prerequisite for a genetic element to be functional but it is not synonymous of that condition (18). Further, if one looks at the term 'function' from an evolutionary standpoint and assumes that a putative undetected function of 98% of the human genome is not a human specific trait, it might be argued that a given DNA element with an important function would possibly show significant signs of selective pressure (i.e., sequential conservation in related organisms) to maintain this functionality over evolutionary time as demonstrated for a very limited number of long ncRNAs (lncRNAs) (19). Comparative studies showed that broadly conserved lncRNAs share a short and 5'-

biased patches of conserved sequence (20). Moreover, lncRNA structure is considerably renewed during evolution, in part due to exonization of transposable elements (20).

Nevertheless, an ever-increasing number of novel classes of small and long ncRNAs are being described, regardless of its homology to that of any related organism or demonstrated function. Driven by recent paradigm shifts in the appreciation of genomic architecture, regulation and transcriptional output, this seems a valid approach to many researchers. Many of these novel ncRNAs are able to interact with DNA, RNA and proteins. Some take part in diverse structural, functional and regulatory activities, controlling nuclear organization and transcription, post-transcriptional and epigenetic regulation (10, 16). This expanding inventory of ncRNAs is implicated in a broad spectrum of processes including organ homeostasis and pathogenesis. This growing index of ncRNAs is fuelled by the discoveries of large-scale consortia aiming to dissect the functional genomic elements such as ENCODE and FANTOM (5). These projects exposed the complexity and plasticity of the genome: it encompasses not only protein-coding genes with multiple transcription start sites, alternative promoter and enhancer elements, splicing initiation and donor sites, as well as variable 3´-untranslated regions (UTRs), but also an unpredictably large number of ncRNAs (5). These display numerous regulatory functions and similarly serve as substrates for transcriptional and post-transcriptional diversification (12, 16). The advent of sequencing technologies revealed that the vast majority of the genome is transcribed either in sense or antisense and it is also expressed in a highly cell type-, subcellular compartment-, and developmental stage-specific manner (21). The current view of RNA transcription is that each nucleotide can contribute to context-dependent transcription, mediated by specific RNA polymerases, ultimately giving rise to numerous and overlapping transcripts (21).

Several reports shed light on the global deregulation of noncoding transcriptome that occurs in cancer cells. One of the best examples is the overexpression of lncRNA *HOTAIR* in breast cancer. *HOTAIR* reprograms breast cells' epigenome in a Polycomb repressive complex 2 (PRC2)-dependent manner, contributing to increased invasiveness and metastasization (22). Recently, it was shown that intronic RNA may serve as molecular scaffold for epigenetic regulation through recruitment of PRC2 proteins to specific gene loci (23). This misregulation of RNA-protein interactions ultimately leads to tumor formation (23). Interestingly, R-loop-formation and head-to-head antisense transcription are known to be involved in transcriptional activation in cancer (24). However, one the first hints on ncRNAs involvement in cancer was the deletion and concomitant down-regulation of miR-15 and miR-16 in chronic lymphocytic leukemia (25). The de-regulation of some PIWI-interacting RNAs may also contribute to breast cancer-specific biology, possibly by remodeling the cancer epigenome (26). Taken together, these body of evidence sets ncRNA as critical components of cancer

biology: ncRNAs are cancer-related genes due to their potential tumor suppressive and/or oncogenic functions (27).

2. The diversity of non-coding RNAs in humans

According to its size, ncRNAs are classified in two main families: lncRNAs, corresponding to transcripts with over 200nt that does not appear to contain a protein coding sequence, and small ncRNAs (sncRNAs), when the RNA sequence contains less than 200nt (12). The ncRNAs localize both to the nucleus and cytoplasm, and may be found in exosomes and other microvesicles, present in bodily fluids such as urine, blood, and seminal fluid, although the abundance and activity of ncRNAs in exosomes remains unclear (28). Exosomes are released from tumor cells and may transfer proteins and RNA across cells. Thus, it is tempting to speculate whether in the PCa microenvironment, miRNAs (the most commonly studied ncRNA in exosomes) may be transferred among stromal cells and cancer stem cells, although it is not clear how miRNAs reassemble into a functional miRISC upon import into other cells.

Moreover, circulating ncRNAs in serum, plasma or urine, although at low levels, may provide new opportunities for biomarker development (29).

a. Small non-coding RNAs

Small ncRNAs differ from lncRNAs by its length and are typically classified according to different biogenesis pathways and genomic origins (Table1). Classically, sncRNAs include all transfer RNAs (tRNAs), some ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs and its derivatives, microRNAs (miRNA), short interfering RNAs and piwi-interacting RNAs (30). More recently, several other small RNAs associated with protein-coding gene transcription and splicing regulation, such as transcription initiation RNAs (tiRNAs), promoter-associated short RNAs, termini-associated short RNAs, 3'-untranslated region-derived RNAs and antisense termini-associated short RNAs have been added to this class (30, 31).

i. microRNAs

Presently, sncRNAs involved in post-transcriptional regulation of target RNAs via the RNAi pathway, such as miRNA, siRNA and piRNA, are considered the most biologically relevant (32). Owing to its involvement in human diseases such as cancer and its potential as disease biomarkers, miRNAs are undoubtedly the best-studied sncRNA class. Mature miRNAs are typically ~22 nucleotide single-stranded RNAs (ssRNA), canonically derived from longer primary transcripts (pri-miRNAs) which are processed to intermediate precursor-miRNAs

(pre-miRNAs) by DROSHA-DGCR8 microprocessor complex (33). These hairpin-precursors are exported from the nucleus to the cytoplasm, via exportin 5 (XPO5), where the terminal loop region of the hairpin is removed by DICER/TRBP2 (34), resulting in a double-stranded mature/star RNA molecule (dsRNA) (Figure 1) (35). Both Drosha and Dicer are RNase-Type III proteins and leave characteristic 2nt offsets on their substrate that can be used for bioinformatics description of miRNAs (36). While the canonical microRNA biogenesis and action model is being constantly refined, Drosha (37) and Dicer (38) independent biogenesis mechanism have been described, but they represent rare exceptions. After the canonical, multistep processing, typically only one of the strands (mature miRNA) is loaded by Argonaute proteins and coupled with diverse components of the RNA-induced silencing complex (RISC). Constrained by the structure of the Argonaute protein, only 7 nucleotides (Position 2-8) of the mature miRNA are exposed (39). This so-called "seed region" defines the range of potential target RNAs, by usually perfect complementarity of these few nucleotides. In the vast majority of cases, target-interaction of miRNAs occurs at the 3'UTR of protein coding genes (40).
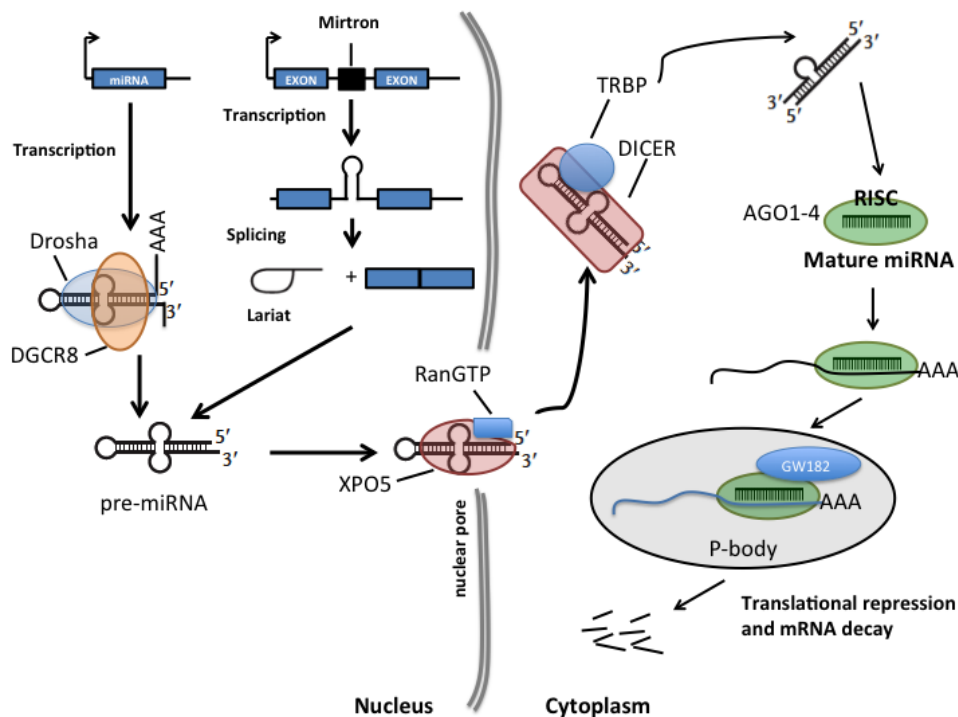
Figure 1. MicroRNA biogenesis in human cells.

In the canonical miRNA biogenesis pathway, primary transcripts are processed by Drosha in the nucleus and by Dicer in the cytoplasm. Both Drosha and Dicer are RNAse III enzymes and produce a hairpin precursor with 2-nt offsets at the 5p and 3p arm. The lncRNA pri-miRNA displays a 7- methylguanosine cap (m7Gppp), ends with a 3 poly(A) tail, and is

transcribed by RNA polymerase II (Pol II). The pri-miRNA contains a stem–loop structure that is cleaved in the nucleus by the endonuclease Drosha together with its double-stranded RNA (dsRNA)-binding protein parner DGCR8, forming a complex called Microprocessor. The output of this trimming is a precursor miRNA (pre-miRNA). It is then exported to the cytoplasm by exportin 5 and further cleaved by the endonuclease Dicer, together with its dsRNA-binding partner TRBP, to produce a miRNA-miRNA* duplex. Further maturation steps reject miRNA* and incorporate the mature miRNA strand into the miRNA-induced silencing complex (miRISC). Alternative biogenesis pathways are also acknowledged. Mirtrons are short introns with hairpin potential that are spliced and disbranched into pre-miRNAs and bypass the Drosha cleavage of the canonical miRNA pathway. It lacks a lower stem and basal single-stranded segments, which are structural features of primiRNA and mediate recognition/cleavage by the DGCR8/Drosha complex. In this pathway, pri-miRNA is generated from a branched mirtron structure that undergoes lariat debranching. Another alternative biogenesis pathway involves pre-miRNA escaping to Dicer processing after nuclear export that is directly loaded into AGO2 protein. AGO2 is responsible for processing the pre-miRNA into a single-stranded miRNA (hsa-miR-451)
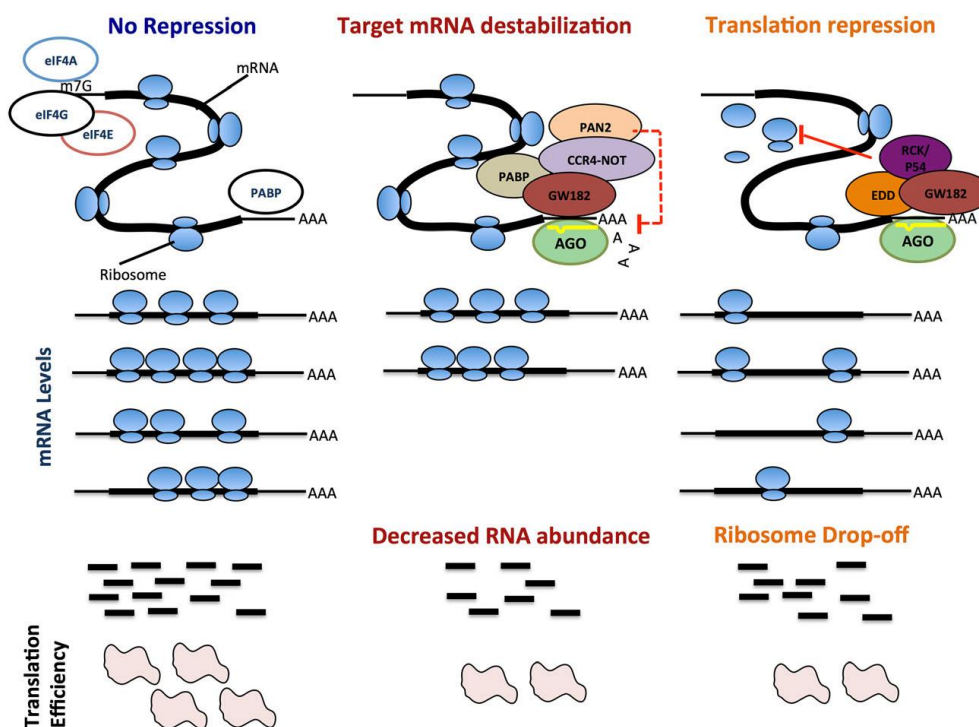
Figure 2. miRNA-mediated gene regulation.

Most plant and a few animal miRNAs direct endonucleolytic cleavage of their mRNA targets by perfect complementarity. However, highly complementary sites in animals' transcriptomes are infrequent. Accordingly, miRNA-directed translational repression is indistinguishable from

mRNA destruction via decapping and 5 -to-3 decay. Thus, it was suggested that miRNAs mainly direct target mRNAs for decay. Nonetheless, the predominant mode of miRNA-mediated repression may be context-dependent. The core RISC complex is formed by Argonaute proteins (1–4 in mamals) and GW182. If mRNA decay and subsequent target mRNA destabilization is observed, it suggests that miRISC interacts with the CCR4–NOT and PAN2 deadenylase complexes to facilitate deadenylation of the poly(A) tail. Following deadenylation, the 5- terminal cap (m7G) is removed by decapping the DCP1–DCP2 complex, and mRNA decay is affected by an exonuclease. The miRISC inhibits translation initiation by interfering with eIF4E-cap recognition and 40S small ribosomal subunit recruitment or by antagonizing 60S subunit joining and preventing 80S ribosomal complex formation. Furthermore, miRISC might inhibit translation at post-initiation steps by inhibiting ribosome elongation. MicroRNA-target interactions might be additionally mapped by Ribosome profiling, providing a "snapshot" of all the ribosomes active in a cell at a specific time point. MicroRNA manipulation would allow systematic monitoring of cellular translation processes and prediction of protein abundance. Coupled with bioinformatic target predictions, this would help determine which mRNA is being translated and which region of the mRNA is being targeted by the miRNA.

miRNA:mRNA interactions that include the RISC-complex then lead to repression or degradation of those transcripts and, ultimately, to a moderate downregulation of the corresponding proteins (Figure 2) (35).

While this seemed a straight forward model when miRNA-function was first discovered, complexity was quickly added when researchers realized that multiple copies of nearly identical and evolutionarily related miRNAs might be found in the genome, that share seed sequences and, consequently, the range of targets (miRNA families). Moreover, single miRNAs have not only one but hundreds of target RNAs and single protein coding genes are targeted by multiple miRNAs (Figure 3).

Consequently, there is redundancy in microRNA targeting. Indeed, one miRNA may have different MRE in the same target RNA. It is, thus, likely that most miRNA act as rheostats, fine tuning the expression of hundreds of genes, in intricate gene networks (41). miRNAs may, in fact, establish thresholds and increase the coherence of the expression of its targets, as well as reduce the cell-to-cell variability in target gene expression (42). miRNAs themselves are also subject to modifications, including post-transcriptional RNA editing (methylation, uridylation and adenylation) (27, 43) and miRNA tailing (35).
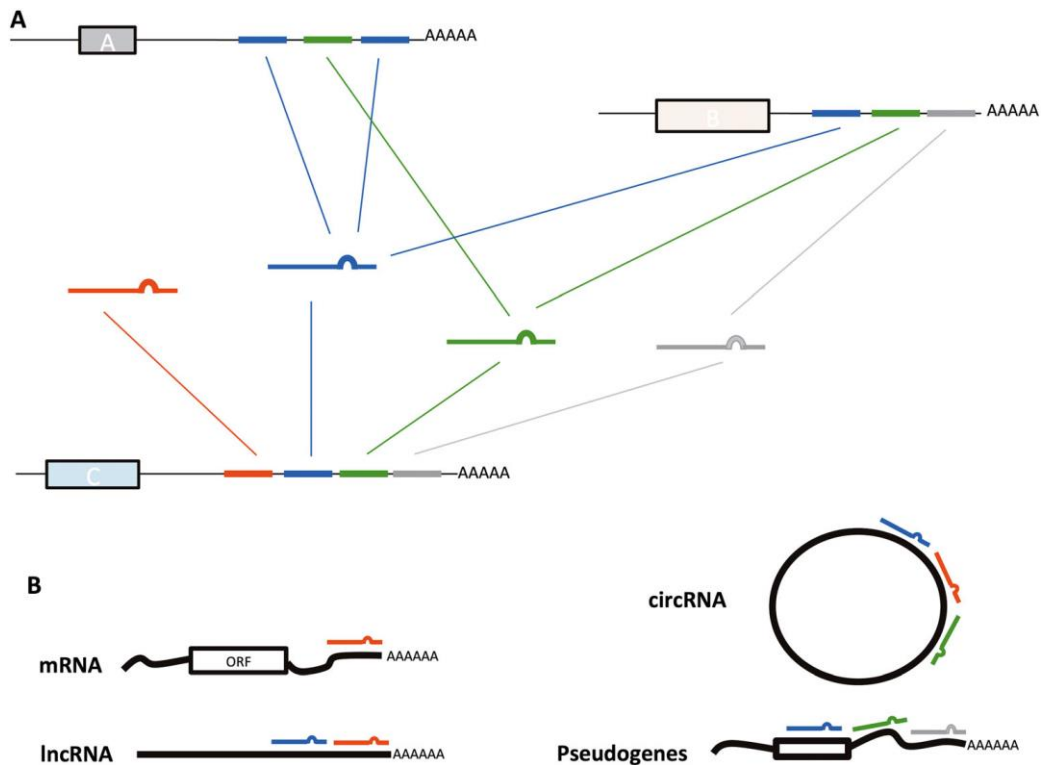
Figure 3. Magnitude of microRNA-mediated gene regulation.

(A) Redundancy of microRNA targeting. Most miRNA act as rheostats, fine tuning the expression of hundreds of genes, in intricated gene networks. A single miRNA can target multiple transcripts and one specific mRNA is able to be targeted by several miRNAs. Indeed, one microRNA may have different MRE in the same target mRNA (miRNA binds to the specific MRE in the same color). (B) Competing endogenous RNAs: natural occurring miRNA decoys. Linear or circular lncRNAs may function as miRNA decoys to sequester miRNAs from their target mRNAs of functionally relevant lncRNAs. Base pairing is also the mode of action of ceRNAs. Those lnc-ceRNAs can mask miRNA-binding sites on a target mRNA to block miRNA-induced silencing through the RNAinduced silencing complex (RISC). The ceRNA comprises both circular RNA (circRNA), lncRNAs, and pseudogenes competing for the complementarity with miRNAs. The ultimate impact of these interactions is that protein-coding RNAs and non-coding RNAs may crosstalk by competing for miRNA binding through their miRNA recognition motifs impairing cell homeostasis. (ORF, open reading frame)

Deep sequencing data revealed that the majority of miRNAs show length and sequence isoforms (isomiRs) with largely unknown functions in cancer biology (43) although it has been shown that they can selectively associate with specific RNA-binding proteins (e.g.,

Argonaute) or exosomes (44) implying context-dependent functional roles. These alterations may, thus, affect miRNA maturation and de-regulate target-genes:miRNA interactions stoichiometry.

Despite growing knowledge on miRNAs biology and the ever-increasing amount of sncRNA sequencing data, defining what is and what is not a miRNA has become a challenge. This uncertainty led to descriptions of several hundreds to several thousands miRNAs in the human genome (45, 46). Remarkably, when structural criteria (Box 1) for annotation and nomenclature of human miRNA genes were recently updated, only a small proportion of the previously reported human miRNAs was recognized, constituting about one third of the 1881 putative miRNA entries in the widely used online repository miRBase (last updated June 2014) were considered *bona fide* miRNAs (36). Consequently, the 523 currently accepted human miRNAs represent a solid foundation for future studies but a reassessment of all published (but not yet in miRBase listed) miRNAs is desirable to further expand our understanding of the human microRNAome.

Box 1. miRNA structural features

- *miRNAs are between 20-26nt long*
- *They are genome-encode and derive from hairpin precursor that shows imperfect complimentarity (~16 nts)*
- *Mature products of both hairpin arms are expressed (mature, co-mature or star sequence)*
- *Show a 5' read homogeneity in 90% of the reads*
- *Display a 2nt offset on both ends which is a consequence of Drosha/Dicer processing*
- *Mature miRNA sequences usually start with A or U.*
- *Flanking region upstream shows UG motif at Position 14, loop shows UGU motif at the 3' end of the 5' arm, and flanking region downstream shows CNNC motif at Position 17-18.*
- *At least some miRNAs of any higher animal taxon are representatives of phylogenetically conserved miRNA families and show very high sequence similarities.*

ii. siRNAs

Endo-siRNAs are double-stranded, 21–26 nt, RNAs (dsRNA) that are cleaved from longer dsRNA intermediates precursors derived from repetitive sequences, sense–antisense pairs (derived from transposons) or long stem-loop structures (47, 48). Endo-siRNAs biogenic pathway in humans is DICER-dependent (although Drosha-independent) and involves

Argonaute proteins (AGO2) (47, 49). Endo-siRNAs originate from diverse genomic locations and have been implicated in post-transcriptional (mRNA cleavage) and epigenetic silencing of protein-coding genes and transposon-derived ncRNAs, respectively, as well as other unclear functions (49). Contrasting with miRNAs, endo-siRNAs bind only RNA molecules containing perfectly complementary sequences (32).

iii. PIWI-interacting RNAs

Another important family of RNAi comprises piRNAs (50), 24–30 nucleotide ssRNAs, derived from single-stranded RNA precursors transcribed from intergenic repetitive elements, transposons or large piRNA clusters (47). The biogenesis is Drosha-/DICER-independent and requires Piwi proteins of the Argonaute/Piwi family (50, 51). During piRNAs biogenesis, piRNA precursors undergo nuclear processing and export, primary or cyclic secondary processing–(the ping-pong cycle, catalyzed by PIWI proteins MILI and MIWI2) and PIWI ribonucleoprotein complex (piRNP) assembly (21, 52). The ping-pong amplification cycle generates antisense piRNAs capable of suppressing the transcript of origin (52). The assembly of the piRNP is essential to establish post-transcriptional regulation and transposon modulation. The piRNAs functions are connected to its origin: if derived from transposons, piRNAs are implicated in regulating cognate transposon activity, whereas piRNAs resulting from piRNA clusters are involved in gene expression control (52, 53). piRNAs were primarily found in germ cells, but recent studies have recognized that piRNAs are expressed in somatic cells, including non-tumorous and tumourous tissues from 11 organs (54).

Current views suggest that both endo-siRNAs and piRNAs are defensive mechanisms against nucleic acid-based parasites, acting as genome's guardian. However, both endo-siRNAs and piRNAs are not considered cancer-related genes and, consequently, additional data is need to ascertain the true relevance of these ncRNA families in tumorigenesis (54, 55). Remarkably, these three major families of sncRNAs associate with different AGO protein subclades to perform sequence-specific gene silencing (35).

iv. snoRNAs

Small non-coding RNAs exert far more biological regulation rather than just RNAi-silencing. One of the firstly described classes of small ncRNAs was snoRNAs. SnoRNAs are 60-300 nt long, mainly localized in the nucleolus, which are encoded by introns of coding and non-coding genes (56, 57). Their function is to guide RNA for post-transcriptional modification of ribosomal RNAs and some spliceosomal RNAs, with a few others involved in nucleolytic processing of the original rRNA transcript (57, 58). Two subdivisions of snoRNAs are known to exist and are involved in two different types of RNA post-transcriptional modification. The

C/D box snoRNAs define the target sites for 2′-O-ribose methylation and H/ACA box snoRNAs demarcate the target sites for pseudouridylation (57, 59). C/D box and H/ACA box snoRNAs are structurally distinct and those differences make the connection with the binding of specific proteins to form the small nucleolar ribonucleoprotein (snoRNP) complexes that identify and modify the cognate targets (60). During processing of rRNA, snoRNA guide sequences hybridize to the target rRNA and lead the snoRNP to direct the modification of ribose 2'-hydroxyl groups or the isomerization of uredines to pseudouridines within pre-rRNAs (58, 61). Dyskerin is the enzyme recruited by H/ACA box snoRNAs to catalyze pseudouridylation at specific ribonucleotides, whereas C/D box snoRNAs activity requires the methyltransferase fibrillarin, to mediate the 2′-O-methylation (59). The reactions occur generally at conserved sites in nascent rRNAs (58, 61). In addition to catalyze nucleotide modification, snoRNP association with pre-rRNAs may also serve to chaperone correct RNA folding for rRNA processing and ribosome assembly (28)

Additionally, snoRNAs have other functions (e.g., Small Cajal body RNAs (62)) and it has recently been found that snoRNA loci may also produce miRNA-like small RNAs (63, 64), uncovering a putative complex crosstalk between snoRNA-guided RNA processing and RNAi pathways. Strikingly, novel evidences implicate snoRNA as controllers of cell homeostasis and snoRNA dysregulation may thus contribute to carcinogenesis (57, 65).

v. smallRNAs incertae sedis

Although the previously described sncRNA families are relatively well understood from a biological standpoint, others are still poorly characterized. These include sncRNAs resulting from gene regulatory regions and gene boundaries [subclasses of promoter-associated small RNAs, such as transcription initiation RNAs (tiRNAs)], termini-associated short RNAs, antisense termini-associated short RNAs and splice-site RNA (spliRNA) (66). Others are structural components of chromosomes – the centromere-associated RNAs and telomere small RNAs. Additionally, some small RNAs are cleavage sub-products of other ncRNAs [e.g., transfer RNA-derived RNA fragments (tRFs)] or are derived from different sources (mitochondrial ncRNAs and miRNA-offset RNAs) (12, 66). TRFs are one of the most abundant sncRNAs, thought to be present in most organisms and generated by ribonucleolytic processing of tRNAs by Dicer and RNAse Z (67). The definition of the multiple tRFs classes is made according to the position of the tRNA cleavage site that gives rise to tRFs. Among the known classes, the most prominent includes 5'- and 3'-tRNA halves (cleaved in the anti-codon loop), 5'- and 3'-tRFs (also known as 3'CCA tRF), and 3'U tRFs (68). The stress-induction of tRFs results in stress granule assembly and inhibition of protein synthesis, linking tRFS to cell homeostasis through control of cell proliferation and mediating RNA inactivation through Argonaute engagement (68).

To further ascertain the specific biological roles of these enigmatic small RNAs functional studies are needed. For instance, when deleting tiRNAs associated with binding sites for RNAPOLII CTCF binding factor, there is a dramatic alteration in CTCF binding and nucleosome density at genomic loci proximal to sites of tiRNA biogenesis (69). Further research is thus required to dissect the evolution, biogenesis and functions of these small ncRNAs classes and explore its potential connections with cancer.

b. Long non-Coding RNAs

According to GENCODE annotation v7, there are 20,687 protein-coding genes and in total, GENCODE-annotated exons of protein coding genes cover 2.94% of the genome or 1.22% of protein-coding exons (5). These data clearly point out that the vast majority of the human genome is transcribed not into a biochemically active RNA but rather into a structural, nc RNA. Transcripts lacking the capacity to code for a protein, are uniformly abundant in all organisms, from yeast to humans (16). There is growing evidence that ncRNA have biologic functions and operate through defined mechanisms. However, this compelling abundance of ncRNAs triggered the discussion whether ncRNA transcription is the output of transcription or ordinary byproducts of the transcriptional system or simply a methodological artifact (12, 70). Thanks to global efforts, it has been possible to assign specific features to define lncRNAs as distinct transcripts: the vast majority of lncRNAs is generated by the same transcriptional machinery, similar to other mRNAs, as emphasized by RNA polymerase II occupancy and histone modifications associated with transcription initiation (promoter, H3K4me3) and elongation (H3K36me3 in the gene body) (16, 30). lncRNAs possess a 5' terminal methylguanosine cap, are often spliced via canonical genomic splice site motifs and some of them are polyadenylated whereas other are not. Alternative pathways also contribute to the generation of lncRNAs such as non-polyadenylated lncRNAs, likely expressed from RNA polymerase III promoters (16). Not only lncRNA regulation is made by well-established transcription factors, but also lncRNA are frequently expressed in a tissue-specific manner (Table 1) (30).

Table 1. mRNA/lncRNA convergent and divergent features

mRNA	lncRNA
Tissue-specific expression	
Form Secondary structure	
Undergo post-transcriptional processing (e.g. 5'CAP, polyadenylation, splicing)	
Important roles in disease	
Protein-coding transcripts	Non-protein coding, regulatory functions
Well conserved across species	Poorly conserved
Present in both nucleus and cytoplasm	Predominately in the nucleus
Around 20-24,000 mRNAs	Predicted 3-100 fold of mRNA in number
Expression level: low to high	Expression level: very low to moderate

Generally, lncRNAs are expressed in lower amounts compared to their protein-coding counterparts, making it difficult to robustly detect in clinical samples (12, 16). Consistent with the many regulatory functions assigned to lncRNAs, the low expression may restrict these lncRNAs to subtle or redundant roles, or reflect incomplete repression in nonspecific cells (16, 66). By comparison to protein-coding genes, lncRNA expression has higher cell specificity than proteins, consistent with their proposed role in architectural regulation in which each cell displays a unique transcriptome (16). The organization of lncRNA loci in the genome revealed transcriptional complexity as lncRNA genes often display large numbers of isoforms. Moreover, lncRNAs are often organized in association with protein-coding genes and half of the protein coding genes have complementary non-coding antisense transcription, further expanding the complexity of genome transcriptional dynamics. lncRNAs may be transcribed from intergenic regions [large intergenic ncRNAs (lincRNAs)]; in antisense, overlapping, intronic and bidirectional orientations relative to protein-coding genes (Figure 4); from gene regulatory regions – UTR, promoters, and enhancers; from specific chromosomal domains (telomere) or derived from the mitochondrial genome (12, 16, 66).

lncRNAs act by a multitude of regulatory mechanisms according to its specific location in the cell. lncRNA play a role as organizing factors in the dynamic nuclear organization that shapes the cell nucleus through nucleosome remodeling (71, 72). Nuclear lncRNAs might be involved in gene-to-gene interactions either locally or in the context of cross chromosome interactions, *i.e.*, *cis*- and *trans*-mediated regulatory roles, respectively (6, 10)].
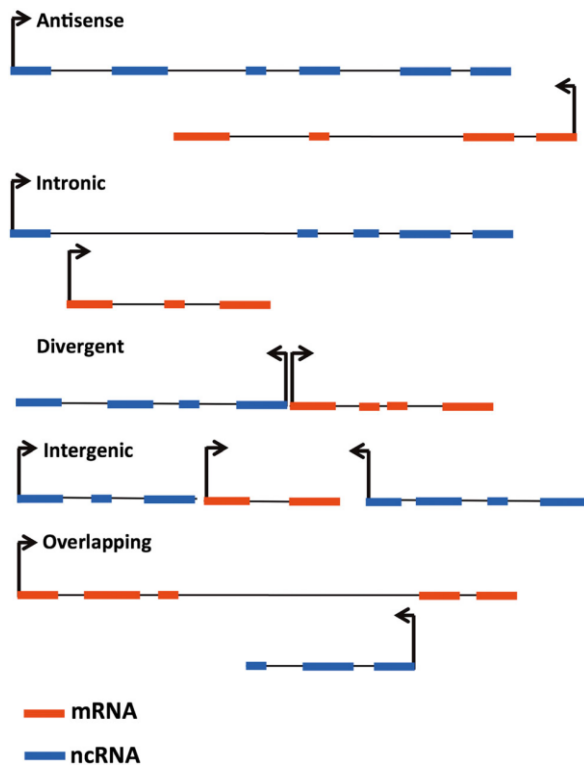
Figure 4. Descriptive structure of a long non-coding RNA loci.

Normally, lncRNAs are defined by their location accordingly to protein-coding genes in the vicinity. Antisense lncRNAs transcription initiate inside or 3' of a protein-coding gene. They are transcribed in the opposite direction of protein-coding genes, overlapping any portion of a mRNA. Intronic lncRNAs initiate inside an intron of a protein-coding gene in either direction and terminate without overlapping exons. Bidirectional lncRNAs are transcripts that initiate in a divergent fashion from the promoter of a protein-coding gene; the precise distance cutoff that constitutes bidirectionality is not defined but is generally within ~100 base pairs. Intergenic lncRNAs (also termed large intervening noncoding RNAs or lincRNAs) are lncRNAs with separate transcriptional units from protein-coding genes. A key structural feature is that lincRNAs need to be 5 kb away from protein-coding genes. LincRNA genes are preferentially found within 10 kb of protein coding genes. These are defined as lncRNA transcripts that encompass a protein-coding gene within the "intron" of a lncRNA or as lncRNAs that overlap the intron of a protein coding gene.

In most cases, nuclear lncRNAs function by recruiting chromatin-remodeling complexes to particular DNA loci (22), as it has been shown to form ribonucleoprotein (RNP) complexes by recruiting DNA methyltransferases, the Polycomb repressive complex (PRC) 2 (promotes H3K27 trimethylation) (22), and H3K9 methyltransferases, resulting in the formation of

repressive heterochromatin and transcriptional inhibition. However, lncRNAs are also associated with transcriptional activation through engaging of chromatin-modifying complexes, including H3K4 methyltransferases, specific transcription factors and recruiting POLII (73-75). Nuclear lncRNAs may also bind and sequester transcription factors away from their target chromosomal regions, thus indirectly impairing gene expression (76).

Nonetheless, a significant number of lncRNAs are transferred to and lodged in the cytoplasm. Functions of cytoplasmic lncRNAs include protein localization, mRNA translation and stability. By recognition of the target by base pairing, they can modulate mRNA at different levels: a) base pairing between *BACE1* and *BACE1-AS* induces stabilization of target mRNA and increases the BACE1 protein expression (77)*; b)* repression of translation (e.g., lincRNA-p21 suppresses target mRNA translation) (78); and c) competition with endogenous RNAs (ceRNAs) for miRNA binding (79). This regulatory system in which multiple RNAs (both coding genes, pseudogenes and lncRNA) may crosstalk and compete for shared miRNA binding, are thought to be relevant for many processes, including cancer (79). Moreover circular RNAs (circRNA) also function as miRNA "sponges" (80), and given that linear ceRNAs have a short half-life, it provides superior stability and its turnover can be controlled by the occurrence of a perfect miRNA binding site (Figure 3B) (81, 82).

Another function of cytoplasmic lncRNA is related to protein localization: lncRNA contain distinct domains that interact with specific protein complexes and through a combination of domains, bring specific regulatory components into proximity, resulting in the formation of a specific functional complex to coordinate gene expression (83). Additionally, lncRNAs not only act as decoys for sncRNAs but they may also function as precursors of sncRNAs, including small nucleolar RNAs (snoRNAs) and miRNAs (84).

i. Natural antisense transcripts

Natural antisense transcripts (NATs) are endogenous RNAs that partially or totally overlap transcripts (either coding or non-coding) originating from the opposite DNA strand (85). NATs can be originated from independent promoters, shared bidirectional promoters or cryptic promoters that are situated within genes (85). Depending on the orientation of the sense transcript, overlapping pairs are classified as: head-to-head (5′-regions overlap, HTH), tail-to-tail (3′-regions overlap, TTT), embedded (one transcript is fully contained within the other) (EMB), or intronic (INT) pairs (85, 86). NATs function locally (in the nucleus, preferentially) or distally (in the cytoplasm) (85), and are usually not abundant (around 10-fold lower abundance than associated mRNA) (85).

Although NATs clearly typify *cis* regulation, affecting alleles on the DNA strand from which they are produced, in a local fashion, they also act in *trans* because they can interact with other loci taking advantage of 3-D organization of chromatin (86)*. Cis*-regulation is due to

antisense transcription in a given locus whereas *trans*-regulation is mediated by the antisense transcript corresponding to the RNA being transcribed (85). *Cis*-acting NATs function either locally (e.g., in promoter–gene interactions) or distally (e.g., in enhancer–gene interactions). Local *cis*-regulation comprises epigenetic alterations proximal to a target gene (e.g., regulation of transcription initiation by affecting DNA methylation), whereas distal *cis*-regulation requires RNA-RNA interactions amid transcripts originated from the same locus (85). Moreover, when NATs remain at the loci of origin, they can mediate *cis* effects due to formation of R-loops, triple helices or stalled polymerases. The functional output of *cis*-regulation by NATs leads to activation or silencing of the corresponding sense mRNA, via transcriptional activation or silencing, mRNA stabilization, alternative splicing or post-translational regulation (86). Because antisense and sense transcripts are transcribed from the same locus, it is suggested that antisense transcripts function recurrently in *cis* whereas other ncRNAs commonly function in *trans*, although there is evidence for *trans*-acting antisense transcripts. Antisense transcription might be far more extensive than previously anticipated, with around 50% of sense transcripts having antisense partners (86). Interestingly, NATs' genomic distribution suggests that they might act as self-regulatory loops that control its own expression.

ii. Enhancer elements and RNAs

Enhancers are non-coding genomic regions that activate transcription of target genes at long distances. Mammalian genomes contain hundreds of thousands of putative enhancer elements, located upstream and downstream of coding target gene promoters, which are critical for cell-specific gene expression programs (87, 88). Enhancers are also considered transcription units, giving rise to transcription of a class of lncRNAs, the eRNAs (88). Histone modification signatures characterize enhancer-like regions, including enrichment of H3K4me1 and H3K4me2 and reduced levels of H3K4me3, compared to promoters (87, 88). eRNAs may be either polyadenylated or non-polyadenilated, and are subdivided into unidirectional and bi-directional transcripts. eRNAs exhibit a 5'cap, usually are not spliced or polyadenylated, and can be produced as unidirectional or bi-directional transcripts. A growing body of evidence suggests that eRNAs are functionally important *per se*, and contribute to enhancer-mediated transcriptional activation of target genes. Primed enhancers are marked with H3K4me1 and H3K4me2 and lack histone acetylation. The repressive histone modification H3K27me3 marks enhancers that are considered to be poised. In contrast, active enhancer regions are enriched for H3K27ac and are bound by actively transcribing RNA Polymerase II (PolII) (88). eRNA expression is a hallmark of active enhancers and it has been used as a signature to identify those regions through transcriptomic profiling. The mechanisms by which eRNAs regulate gene expression are not completely clear but it has

been hypothesized that it may stabilize enhancer-promoter looping, facilitate PolII recruitment and its transition into productive elongation (87). As such, eRNAs are likely to have important functions in many regulated programs of gene transcription including those mediated by Androgen receptor (89), p53 and ERα (ESR1) (90).

iii. Long intervening noncoding RNAs

lincRNAs (long intervening noncoding), also called long intergenic noncoding RNAs, are lncRNAs that do not overlap exons of either protein-coding or other non-lincRNA types of genes (19). They are transcribed from multiple loci in the human genome, are located in nucleus although are more frequently reported in cytoplasm. lincRNAs lack defining sequence or structure characteristics as they combine multiple classes of noncoding RNAs (such as intronic and intergenic genes) (Figure 4). However, a few common features are observed, including being composed of few exons (normally 2-3) which makes them shorter than mRNAs (19). The average length of lincRNAs exons is no larger than its counterparts in PCG. Although the transcriptional regulation, chromatin modifications and splicing signals are similar to PCG, lincRNAs seem to be less efficiently spliced. Interestingly, lincRNAs significantly overlap repetitive elements, probably due to the fact that lincRNA functions are more tolerant to retrotransposon insertions. Repetitive elements were reported to play important mechanistic roles in lincRNAs roles, enabling base pairing with other RNAs containing repeats from the same family. Finally, the median lincRNA levels are only about a tenth of that of mRNAs, as lincRNA expression is typically more variable among tissues and enriched in testis and brain. LincRNAs' functions include co-transcriptional regulation, regulation of gene expression (both in *cis* and *trans*) by bridging proteins and chromatin, scaffolding nuclear and cytoplasmic complexes, and RNA-RNA interactions. Consequently, lincRNAs are believed to play a widespread role in gene regulation and maintenance of cell's homeostasis (19).

iv. Pseudogenes

Pseudogenes are ancestral copies of protein-coding genes that arose from genomic duplication or retrotransposition of mRNA sequences into the genome, followed by accumulation of deleterious mutations due to loss of selection pressure (91). A pseudogene shares an evolutionary history with a functional protein-coding gene but it has been mutated through evolution to contain frameshift and/or stop codon(s) that disrupt the open reading frame (92). Pseudogenes pervade the genome in close sequence similarity with their cognate genes. There are three main types of pseudogenes: (a) unitary pseudogenes - species specific unprocessed pseudogene without a parent gene in the same species but with an active orthologue in another species; (b) processed pseudogenes – which appear to

have been produced by integration of a reverse transcribed mRNA into the genome; and (c) unprocessed pseudogene - those that may contain introns as it resulted from gene duplication (92). Pseudogenes are of capital importance owing to its competing endogenous RNAs (ceRNAs) action as natural miRNA "sponges" (79). Moreover, they may also regulate the expression of their parent gene by decreasing mRNA stability of the functional gene through its own over-expression (93). Interestingly, pseudogenes are also a source of small interfering RNAs, impacting on gene expression by means of RNAi pathway (94) and by generating antisense transcripts (95).

3. Non-coding RNAs in Prostate Cancer

The constantly expanding inventory of ncRNAs has been implicated in a broad spectrum of processes including prostate homeostasis and pathogenesis. The emergence of ncRNAs is of crucial importance for prostate biology because prostate cells are transcriptionally active and numerous reports documented the deregulation of ncRNAs in prostate cancer (PCa) (27, 30). This is the most commonly diagnosed cancer among men worldwide, and a major cause of morbidity and mortality (96). Although radical prostatectomy reduces mortality among men with localized prostate cancer, up to 40% of patients experience disease progression and recurrence (97). Numerous studies using mRNA-based techniques contributed to a better understanding of the molecular pathways involved in prostate carcinogenesis (98). It is likely, then that unraveling the biological functions of ncRNAs in PCa will provide new insights into their functions, mechanisms of action and potential usefulness as tools for PCa management (27). Despite the myriad of ncRNA families described thus far, only a small proportion are known to be involved in PCa. The best examples include small RNA families, mainly miRNAs and some lncRNAs, including eRNAs and antisense RNAs. Other classes are under active investigation, including pseudogenes, lincRNAs and tRNAs. These ncRNAs not only control functional pathways of cell biology, but it may also constitute novel therapeutic targets or diagnostics biomarkers. In the following sections, we review the rapidly growing knowledge on ncRNAs as key players in prostate tumorigenesis and highlight their translational potential into the clinics.

a. Small non-coding RNAs

To date, the most extensively studied sncRNAs in PCa are miRNAs. These are classified as oncomirs (when miRNA expression favors tumor development) or tumor suppressor miRNA (i.e., when its expression normally counteracts tumor initiation and/or development) and play a critical role in PCa [58]. Dysregulation of miRNAs in cancer may occur through epigenetic changes (commonly, promoter CpG island hypermethylation) or genetic alterations, as well as miRNA biogenesis machinery dysfunction, which subsequently affects transcription of

primary miRNA, its processing to mature miRNAs and/or interactions with mRNA targets [59].

i. Dysregulation of microRNAs in PCa

NGS-based profiling, which enables high-throughput analysis of the miRNAome with single-base resolution [99], revealed common downregulation of miR-205, miR-143 and miR-145, and upregulation of miR-375 and miR-148, among others (Table 2). However, due to the inherent heterogeneity of PCa, sample selection and technological platforms used, some discrepancies in results are apparent. Down-regulation of miR-15a-miR-16-1 cluster (putative tumor suppressors through targeting of *BCL2*, *CCDN1* and *WNT3A*) in PCa due to 13q14 deletion is commonly acknowledged [100]. Deletion of this cluster fuels survival, proliferation and invasion of PCa cells, whereas in vivo overexpression results in growth arrest, apoptosis and marked regression of PCa xenografts [100]. Strikingly, in vitro blockade of miR-15a and miR-16-1 promotes survival, proliferation and invasiveness of previously untransformed prostate cells, which become tumorigenic in immunodeficient NOD-SCID mice [100]. On the other hand, loss of miR-101 contributes to overexpression of EZH2, linking PCa progression and altered of epigenetic reprogramming [101]. Indeed, one or both genomic loci encoding for miR-101 [located in chromosome 1 (MIR101-1) and in chromosome 9 (MIR101-2)] are lost in a sizeable proportion of primary PCa and up to 2/3 of metastatic PCa [101]. In DU145 cells, forced expression of miR-101 impairs cell invasion and reduces tumor growth in a mouse xenograft, whereas miR-101 re-expression globally decreases H3K27me3 histone mark levels at PRC2 target genes' promoters, demonstrating that manipulation of miR-101 expression may be of therapeutic usefulness [101].

Mir-34a, a p53 target [103], is downregulated due to promoter methylation [102] and underexpressed in CD44+ PCa cells purified from xenograft and primary tumors [104]. Its overexpression in cell pool or purified CD44+ PCa cells inhibits clonogenic expansion, tumor regeneration, and metastasis, whereas delivery miR-34a antogomirs to CD44- PCa cells has the opposite effects [104]. These effects are mediated by CD44, a target of miR-34a, and *CD44* silencing phenocopied miR-34a overexpression [104]. Additionally, miR-34 cooperates with p53 to counteract cancer progression, and jointly regulate prostate stem/progenitor cell activity [104]. This action is also mediated by *MET*, a mutual p53/miR-34 downstream target and a critical regulator of stem cell compartment [104]. This suggests a therapeutic potential for miR-34a against PCa by directly acting on cancer stem cells [104].

One of the main focus on miRNA research in PCa is AR-signaling pathway. Not only AR is targeted by multiple miRNAs, but it also modulates miRNA expression, mediated by androgen-responsive elements within the promoter region [105]. MiR-21 is overexpressed in primary PCa and DU145 and PC3 PCa cell lines [107] and AR binding to its promoter

enhances transcriptional activity, promoting hormone-dependent and hormone-independent PCa growth [106]. miR-21 inhibition using antisense oligonucleotides does not affect proliferation although it increases sensitivity to apoptosis and inhibits cell motility and invasion by targeting *MARCKS*, a gene with a role in cell motility [108], whereas miR-21 overexpression represses *BTG2*, which induces expression of luminal markers and promotes epithelial-mesenchymal transition (EMT) [109]. Increased miR-21 expression is associated with shorter biochemical recurrence-free survival and predicts biochemical recurrence in PCa patients submitted to radical prostatectomy [110]. The list of AR-regulated miRNA also includes miR-27a, miR-141, miR-101 and miR-125b [111]. Conversely, miR-135b, miR-185, miR-297, miR-34a, miR-34c, miR-421, miR-634, miR-654-5p, and miR-9 influence androgen signalling by targeting AR [105]. Thus, miRNAs are involved in hormone-dependent and hormone-independent PCa growth, constituting putative therapeutic strategies to inhibit AR function and androgen-dependent cell growth in PCa.

PTEN is a tumor suppressor that antagonizes PI3K/AKT signalling and its expression is frequently abrogated in PCa. Decreased *PTEN* abundance due to up-regulation of miR-106b~25 cluster (due to genomic amplification) and miR-22 in PCa is critical for malignant transformation of prostate cells [112]. In DU145 cells, stable over-expression of pri-miR-22 markedly increases colony formation and caused increased proliferation and tumor growth, as well as over-stimulation of AKT pathway in xenografts [112]. The same effects are apparent when miR-106b~25 cluster is stably expressed in PCa cells, leading to decreased PTEN abundance and activity [112]. Strikingly, that miRNA locus also collaborates with its host gene, *MCM7*, to promote malignant transformation. In nude mice, larger tumors were formed, compared to control cells, when miR-106b~25 cluster was overexpressed [112]. Moreover, miR-22 and miR-106b~25 clusters cooperate with c-MYC, further emphasizing its proto-oncogenic properties. Indeed, *MCM7*, and, consequently, miR-106b~25 cluster transcription is enhanced by c-MYC, suggesting that its oncogenic activity may also involve transcriptional activation of *PTEN*-targeting miRNAs [112].

MicroRNAs might be also involved in development of PCa bone metastasis, as loss of miR-15 and miR-16 and increased miR-21 expression stimulate dissemination and bone marrow colonization, through aberrant TGF-β and Hedgehog signalling [107].

In exosomes derived from PCa bulk and cancer stem cells (CSC), miR-100-5p and miR-21-5p were the most abundant miRNAs in both cell types, among 1839 miRNAs isolated [113]. Strikingly, biological processes controlled by the differentially expressed miRNAs in bulk exosomes were related to fibroblast growth, epithelial proliferation, and EMT, through MMPs activation, whereas those from CSCs exosomes controlled proliferation, epithelial differentiation and angiogenesis [113]. Overexpression of miR-100-5p, miR-21-5p, and miR-139-5p in a normal prostate fibroblast cell line (WPMY-1) resulted in increased expression of

MMPs [113], with a predominant effect of miR-21-5p on *MMP9*, and of miR-100-5p on *MMP2* and *MMP13*, whereas miR-139 induced expression of all MMPs. Ultimately, transfection of those miRNAs significantly increased *RANKL* expression, which induces cell proliferation, emphasizing that miRNAs contained in exosomes may play a significant role in cancer invasion and metastasis [113].

Table 2. Representative microRNA in PCa biology

miRNA	Function(s)	Disruption	PCa hallmarks	Target(s)	Ref
miR-1	TS-miRNA	Epigenetic silencing	Cell cycle control, mitosis, DNA replication/repair and actin dynamics. Suppresses xenograft growth of PCa cells	*LASP1, PTMA, XPO6, NOTCH3*	[100]
miR-31	TS-miRNA	Epigenetic silencing	Suppresses androgen receptor and inhibits prostate cancer growth *in vivo*	*AR, CDK1, E2F2, EXO1, FOXM1, MCM2*	[101]
miR-34b/c	TS-miRNA	Epigenetic silencing	Suppresses cell proliferation, colony formation, migration/invasion, cell-cycle arrest and apoptos is due to demethylation, active chromatin modifications, and AKT pathways Decreases tumor growth in nude mice	*cMYC, DNMT1, HDAC1, HDAC2, HDAC4, AKT2*	[102, 103]
miR-145	TS-miRNA	Epigenetic ilencing and p53 mutations	Regulates androgen-dependent cell growth *in vitro*. Promotes cell cycle arrest. Suppresses PCa tumor growth *in vivo*	*ERG, AR, SENP1*	[104–107]
miR-193b	TS-miRNA	Epigenetic silencing	Regulates anchorage independent growth and controls cell cycle	*CCND1, uPA*	[108–110]
miR-203	TS-miRNA	Epigenetic silencing	Controls proliferation, migration and invasive potential. Suppresses PCa metastasis *in vivo*. Loss of miR-203 promotes tyrosine kinase inhibitors resistance	*LASP1, Rap1A, GOLM1, AREG, EREG, TGFA*	[111–115]
miR-205	TS-miRNA	Epigenetic silencing	Cell viability impairment, Neutralizes epithelial-to-mesenchymal transition and reducing cell migration/invasion. Inhibits metastasis *in vivo*	*MED1, PKC1, AR, ZEB1*	[116–119]
miR-224	TS-miRNA		Suppresses PCa cell proliferation, apoptosis, invasion, and migration	*TRIB1, TPD52*	[120, 121]
miR-21	Onco-miR	AR-regulated	Promotes hormone-dependent and hormone-independent prostate cancer growth. Induces EMT and luminal markers expression. Enhances PCa tumor growth *in vivo*	*BTG2, FBXO11*	[122–124]
miR-32	Onco-miR	AR-regulated	Regulates autophagy and induces radioresistance. Conferees growth advantage and blocks apoptosis	*BTG2, PIK3IP1, DAB2IP*	[125, 126]
miR-96	Onco-miR		Regulates autophagy under hypoxia, enhances growth and cellular proliferation, promotes prostate bone metastasis *in vivo*.	*MTOR, ATG7, FOXO1, AKT1S1*	[127–129]
miR-183	Onco-miR		Involved in zinc homeostasis, regulates cell growth and motility, positively controls synthesis and serum levels of PSA	*hZIP1, Dkk-3, SMAD4, PSA*	[130–132]
miR-375	Onco-miR		Stimulates cell growth, invasion ability, and impairs apoptosis in a cell-specific context	*SEC23A, CCND2*	[133, 134]

It was recently demonstrated that PCa-derived adipose stem cells (pASCs) stimulated with conditioned media or exosomes (isolated from PC3, and C4-2B cells) induced prostate-like neoplastic lesion *in vivo* (99). The oncogenic stimulation of pASCs might be a consequence of the RNA transfer by PCa-derived exosomes and activation of oncomiRNAs (e.g. miR-125b, miR-130b, and miR-155), along with oncogenic factors (e.g. *H-RAS* and *K-RAS*) (99). In fact, expression of miR-125b and miR-130b promoted downregulation of tumor suppressors *Lats2* and *PDCD4* in pASCs exposed to PCa-derived exosomes. Functionally, pASC tumors acquire cytogenetic aberrations, mesenchymal-to-epithelial transition features and expressed neoplastic markers reminiscent of molecular features of PCa xenografts (99).

Due to its plasticity and cargo potential, PCa-derived exosomes might play a critical role in clonal expansion of tumors through neoplastic reprogramming of tumor-ASCs in cancer patients. This also emphasizes that deregulated expression of oncomiRs cause oncogenic transformation of pASCs due to disruption of transcriptional networks of tumor suppressor genes (99). Further research on other ncRNA families and different prostate cells (e.g., basal, luminal and fibroblasts) might help understand how exosomes are involved in crosstalk between tumor and stromal cells to synergistically promote tumor progression and drug resistance.

ii. Small nuclear and nucleolar RNAs in PCa

The role of other small ncRNAs in prostate tumorigenesis has been also investigated. SnoRNA U50 is mutated and downregulated in PCa, and a homozygous 2-bp (TT) deletion was identified both in PCa cell lines and primary tissues. Ectopic expression of snoRNA U50 abrogates colony formation, a feature associated with tumor-suppression. (100).

The nucleolar protein dyskerin (DKC1) catalyzes pseudouridylation of rRNA and it is also required for the formation of hTR, the RNA component of telomerase. Compared to benign tissues, *DKC1* mRNA levels were higher in PCa samples, especially in lymph node metastases (101). SiRNA-mediated depletion of *DKC1* decreased cell proliferation of prostate cells (101), suggesting that deregulation of snoRNA machinery is important for prostate carcinogenesis.

Using a deep sequencing approach to characterized small non-coding RNA transcriptome, an increase in both global snoRNAs and tRNA expression in PCa metastatic to lymph node compared to that of primary PCa was shown, suggesting a possible oncogenic role for snoRNAs, particularly in more advanced tumors (102). In addition, there is a strong differential expression of snoRNAs and tRNAs, comparing PCa and normal prostate samples (102). Additionally, snoRNA-derived RNAs (sdRNAs) display higher differential expression than miRNAs and they are greatly upregulated in PCa. Using qPCR, *SNORD44*, *SNORD78*, *SNORD74* and *SNORD81* and sdRNAs were shown to be upregulated in PCa. The higher expression levels of *SNORD78* and its sdRNA - sd78-3' – were associated with metastatic PCa (102).

The ribosome biogenesis begins in the nucleolus (103). Here the ribosomal RNA (rRNA) is transcribed, processed, and assembled into ribosomal subunits (104). It hosts a transcriptional unit encoding a 45S ribosomal RNA precursor that is processed into the mature 18S, 5.8S and 28S RNA species (105). 45S percursor rRNA and mature rRNAs 28S, 18S and 5.8S are overexpressed in PCa samples compared to morphologically normal prostate tissues (104). The mechanism leading to the aberrant expression is not well characterized, but apparently, overexpression is not associated with rDNA promoter

hypomethylation (104). In fact, 45S, 18S and 5.8S rRNA expression levels, altered nucleolar structure and function are more closely associated with *MYC* mRNA levels (104) suggesting that *MYC* might be involved in rRNA biogenesis. In a different report, *MYC* was found to be required for rRNA transcription and processing (106). In PCa cells, MYC binds to the 5′ upstream region of *Fibrillarin* (*FBL*), a gene required for rRNA production and processing (106). *FBL* is overexpressed in PCa samples and siRNA-mediated depletion of *FBL* suppressed cell proliferation and clonogenic survival. Moreover, *FBL* knockdown decreased the levels of 5.8s, 18s and 28s rRNAs, whereas only a modest reduction in 45S pre-rRNA was observed (106). Conversely, *MYC* knockdown associated with decreased levels of pre-rRNA as well as of processed rRNAs, indicating that *MYC* is required for rRNA transcription and processing (106). Genome-wide analysis of *MYC* depletion revealed down-regulation of 133 nucleolus-associated genes and of 64 genes associated with rRNA processing (106). Those comprised *fibrillarin*, *nucleolin*, *UBF*, and *nucleophosmin*. In addition, overall nucleolar size was reduced after *MYC* depletion *in vitro* (106). Considering these findings, *MYC* overexpression in PCa cells can drive enhanced *de novo* nucleolar and ribosomal gene expression, thus fostering the malignant phenotype.

rRNA is crucial for both androgen-dependent and -independent growth of PCa cells. The androgen-AR signaling leads to the accumulation of rRNA in androgen-dependent prostate cells and angiogenin (ANG) is upregulated in PCa cells, mediating androgen-stimulated rRNA transcription (107). In androgen-dependent cells, androgen stimulation promotes ANG nuclear translocation, where it binds to rDNA promoter, stimulating rRNA transcription (107). Blocking ANG leads to inhibition of androgen-induced rRNA transcription. Moreover, ANG signalling is not only critical for androgen-dependent growth but also for the castration-resistant phenotype. In an androgen-independent context, ANG stimulation leads to constitutive nuclear translocation in androgen-insensitive cells, ensuing a continuous rRNA overproduction and thereby stimulating cell proliferation (107).

iii. tRNA-derived RNA fragments

Global expression profile of prostate cell lines revealed that the second most abundant group of sncRNA was that of fragments derived from tRNA, the tRNA-derived RNA fragments (tRFs) (108). Deep sequencing characterization of LNCaP and C4-2 cell lines, disclosed 17 tRNA-related small RNAs, including the most abundant: tRF-1, tRF-3, and tRF-5. For downstream validation, tRF-1001, a member of tRF-1 series, was selected. tRF-1001 is derived from the 3' end of a Ser-TGA tRNA precursor transcript, which is not retained in the mature tRNA (108).

The tRF-1001 is expressed more abundantly in cell lines than in tissues, but its expression decreases either upon starvation or high cell density in DU145 and LNCaP cells. Reduction

of cellular metabolism also decreased expression of tRF-1001 precursor but the corresponding mature tRNA levels were unaffected. The tRF-1 series of small RNAs are 3′ sequences from pre-tRNA, released through a cleavage by tRNA endonuclease ELAC2 during the 3′-end maturation of tRNA. Knockdown of *ELAC2* decreased tRF-1001 expression, leading to accumulation of the pre-tRNA (108). tRF-1001 and its precursor tRNA are exclusively localized in the cytoplasm, providing evidence that biogenesis occurs in the cytoplasm, rather than in the nucleus as it happens for tRNAs. These data sustain a functional role for tRFs, putting aside the idea of a mere byproduct of tRNA biochemical processing (108).

Recently, RNA-sequencing was used to profile tRFs in fresh frozen tissue samples derived from normal adjacent prostate and PCa at different stages (109). A total of 598 unique tRFs were identified and several are deregulated in PCa. Strikingly, 5'-tRFs constitute approximately 75% of all tRFs detected in prostate tissues. Notably, most of the identified tRFs are derived from 5'- and 3'- of mature cytosolic tRNAs. Nonetheless, tRFs derived from different segments of tRNAs, including pre-tRNA trailers and leaders, as well as tRFs from mitochondrial tRNAs were catalogued. Globally, 110 tRFS were found deregulated (72 upregulated, 24 downregulated and 13 upregulated in one group but downregulated in the other group (109)). Most of the upregulated tRFs were 5'-tRFs and most of downregulated were 3'-tRFs. Downstream qPCR validation of 6 different tRFS revealed that 4 tRFs (three 5'-tRFs and one D-tRF) were upregulated, and 2 tRFs (3'-tRF class) were downregulated in PCa. tRF-544 (isotype Phe, anticodon GAA - tRNA^{PheGAA}) is thought to be associated with aggressive forms or advanced stages of PCa. Interestingly, high expression level ratio tRF-315/ tRF-544 significantly associated with poorer progression-free survival and shorter time to disease relapse.

Sex hormone-dependent tRNA-derived RNAs (SHOT-RNAs) are commonly expressed in AR positive PCa cancer cell lines (110). In LNCaP-FGC cells, both 5′- and 3′-tRNA halves from SHOT-RNA^{AspGUC} and SHOT-RNA ^{HisGUG} are detected by northern blot, but not in DU145 or PC3 cells, and AR knockdown reduced tRNAs expression levels. One of must abundant SHOT-RNAs detected by Honda *et al* - 5′- SHOT-RNA RNA^{LysCUU} – was knocked-down using siRNAs in LNCaP-FGC and cell growth rate was decreased compared to control-siRNA (110). Because levels of mature tRNA were not changed by siRNA transfection, reduced proliferation seems to be solely attributable to the change in SHOT-RNA RNA levels. This strategy was also applied to SHOT-RNA^{AspGUC} and SHOT-RNA^{HisGUG} and depletion of each SHOT-RNA impaired cell growth, as well. Nevertheless, 3′-SHOT-RNA^{AspGUC} depletion failed to impair cell growth (110). Overall, these data support SHOT-RNAs as functional RNA molecules and different species of 5′-SHOT-RNA are involved in cell proliferation (110). To

determine whether 3′-SHOT-RNA holds functional relevance or not, additional studies are required.

The current understanding of tRFs, however suggests that it are not merely byproducts of random cleavage of tRNAs, but might act as mediators of translational and/or gene regulation. Although some isolated functions have been indicated, the vast majority of tRFs appear to operate via uncharacterized mechanisms. It has been proposed that 5′- but not 3′-derived tRFs, play a role in stress granule assembly or inhibition of protein synthesis *in vitro* (111). However, 3′-derived tRFs are able to repress their mRNA targets in a miRNA-like fashion and may exert tumor suppressive functions (112).

iv. Other short RNAs

Although no direct involvement of piRNA in PCa has been reported, some genes implicated in piRNA biogenesis are deregulated in PCa. Defects in Tudor-domain proteins significantly impair piRNA pathway, especially its ping-pong components, although not abolishing it. Because multiple Tudor-domain-containing proteins exists, one may argue that it exhibit overlapping or redundant roles in the piRNA pathway, explaining the somewhat minor phenotypes of the individual mutants (113).

Tudor domain-containing protein 1 (TDRD1), is a direct target gene of ERG, strongly correlating gene with ERG overexpression (114). Mechanistically, ERG is able to disrupt tissue-specific DNA methylation pattern at the *TDRD1* promoter, resulting in *TDRD1* transcriptional activation (114). Piwil2 has been recently described as an oncogene able to modulating invasion and metastasis, as well as EMT (115). Of note, global piRNAs levels were not assessed to quantify the deregulation caused by TDRD1 and Piwil2 aberrations.

b. Long non-coding ncRNA

Although ncRNAs research, and specially lncRNAs, is still in its infancy, significant roles have been ascribed to some lncRNAs in PCa and these are summarized in Table 3.

i. Antisense-Regulatory lncRNAs in PCa

The role of dysregulated antisense transcript expression is under investigation in PCa. The polyadenilated antisense transcript *ANRIL* (encoded by CDKN2B-AS1) is expressed from the tumor-suppressor locus INK4b-ARF-INK4a (9q21.3). *ANRIL* and *CBX7* (member of Polycomb Repressor Group 1) are both up-regulated in PCa samples (116). Furthermore, CBX7 is responsible for maintaining silenced chromatin states through recognition of H3K27me3 (116). CBX7 binds to H3K27me3 and interacts with *ANRIL* at the INK4b-ARF-INK4a locus. CBX7 employs different regions within its chromodomain for binding to H3K27me3 and *ANRIL* RNA, suggesting that both interactions are important for the

sustained *cis*-repression of the locus (116). Thus, RNA-protein interaction underlies the ability of PRC1 to repress the INK4b-ARF-INK4a cluster and its disruption contributes to PCa development by reducing senescence (116). Interestingly, these data might indicate that the frequent promoter hypermethylation observed at this locus occurs as a secondary event after cell differentiation.

Another NAT with critical impact in PCa cells is *CTBP1-AS*, an androgen-responsive lncRNA that promotes PCa growth through sense-antisense repression of the transcriptional co-regulator *CTBP1* and global epigenetic regulation of tumor suppressor genes (117). The up-regulation of *CTBP1-AS* is inversely correlated with *CTBP1* in primary and metastatic PCa, associating with high AR expression status. Depletion of *CTBP1-AS* mRNA abolished the androgen-dependent reduction of *CTBP1*, indicating that *CTBP1-AS* directly regulates *CTBP1* at RNA level (117). Silencing *CTBP1-AS* reduced LNCaP cell proliferation, and *in vivo* tumor growth was also reduced, concomitantly with an increased CTBP1 expression. Microarray analysis showed that transcriptional activation of androgen-induced genes was diminished by siCTBP1-AS (117). Interestingly, *CTBP1-AS* overexpression stimulated cell growth and promoted resistance to growth inhibition by bicalutamide, ultimately rendering *in vivo* tumor growth after castration. Mechanistically, *CTBP1-AS* coordinates *cis*-repression of *CTBP1* promoter, reducing H3Ac and H4K4me levels but not altering repressive marks (117). *CTBP1-AS* binds to HDAC-Sin3A complex and coordinates HDAC-mediated repression by chromatin deacetylation within *CTBP1* promoter's in the AR-dependent system. Moreover, *CTBP1-AS* also interacts with PSF, which binds at *CTBP1* promoter to induce histone deacetylation by HDACs to promote transcriptional repression of CTBP1. Additionally, *CTBP1-AS* may also act as trans-acting regulator of androgen-regulated genes by recruiting the HDAC/Sin3A repressor complex via PSF, prompting cell cycle progression by repressing cell cycle regulators and modulating global androgen signaling (e.g., p53, SMAD3) (117).

Another example of antisense gene regulation is the transcriptional control of tumor-suppressor gene *RASSF1A* by *RASSF1A*-antisense RNA 1 (118). *RASSF1A-AS1* is upregulated is PCa cell lines, inversely correlating with *RASSF1A* expression (118). *RASSF1A* and RASSF1A-AS1 form a RNA-DNA hybrid at the *RASSF1A* promoter and recruits the polycomb repressor complex PRC2. PRC2 contributes to chromatin compaction by catalyzing the methylation of histone H3 at lysine 27, which is enriched at *RASSF1A* promoter, and specifically blocks *RASSF1A* expression (118).

ii. Enhancers and enhancer RNAs (eRNAs) in PCa

Cancer cells display altered expression patterns and enhancer usage in comparison with their normal counterparts (119). In PCa, eRNAs have been implicated in assisting AR-

mediated signaling, as mediators of enhancer-promoter looping and in altering transcription factor binding (Figure 5).
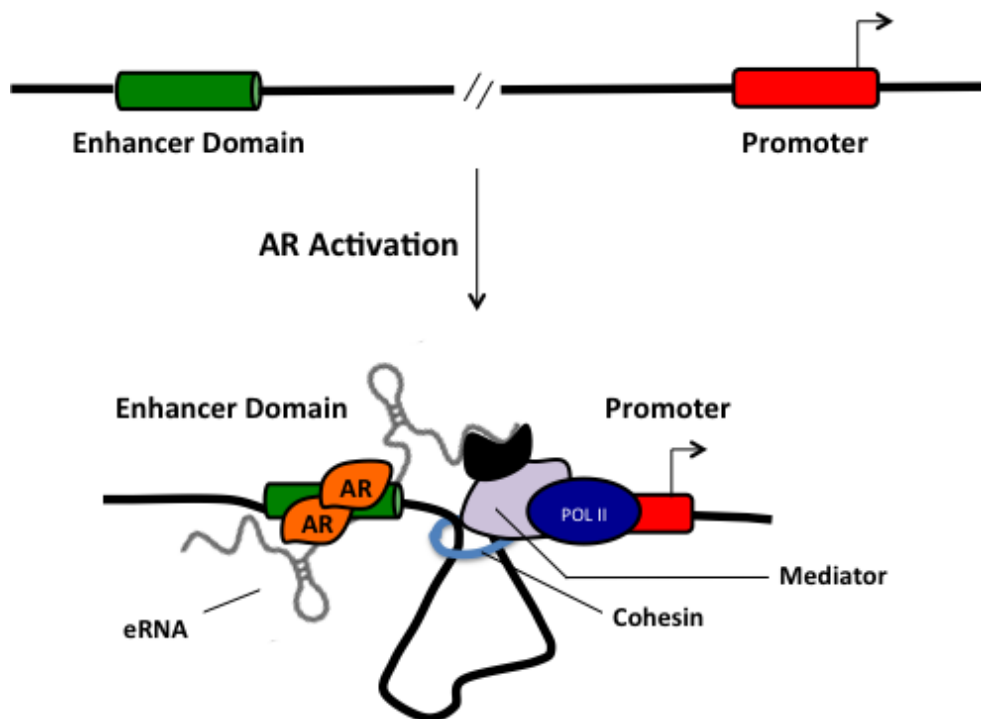
Figure 5. Transcription derived from enhancer is important for long-range transcriptional control.

eRNAs are lncRNA derived from short regions of DNA that enhance the expression of genes at varying distances. Effects can be mediated by transcription factor binding to these sites, such as androgen receptor (AR). AR controls PCa cell-specific gene expression programs through interactions with diverse co-activators and the transcription machinery. Gene activation may involve DNA loop formation between enhancer-bound AR and the transcription machinery at the core promoter. This interaction seems to be mediated by mediator complex and cohesin, as they have been reported to interact physically and functionally connect the enhancers and core promoters of active genes. The eRNAs produced from AR-binding DNA segments, facilitate the spatial interaction between enhancer and promoter, ultimately enhancing long-distance transcriptional regulation. Moreover, specific eRNA might encompass androgen response elements (ARE), supporting AR and mediator interactions. This mechanism is critical for PCa cells, as androgen-induced eRNAs scaffolds the AR-associated protein complex that modulate chromosomal architecture and selectively enhance AR-dependent gene expression involved in PCa initiation and progression

FoxA1 has been reported to contribute to the enhancer code in PCa cells, as FoxA1 regulates AR genomic targeting by simultaneously anchoring AR to cognate loci and restricting AR from other ARE-containing loci in the human genome (72). In addition, knockdown of *FoxA1* markedly elevated di-hydro-testosterone (DHT) response and caused AR binding to a distinct cohort of enhancers. Global nuclear run-on sequencing (GRO-seq) was applied to understand how differential AR binding is translated into hormonal gene-response (72). After DHT treatment, GRO-seq detected ncRNA expression from a subset of H3K4me1-positive and H3K4me3-negative regions. These differentially expressed eRNAs are largely symmetrical and bidirectional (as depicted for the KLK3 enhancer). Moreover, these AR-activated enhancers marked by increased eRNA expression are responsible for activation of nearby coding transcription units (72). Chromosome conformation capture (3C), suggested that eRNA induction per se is the most precise mark of the functional looping between an activated enhancer and its regulated gene promoter, rather than p300 or MED12 binding (72). Moreover, both DHT and FoxA1 knockdown demonstrated a strong H3K4me2-marked central nucleosome, suggesting that nucleosome remodeling is not required to induce specific enhancer-promoter looping and subsequent target gene activation (72).

Furthermore, it has been reported that PolII binds to a large number of intergenic AR-bound enhancers, marked by H3K4me1 and H3K27ac, which produce eRNAs that may regulate neighbor or distantly located genes (89). This evidence suggests than eRNAs may contribute to AR-driven looping complex that enhances spatial communication of distal enhancers and target promoters, leading to transcriptional activation of specific genes (89). The *KLK3* enhancer is marked by AR binding, H3K27ac and H3K4me1, and produces a bidirectional eRNA named KLK3e (89). Both *KLK3* and *KLK3e* expression is induced by DHT treatment and blocked by bicalutamide, indicating a high correlation of activity-dependent induction between eRNAs and adjacent protein coding genes.

KLK3e sense strand gives rise to a >2kb polyadenylated transcript that is substantially more expressed than the antisense transcript. *KLK3e* facilitates the spatial interaction of the *KLK3* enhancer and the *KLK2* promoter, enhancing long-distance *KLK2* transcriptional activation (89). *KLK3e* contains the core enhancer element derived from the androgen response element III (AREIII) required for the interaction of AR and Mediator 1 (*MED1*). Suppression of either *KLK3e* or *MED1* reduced the interaction of KLK3/2 loci, supporting a role for *MED1* as a mediator of the long-range chromatin looping and cooperating with *KLK3e* in the enhancer target-promoter interaction. Globally these data suggests that *KLK3e* forms a functional complex with AR and MED1 that facilitates the association of AR-bound enhancers with promoters, resulting in transcriptional activation of target genes (89). Supporting this hypothesis, *KLK3e* expression is significantly correlated with *KLK3* and *KLK2* (R2=0.62;

0.59, respectively). Further understanding of how AR-induced eRNAs act as a scaffold for AR-associated protein complex that selectively modulate chromosomal architecture and gene expression may translate into new RNA-based therapy to improve response to androgen deprivation therapy (89).

Recently, a new role for single-strand nicks was identified, mediated by DNA topoisomerase 1 (TOP1), in relaxing supercoiled DNA at gene enhancers to promote enhancer dependent transcription (120). In LNCaP cells, TOP1 was recruited to AR-regulated enhancers in response to androgen treatment. Using ChIP-seq, of the 6,545 putative AR-bound enhancers, 96% were occupied by TOP1. Of these, 60% revealed an androgen-stimulated increase in TOP1 binding as well as in RNAPOL II occupancy, indicative of active transcriptional activity (120). GRO-seq analysis of serum-starved LNCaP cells treated with DHT identified 644 putative enhancers (74% of them showed increased TOP1 occupancy) with significantly up-regulated eRNA expression. Knockdown of endogenous *TOP1* resulted in decreased eRNA expression of 79% of AR-regulated enhancer, accompanied by lower expression levels of 368 protein-coding mRNAs (including *KLK3*, *KLK2*, *TMPRSS2* and *NDRG1*) (120). Having proved that TOP1 reduces both eRNA and mRNA production of most AR-regulated target genes, the authors found that prior binding by NKX3.1 was required to recruit *TOP1* to enhancers following androgen treatment. siRNA-depletion of *NKX3.1* inhibited recruitment of *TOP1* and reduced DHT-dependent upregulation of eRNA expression (120). Strikingly, depletion of both *TOP1* and *NKX3.1* reduced DHT-mediated eRNA upregulation at the same AR-bound enhancers, apparently without affecting AR recruitment. This reveals that NKX3.1 and TOP1 occupy the same binding sites at enhancer elements and co-regulate an AR transcription program. The Y723F *TOP1* mutant did not block transcriptional activity in *TOP1*-depleted cells, suggesting that the nicking activity of *TOP1* is required for its effects on enhancer activation (120). Given that single-strand nicks might lead to the formation of DNA double-strand breaks, several components of the DNA damage response pathway - MRE11, RAD50 and ATR – are recruited to AR-regulated enhancers after DHT treatment and are required for eRNA and protein-coding mRNA transcription. Taken together, these data suggest a common usage of the DNA damage repair machinery to regulate AR-mediated gene transcription, highlighting the complexity of PCa (120).

In a recent study, using Chem-seq (121), a compound that inhibits cell proliferation *in vitro* and tumor growth *in vivo* – SD70 – was identified. SD70 binds to AR-bound functional enhancers, regulating DHT-induced gene transcriptional programs (121). Moreover, it was found that KDM4C binds at AR-regulated enhancers and is recruited in a DHT-dependent fashion. *In vitro*, SD70 inhibits KDM4C demethylase activity causing elevated H3K9me2 levels at enhancer and promoter regions - a plausible component of the inhibitory effects on

DHT target gene expression (121). These results suggest that targeting enhancer regions has potential therapeutic value for PCa.

iii. lncRNA as master regulators of alternative splicing and translation

Recently, it has been shown that lncRNA are required to assemble nuclear domains specialized in RNA processing, such as the nuclear speckle and the paraspeckle. The oncogenic lncRNA *MALAT1* (also known as *NEAT2*, located at 11q13.1) is present within the nuclear speckle and spatially re-organizes the actively transcribed genes closed to the nuclear speckles, a domain know for its abundance in pre-mRNA splicing factors (10). Knockdown of *MALAT1* disclosed that this lncRNA regulates alternative splicing of multiple genes by controlling the availability of serine/arginine-rich splicing factors in active transcription sites (122). Interestingly, during post-transcriptional processing of *MALAT1*, a conserved 3' tRNA-like sequence generates a short tRNA-like ncRNA called MALAT1-aasociated small cytoplasmic RNA (*MASCRNA*), whose function is still unclear. *MASCRNA* is a 61-bp short tRNA-like ncRNA of unknown function, generated by RNase-P cleavage and then exported to the cytoplasm (123).

On the other hand, the lncRNA *NEAT1* is an essential structural element to initiate *de novo* assembly of paraspeckles, which are believed to be nuclear domains specialized in retention of adenosine-to-inosine edited mRNAs (124). Inducing *NEAT1* transcription locus is sufficient to form new paraspeckles at the integration locus. However, active transcription of *NEAT1* is necessary to tether the lncRNA to its own transcription locus and carry out this role (124). Taking into account that *MALAT1* and *NEAT1* are separated by approximately 70kb, it is conceivable that coordinated deregulation of both loci may hinder alternative splicing by controlling the nuclear localization of splicing factors as well the control of RNA editing and export, further contributing to prostate carcinogenesis (10). Indeed, both *MALAT1* (125) and *NEAT1* (126) are overexpressed and possess pro-tumorigenic activity in PCa. *MALAT1* overexpression in primary PCa is associated with higher Gleason score, pathological stage and serum PSA >20ng/ml (127). Besides it association with poor prognosis, MALAT1 expression is significantly increased in castration-resistant PCa (CRPC) compared to hormone sensitive PCa (127). Functional assays using siRNA specific to knock down *MALAT1* expression in 22RV1 and LNCaP-AI cells inhibited cell cycle at G0/G1 phase, migration and invasion (127). RNAi silencing of *MALAT1* in PCa xenografts of castrated male nude mice resulted in significant reduction of tumor volume and metastasis number, increasing survival time (127). Whether these alterations are specific of MALAT1 or are the combined effect in downstream genes (e.g., RNA splicing deregulation) controlled by MALAT1 is still a matter under study. Using EZH2 antibody-based RNA immunoprecipitation combined with next generation sequencing (RIP-seq), EZH2 was found to bind to *MALAT1*

(125). Both GST pull-down and RIP assays showed that the 3' end of *MALAT1* interacts with the N-terminal of EZH2. Moreover, *MALAT1* and *EZH2* are positively correlated, in CRPC samples. Moreover, depletion of *MALAT1* impaired EZH2 recruitment to its target loci (*DAB2IP* and *BRACHYURY*) and caused its upregulation, suggesting that *MALAT1* mediates EZH2-enhanced migration and invasion in CRPC cell lines (125). Moreover, *MALAT1* enhances expression of PRC2-independent target genes of EZH2 both in vitro and in patient-derived xenografts (TMEM48 and KIAA0101) (125).

NEAT1 is an ERα-regulated lncRNA, upregulated in PCa, producing two RNA isoforms that overlap completely at the 5'-end. The shorter isoform is 3.7 kB in length and more abundant than the longer, 23 kB, isoform (NEAT1_2) (126). NEAT1 expression is a prognostic biomarker for aggressive PCa independent of standard clinical and pathologic parameters (126). Oestrogen treatment upregulates NEAT1 transcript levels in a time-dependent manner and in VCaP cells results in re-distribution of NEAT1 from paraspeckles to an enhanced distribution throughout the nucleus (126). Knockout of *NEAT1* compromised the expression of ERα target genes, suggesting that NEAT1 is not only a downstream target but also a mediator of ERα signalling in PCa cells. NEAT1 transcriptionally regulates a compendium of genes known to be involved in PCa progression, including *PSMA* and *GJB1* (126). Overexpression of NEAT1_1 significantly increased active chromatin marks H3K4Me3 and H3AcK9 at the *PSMA* promoter and induced subsequent recruitment of NEAT1_1 and ERα to the same promoter. RNA immunoprecipitation revealed that NEAT1 directly interacts with histone H3, favoring a chromatin landscape for active transcription through active histone marks (126). Phenotipically, knockdown of *NEAT1* in VCaP cells significantly decreased proliferation and the invasive properties of cells. Overexpression of *NEAT1* resulted in a significantly higher number of viable colonies, establishing an oncogenic role for *NEAT1* (126). In athymic nude mice, injection of either VCaP or NCI-H660 overexpressing *NEAT1*, resulted in a significantly higher tumour growth rate, compared to scramble cells. Moreover, in vitro NEAT1 expression is inhibited when cells are treated with ERα antagonists in combination with E2. Similar results observed with AR antagonists enzalutamide and bicalutamide, suggest that *NEAT1* is associated with resistance to therapeuty (126). Thus, these data suggest a role for paraspeckles in the lncRNA-mediated regulation of gene expression in PCa.

iv. lincRNAs deregulation in PCa

Long intervening noncoding RNAs (lincRNAs) are emerging as key regulators of diverse cellular processes but determining their individual function remains a challenge. lincRNAs are also called long intergenic noncoding RNAs, although lincRNAs derive from genes and

are thus genic, which do not overlap with exons of either protein-coding or other non-lincRNA types of genes (19).

Ab initio transcriptome sequencing of polyA+ RNA from 102 PCa tissues and cell lines revealed a total of 1,859 unannotated lincRNAs throughout the human genome (128). A set of 121 of those transcripts accurately distinguished benign, localized and metastatic PCa by unsupervised clustering. *PCAT-1* (located in the 8q24 gene desert) is predominantly cytoplasmic and was upregulated in PCa samples especially in high-grade (GS≥7) and metastatic tumors. Strikingly, *PCAT-1* and *EZH2* expression were nearly mutually exclusive, suggesting that their expression may define two subsets of high-grade disease. However, upregulation of *PCAT-1* was not dependent of 8q24 amplification (128). Inhibiting *EZH2*, using either shRNAs or DZNep, caused a dramatic up-regulation of *PCAT-1* in VCaP cells. ChIP assay showed that SUZ12, a core component of PRC2, directly binds to *PCAT-1* promoter ~1kb upstream of TSS (128). By RNA immunoprecipitation, it was demonstrated that *PCAT-1* binds to SUZ12 protein in VCaP cells, a feature that was abolished by RNase A, RNase H or DNase I treatment. This suggests that *PCAT-1* exists primarily as a single-stranded RNA and secondarily as a RNA/DNA hybrid. Moreover, *PCAT-1* stable overexpression in RWPE cells promoted cell proliferation, and RNAi silencing decreased cell proliferation in LNCaP but not in DU145 (lacks *PCAT-1* expression) or VCaP cells (*PCAT-1* is repressed by PRC2) (128). Genomewide expression analysis of LNCaP cells after treatment with siRNAs against *PCAT-1* disclosed upregulation of 255 genes and repression of 115 genes, revealing that *PCAT-1* is predominantly repressive. Additionally, the upregulated genes showed enrichment for mitosis and cell cycle (128). Specifically, PCAT-1 targets *BRCA2*, *CENPE* and *CENPF*, whose expression is upregulated upon *PCAT-1* silencing in LNCaP cells. Further research demonstrated that PCAT-1 overexpression decreased RAD51 foci formation (a component of homologous recombination, HR) after therapy with PARP1 inhibitors and *PCAT-1* knockdown increased foci formation upon therapy, in PCa cells (129). *BRCA2* inactivation impairs both HR and double stranded DNA break repair (DSB). *PCAT-1* expression is correlated with decreased *BRCA2* levels, and *in vitro*, the 5' end of *PCAT-1* is able to directly repress the activity of *BRCA2* 3'UTR (129). PCAT-1 overexpression produces a functional deficiency in HR through post-transcriptional repression of *BRC2* tumor suppressor, which, in turn, reveals a high sensitivity to small molecule inhibitors of PARP1, both *in vitro* and *in vivo* (129). Whether PCAT-1 may act as predictive biomarker for patient response to PARP1 inhibitor therapy is still to be proved.

PCAT-1 is located 725kb upstream of the *MYC* oncogene (130). Overexpression of *PCAT-1* in DU145 and RWPE increased c-MYC protein levels, while silencing of *PCAT-1* in LNCaP decreased c-MYC protein, suggesting a *cis*-regulation involving these loci (130). Strikingly, c-MYC silencing fully abrogated the proliferative effects of *PCAT-1* overexpression in DU145

and RWPE, indicating that *PCAT-1* mediated cell proliferation is dependent of c-MYC overexpression. Luciferase assay revealed that *PCAT-1* overexpression increased *cMYC* 3'UTR activity, whereas silencing of *PCAT-1* decreased *c-MYC* 3'UTR activity. Mechanistically, this suggests that *PCAT-1* regulates *c-MYC* in a post-transcriptional manner by 3'UTR activation, which can result in gene activation and increased protein abundance [95].

Another important lincRNA in PCa is *SChLAP1* (second chromosome locus associated with prostate-1; also designated LINC00913) (131). *SChLAP1* is located in a 'gene desert' on chromosome 2q31.3 and is highly expressed in ~25% of PCa, being more frequently expressed in metastatic compared to localized PCa. Its expression was associated with ETS gene fusions and *PTEN* deletions in localized PCa (131). Moreover, *SChLAP1* levels independently predict poor outcome, including metastasization and PCa–specific mortality (131). Knockdown of *SChLAP1* dramatically impaired cell invasion and proliferation *in vitro* and, in turn, overexpression of a siRNA-resistant *SChLAP1* isoform rescued the *in vitro* invasive phenotype of 22Rv1 cells treated with siRNA. Overexpression of the three *SChLAP1* isoforms in RWPE cells dramatically increased the ability of these cells to invade in vitro but did not affect cell proliferation. *In vivo*, *SChLAP1* depletion impaired metastatic seeding and growth. Overall, *SChLAP1* seems to control tumor invasion and metastasis by influencing cancer cell intravasation, extravasation and subsequent tumor cell seeding (131). Using Gene Set Enrichment Analysis of 22Rv1 and LNCaP cells with *SChLAP1* knockdown, *SChLAP1*-regulated genes were correlated with the SWI/SNF complex, a multiprotein complex know to physically rearrange nucleosomes at gene promoters, thus controlling transcription (131). Mechanistically, *SChLAP1* co-immunoprecipitates with SNF5 and attenuates SNF5 genome-wide localization. Upon knockdown of *SChLAP1*, 9 of 12 target genes disclosed a substantial increase in SNF5 binding. These data sustain that oncogenic *SChLAP1* overexpression antagonizes the tumor-suppressive role of SWI/SNF complex function by attenuating the genomic binding of this complex, thereby impairing its ability to properly regulate gene expression (131).

Prostate cancer antigen 3 (*PCA3*) is a spliced intronic antisense lncRNA embedded within intron 6 of the corresponding sense gene *PRUNE2* and upregulated in PCa samples, holding promise as biomarker for PCa detection (132). *PCA3* controls *PRUNE2* levels via a unique regulatory mechanism involving formation of a *PRUNE2*/*PCA3* double-stranded RNA that undergoes ADAR-dependent adenosine-to-inosine RNA editing (132). Because Drosophila behavior human splicing (DBHS) protein P54NRB binds to inosine-containing RNA (RNA-I), regulating gene expression, it was found, using RNA-ChIP, that *PCA3* and *PRUNE2* pre-mRNA species associate with P54NRB protein, suggesting that DBHS proteins also contribute for *PRUNE2*/*PCA3* regulation (132). *In vitro* stimulation with a synthetic

testosterone homolog induced *PCA3* expression and decrease PRUNE2 levels [97]. *PCA3* silencing or ectopic *PRUNE2* expression decreased cell proliferation and transformation *in vitro*; in contrast, *PRUNE2* silencing or ectopic *PCA3* expression increased cell proliferation and transformation (132). PRUNE2-deficient PC3 cells stably expressing ectopic *PRUNE2* display lower levels of proliferation and transformation *in vitro*, consistent with the negative regulation of *PRUNE2* by *PCA3* (132). In SCID mice, *PRUNE2* silencing and ectopic *PCA3* expression yielded markedly larger tumor xenografts than controls; in contrast, tumor growth was significantly diminished compared to controls when *PCA3* was silenced, further illustrating the oncogenic activity of *PCA3* (132). Serum PSA was increased in SCID mice injected with LNCaP cells with ectopic PCA3 expression or *PRUNE2* silencing, compared to controls (132). In human PCa samples, *PCA3* and *PRUNE2* levels inversely correlate. Moreover, A>G/T>C alterations were the most frequent substitutions, indicative of A-to-I editing in both *PCA3* and *PRUNE2* pre-mRNA strands (132). These results establish *PCA3* as a dominant-negative oncogene and *PRUNE2* as a tumor suppressor gene in PCa, and their regulatory axis represents a putative target for clinical intervention (132).

v. Pseudogenes

CXADR-ψ, a processed pseudogene on chromosome 15, parental of the tumor-suppressor *CXADR*, was found overexpressed in PCa tissues compared to benign tissue samples (91). CDNA cloning from two PCa samples positive for *CXADR-ψ* showed perfect sequence similarity to the pseudogene *CXADR-J* and only 84% to CXADR wild-type gene (91). No correlation was depicted for *CXADR* and *CXADR-ψ*. Interestingly, *CXADR-ψ* expression was nearly restricted to PCa lacking an ETS gene fusion, with few ETS-positive samples exhibiting expression of this pseudogene (91). On the other hand, *CXADR* gene expression was found in both ETS-positive and ETS-negative samples (91). In the same study, a PCa-specific readthrough transcript involving *KLK4*, an androgen-induced gene, and *KLKP1*, an adjacent pseudogene, was identified. *KLK4-KLKP1* transcript was highly expressed in 30%–50% of PCa tissues, and this expression was lineage and cancer specific, with low expression detected in benign prostate and other tissues (91). *KLK4-KLKP1* transcript was previously described in LNCaP as a *cis* sense-antisense chimeric transcript (91). This chimeric transcript is composed of the first two exons of *KLK4* and the last two exons of *KLKP1*. It retains an open reading frame incorporating 54 amino acids encoded by the KLKP1 pseudogene in the putative chimeric protein (91). Additional studies are needed to understand the biological role of the chimeric transcript *KLK4-KLKP1* in PCa biology.

Pseudogene transcription has also been shown to regulate cognate wild type gene expression by sequestering miRNA acting endogenous miRNA sponges, or competing endogenous RNAs (ceRNAs) (79). ceRNAs communicate and co-regulate each other by

competing to bind to a common pool of miRNAs, thus altering miRNA availability and stoichiometry (79). *PTENP1* pseudogene has been reported to regulate levels of its cognate gene, *PTEN*, by competing for shared miRNAs (79). Both miR-19b and miR-20a (normally over-expressed in PCa) suppressed both *PTEN* and *PTENP1* mRNA abundance. Blocking miR-17 and miR-19 family increased *PTEN/PTENP1* levels, highlighting a shared miRNA-mediated regulation between these two genes and highlights the role of *PTENP1* as a tumor suppressor acting as a decoy for oncogenic miRNA-targeting of PTEN (79). Additionally, *KRAS/KRAS1P* transcript levels are positively correlated in PCa and *KRAS1P* 3'UTR overexpression in DU145 cells resulted in increased *KRAS* mRNA abundance and cell growth. These data support a role for *KRAS1P* in PCa, being targeted by *KRAS*-targeting miRNAs. *In silico* analysis revealed that *KRAS1P* maintains the validated binding sites for miR-143 and let-7 family previously reported for *KRAS* [44]. These data provide a framework of pseudogenes as natural miRNA decoys in PCa development.

vi. Transcribed ultraconserved region (T-UCR)

Ultraconserved regions (UCR) are genomic sequences with 100% conservation between human and rodent genomes, more than 200 base pairs in length but not harboring any known gene (133). Due to the high levels of sequence conservation, UCR must have biological functions essential to mammalian cells, although still largely enigmatic. Some UCR have been functionally implicated in transcriptional enhancement, alternative splicing, nonsense mediated decay mechanisms or miRNA-binding decoys (133). There are 481 UCRs described, some of which overlap with coding exons, although it is believed that more than half of them do not encode any protein. Surprisingly, 68% of UCRs (i.e., 325) are transcribed, defining a new class of long non-coding RNA: T-UCRs (134). Many transcripts from T-UCRs are polyadenylated and enriched for H3K4me3 at the TSS (135). Although UCRs range from 200 to 779 bp in length, the transcriptional units of T-UCRs (the non-spliced, full-length cDNAs) are usually up to 2 kb for known T-UCRs (134, 136). T-UCRs are expressed in normal tissues both ubiquitously or in a tissue-specific pattern.

The expression profile of the 481 known UCR revealed that particular T-UCRs are deregulated in PCa, including uc.106+, uc.477+, uc.363+A, uc.454+A, associating with cancer progression, Gleason score, and extraprostatic extension (133). Modulation with the epigenetic drugs TSA and 5-AzaC increase uc.283+A expression while treatment with R1881 increased the expression of uc.287+ and repressed uc.283+A expression, indicating that both epigenetic factors and androgens are responsible for regulation of T-UCRs. Genomewide expression analysis of LNCaP cells treated with a specific siRNA against uc.106+ or sicontrol indicated that uc.106+ might impair cellular transcription of genes involved in cell proliferation and cell death, as well as immune response. Although the

experimental concept of this work (133) was not the most clear, it showed, for the first time, differential expression of T-UCR in prostate tissue samples.

The SNP rs8004379 in the UCR uc.368 is significantly associated with BCR (137). Interestingly, the variant allele, C, for rs8004379 indicates a decreased risk of BCR in a dose-dependent manner after adjusting for age, PSA level, pathologic Gleason score, and stage (137). RNA secondary structure prediction reveals that rs8004379 has a marked effect on uc.368 RNA structure, with a slight reduction in the free energy of the C allele compared to the A allele. Moreover, this SNP is located in the intron of *NPAS3* gene, and C allele in rs8004379 is correlated with increased *NPAS3* expression (137).

More detailed investigation is needed to establish a role for T-UCR in PCa.

Table 3. lncRNA manipulation and consequential phenotypes in PCa

lncRNA	Biotype	Function	Phenotype	Ref
MEG3	lincRNA	Tumor suppressor	Promotes apoptosis by blocking BCL2, enhancing BAX and activating CASP3. Induces G0/G1 arrest by inhibiting CCND1	[151]
PCAT-29	lincRNA	Tumor suppressor	Androgen-regulated lincRNA. Suppresses PCa growth and metastases in chick chorioallantoic membrane assays. Identifies a subset of patients at higher risk for disease recurrence	[152]
NANOGP8	Pseudogene	Tumor suppressor	Decreases clonogenic and migratory potential of PCa cell lines ultimately impairs tumor development *in vivo*. *NANOGP8* knockout increases sensitivity to docetaxel	[153]
DRAIC	lincRNA	Tumor suppressor	Activated by FOXA1 and NKX3-1, and repressed by AR, promoting downregulation of *DRAIC* during prostate cancer progression. Impairs cell invasion and migration, blocking the acquisition of fibroblast-like morphology in PCa cells	[154]
PCAT29		Tumor suppressor	Repressed by AR and induced by FOXA1. Migration and metastasis suppressor	[154]
GAS5	lncRNA/ Retained intron	Tumor suppressor	Involved in cellular growth arrest and apoptosis probably due to GAS5 encoded transcript mimics glucocorticoid response element (GRE). Hosts multiple snoRNA, containing multiple C/D box snoRNA genes in its introns	[138, 155]
PCGEM1	lincRNA	Oncogene	Transcriptional regulator of key metabolic pathways in PCa cells acting as a coactivator for both c-Myc and AR, providing growth advantage. Also recruits PYGO2 and enhances selective looping of AR-bound enhancers to target gene promoters in PCa cells	[156, 157]
PlncRNA-1 (CBR3-AS1)	Antisense	Oncogene	Regulates cell proliferation and apoptosis, by targeting AR	[158]
HOTAIR	Antisense	Oncogene	Androgen-repressed ncRNA upregulated following androgen deprivation therapies and in CRPC. Binds to the AR protein to block its interaction with MDM2, thereby preventing AR ubiquitination and protein degradation.Involved in cell growth and invasion	[159]
PRNCR1	lincRNA	Oncogene	Binds to the carboxyterminally acetylated AR on enhancers, and binds DOT1L. Promotes transcriptional activation by AR and ultimately cell proliferation	[157]
PCAT5		Oncogene	Implicated in cell growth, migration/invasion, colony-forming, and apoptosis. PCAT5 is a regulatory target of ERG	[160]
PCAT18		Oncogene	Involved in cell proliferation and apoptosis, migration and invasion. Activated by AR	
TRPM2-AS	Antisense	Oncogene	Associated with poor clinical outcome. Depletion of *TRPM2-AS* induces apoptosis both *in vitro* and *in vivo* in androgen-independent PCa cells. Critical to maintain the cell cycle progession	[161]

## 4. Clinical utility of ncRNA in PCa management

a. Diagnostic and Prognostic Biomarkers

The emergence of regulatory RNA offers several putative benefits, due to its tissue- and cancer-specific expression and involvement in the regulation of PCa hallmarks (Figure 6). Serum PSA is currently in widespread clinical use, increasing prostate cancer early detection. However, its lack of specificity results in high negative biopsy rate, overdiagnosis and overtreatment of PCa (138). NcRNAs may, thus, provide new biomarkes to accurately diagnose PCa, improve disease management and reduce overtreatment. Given that sncRNAs are resistant to variations in temperature and pH as well as to endogenous RNase activity, they offer unprecedented potential to become blood/urine-based biomarkers (139). Serum samples from men with low-risk, localized PCa and metastatic CRPC have been shown to exhibit distinct circulating miRNA signatures (140). Indeed, miR-21 (141), miR-141 (139, 140), and miR-375 (140) expression levels are increased in the plasma/sera and discriminate patients with advanced PCa from healthy controls, associating with poor prognosis. Moreover, miR-21 serum levels are particularly elevated in patients resistant to docetaxel-based chemotherapy (141).

Indeed, miR-21 (141), miR-141 (139, 140), and miR-375 (140) expression levels are increased in the plasma/sera and discriminate patients with advanced PCa from healthy controls, associating with poor prognosis. Moreover, miR-21 serum levels are particularly elevated in patients resistant to docetaxel-based chemotherapy (141). In two independent cohorts, promoter hypermethylation of GABRE~miR-452~miR-224 predicted biochemical recurrence after radical prostatectomy (142). Moreover, GABRE~miR-452~miR-224 methylation levels also accurately distinguished non-malignant from PCa samples (AUC: 0.98), suggesting that this locus might be suitable for urine-based PCa detection. No only GABRE~miR-452~miR-224 has biomarker potential, but re-expression of miR-224 and miR-452 impaired cell viability, migration, and invasion capabilities (142).

The lncRNA *PCA3* is markedly overexpressed in more than 95% of primary PCa (143). Due to its PCa-specificity, urinary detection of *PCA3* has been developed as a PCa detection test with superior tumor specificity compared to PSA (138). FDA approved this test for clinical use under the name of Progensa *PCA3* with the ultimate goal of aiding in the decision of repeat prostate biopsy. However, correlations between *PCA3* expression and clinical and pathological parameters are conflicting, although some studies reported that *PCA3* test is negative in men with indolent PCa (144). To improve its performance as a prognostic biomarker, *PCA3* was combined with other de-regulated genes, such as *TMPRSS2-ERG*. In

two independent prospective, multicentric, evaluations the panel composed of *PCA3* and *TMPRSS2-ERG* showed superior PCa specificity over serum PSA. This finding might help reduce the number of excessive prostate biopsies (145) and could also have utility for risk stratification in an active surveillance setting (146).
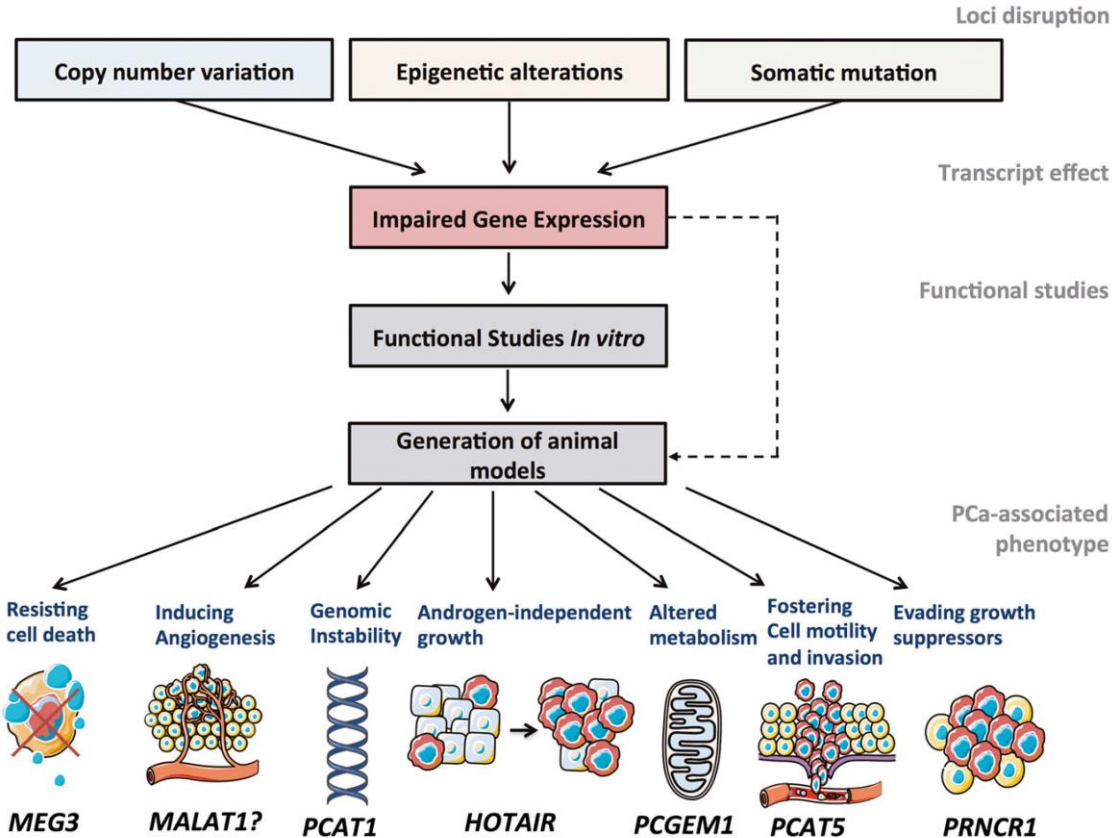
Figure 6. lncRNAs as master regulators of PCa phenotype.

Alterations in genomic sequence and/or expression levels in PCa cells led to initial identification of PCa-associated lncRNAs. Subsequent functional studies directly connected some of the identified lncRNAs with prostate carcinogenesis. Those not only control some of the hallmarks of cancer but also contribute to androgen-independent growth, transcriptional regulation and may be of value for clinical management of PCa patients

ncRNAs may also be detected in exosomes secreted into blood stream or urine. Exosomes are membranous vesicles containing various biomolecules, including lncRNAs, involved in cellular communication and are secreted from many cells, including cancer cells. Combining sncRNA-sequencing and qPCR validation in exosomes derived from CRPC patients, increased expression of miR-1290 and miR-375 was found in exosomes and associated with

decreased overall survival in CRPC patients (147). A multivariate model that included miR-1290 and miR-375 levels, ADT failure time, and PSA levels at the time of CRPC stage, concluded that patients with a high risk score had a 2.58-fold higher risk of death than patients with a low-risk score (HR: 2.58; 95% CI, 1.51–4.41) (147). In exosomes purified from urine samples either from PCa patients and individuals with benign prostatic hyperplasia (BPH), the expression levels of lincRNA-p21 were significantly higher in PCa, discriminating from BPH (148). The biomarker performance of lincRNA-p21, however, was disappointing (67% sensitivity and 63% specificity). Combination with serum PSA increased specificity to 94%, but sensitivity decreased to 52%. Testing in larger cohorts is needed to fully disclose the biomarker potential of exosomal ncRNAs in PCa.

b. ncRNAs as tools for genomic epidemiology and risk prediction

Over the last years, genomewide association studies have become a routine tool to identify germline SNPs and cancer-associated genetic variations that map to non-coding coordinates (149). The vast majority of those SNPs are located whithin enhancers, but others are localized within ncRNA-gene body (150). Although PCa risk-related loci were enriched in lncRNAs, the SNP density in regions of lncRNA was similar to that of protein-coding regions (151). The 8q24 region has been identified as the most important susceptibility region for PCa (152). This 1.2 Mb stretch of the genome is enriched for lncRNAs, including *PCAT1*, *PRNCR1* and *PVT1* and it also harbors the *c-MYC* gene. The eight SNPs detected at 8q24 account for approximately 8% of the 2-fold increased risk of PCa in first-degree relatives of men with the disease (152). The link between 8q24 SNPs and PCa risk is, however, not clear although the proximity to *c-MYC* oncogene suggests that these SNPs might be involved in long-range control of *MYC* expression, notwithstanding the lack of experimental data to support this speculation (152).

Mapping of DNase I hypersensitive sites identified a variant called rs378854, which is in complete linkage disequilibrium with rs620861, as a novel functional PCa-specific genetic variant (153). *In vitro*, the risk allele (G) of rs378854 reduces binding of the transcription factor YY1 (a putative tumor-suppressor in PCa). Chromatin conformation capture experiments depicted that the region surrounding rs378854 interacts with *MYC* and *PVT1* promoters. Moreover, expression of the *PVT1* oncogene in normal prostate tissue increased with the presence of the risk allele of rs378854, whereas expression of *MYC* was not affected (153).

Collectively, clinical use of some SNPs may help to identify patients at risk for PCa and may stratify patient phenotypes (such as clinically aggressive *vs.* indolent) and outcome. The use of specific SNPs may also be useful to predict patients' response to therapy.

5. Discussion & Conclusions

RNA is not only functional as a messenger between DNA and protein but it is also involved in the regulation of genome organization and gene expression, which is extremely elaborated in complex organisms. Among the challenges in the coming years, depiction of the crosstalk between different types of structural RNAs as well as the hierarchy of RNA- and protein-mediated regulation of gene expression that contribute to PCa are capital. Additionally, characterization of the mechanisms mediating RNA communication between PCa cells and mapping the genomic locations of RNA-binding sites (66) are mandatory to further understand the how gene expression control and cell state decisions are accomplished in PCa. Will ncRNA help on achieving a better definition of PCa as single pathological entity or ncRNA profiling may render a subclassifcation of PCa?

Cellular RNAs contain more than a hundred structurally distinct post-transcriptional modifications at different sites (154). These RNA modifications may play an adaptive role that can fine-tune the structures and functions of mature RNAs to influence gene expression (154). Some post-transcriptional RNA modifications can be dynamic and might have regulatory roles equivalent to those of post-translational protein modifications. Therefore *RNA epigenetics* will help determine both mechanisms and functions of these dynamic RNA modifications and ultimately define the "prostate cancer epitranscriptome".

Genome-editing using CRISPR approaches will offer the capability to dissect ncRNAs functions. Moreover, it will provide the ability to directly modify or correct critical PCa-associated alterations by targeting a genomic locus with an engineered guide RNA, offering new therapeutic options for PCa.

During prostate epithelial transformation, AR cistrome undergoes extensive reprogramming. Accordingly, androgen-induced eRNA scaffolds AR-associated protein complexes that modulate chromosomal architecture, suggesting that eRNAs are the most critical RNAs involved in PCa.

Translating the developments in RNA biology and technology updates into deeper understanding of prostate carcinogenesis may assist in the advance of precision medicine, providing not only new and more robust biomarkers (either single or panel ncRNA) but also paving the way for patient-tailored RNA-based therapies, as an alternative to currently available therapeutic strategies. The age of RNA has come.

6. Acknowledgments

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2.2. Epigenetic regulation of miRNA biogenesis

Epigenetics represents a new molecular dimension in cancer research and directly controlls the key hallmarks of cancer cells (48). These alterations are dynamic in their nature and are major determinants of chromatin architecture to allow access of condensed genomic DNA to the regulatory transcription machinery proteins, and thereby control gene expression (48). MiRNA's genes can be epigenetically silenced through DNA methylation and due to covalent histone modifications (49).

Cytosine methylation is a DNA modification generally associated with transcriptional silencing. The cytosine base in DNA can be methylated to become 5-methylcytosine (5mC), and are sometimes referred to as the "5th nucleotidic base". Approximately, half of the genes in vertebrate genomes contain small (around 1kb) CpG-rich regions recognized as CpG islands (CGIs), and the rest of the genome is depleted for CpGs (50). Another important epigenetic mechanism is histone modifications and chromatin alterations. Alterations in the patterns of histone Post-Translational Modifications (PTMs) have been extensively linked to cancer, both at the global level across the genome (51). It is clear that histones' PTM mediates a variety of critical biological processes, generally via chromatin modification that is conducive to the expression or repression of target genes, including miRNA genes (51). Promoter methylation and histone marks represent significant means of silencing various tumor-suppressor genes in the cell, an important contribution to gene expression regulation (48). Thus, it is now accepted that miRNA genes can be epigenetically regulated through epigenetic alterations. Like to other RNA POLII transcripts, miRNA genes have TATA boxes and Transcription Factor II B (TFIIB) binding sites upstream of the transcription start site. Moreover, the DNA sequence of miRNA genes contains upstream regulatory elements (e.g. enhancers), and promoter regions. This is an additional proof that miRNAs are subjected to dynamic methylation regulation via DNA methyltransferases or active DNA demethylation, histone modifications, or binding of transcription factors to canonical sites within miRNA promoters to either promote or repress transcriptional activation (49). Since DNA methylation is a tissue-specific trait as well as a tumor-specific one, its potential as a biomarker has also been extensively explored. Methylated genes appear to have a higher specificity for cancer. DNA methylation is an interesting alteration for laboratory testing since it is far more stable and easier to work with than RNA and its isolation and detection is rather straightforward (52).

The book chapter entitled "DNA Methylation Alterations as Biomarkers for Prostate Cancer" covers the basic concepts of DNA methylation gene regulation in PCa. Moreover, it discusses whether altered epigenetic patterns may serve as useful biomarkers for PCa management, and reviews the effectiveness of miRNA's promoter methylation as novel PCa biomarkers for routine clinical practice.

2.2.1. BOOK CHAPTER: Chapter 14 - DNA Methylation Alterations as Biomarkers for Prostate Cancer.

João Ramalho-Carvalho, Rui Henrique, Carmen Jeronimo:

DNA methylation alterations as biomarkers for Prostate Cancer.

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Abstract

Prostate cancer (PCa) is the most common noncutaneous malignancy and a leading cause of cancer-related death among men. During prostate cancer development and progression, tumor cells undergo abnormal epigenetic modifications, including alterations in DNA methylation patterns. DNA methylation, the most studied epigenetic alteration in human cancer, is associated with transcriptional repression of both coding and non-coding regions of the genome. The relevance of aberrant DNA methylation for cancer risk assessment, diagnosis and therapy monitoring in different cancer types, including prostate cancer, is increasingly acknowledged. Aberrant DNA hypermethylation, especially at gene promoters, is widespread during prostate carcinogenesis, suggesting that restoration of a normal prostatic cell epigenome through treatment with demethylating drugs could be clinically beneficial. Herein, we discuss the role of DNA methylation changes in PCa and how these may translate into the clinical management of PCa patients.

Key words

Epigenetics, DNA Methylation, Biomarker, Prostate Cancer, Clinical Management.

Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer in men, with an estimated 250,000 new cases in the United States diagnosed each year, ranking second in cancer-related mortality. The risk factors for PCa include aging, race (e.g., Afro-American men are more prone to develop PCa whereas native Japanese are at low risk for developing PCa) and family history of PCa (susceptibility genes may contribute up to 5-10% of all detected PCa cases) (1).

Presently, most patients are diagnosed after detection of elevated serum PSA levels or abnormal digital rectal examination, which entail diagnostic prostate biopsy. Clinically localized PCa, which is potentially curable, is usually treated with radical prostatectomy or radiation, although patients with low-risk disease or short life expectancy may be managed expectantly (e.g., through periodic serum PSA measurements and repeat biopsies, if required, to assess disease progression). Conversely, patients with locally advanced or metastatic (i.e., mostly incurable) disease are initially treated with androgen-deprivation therapy (ADT). However, almost all advanced PCa cases, after a period of ADT, progress to castration-resistant disease, an aggressive and highly lethal form of PCa (1, 2).

Prognostication of PCa behavior mostly relies in histological grading, staging and baseline serum PSA levels. PCa histological grading is based on the Gleason grading system, which combines five simple grades [from grade 1 (most differentiated) to 5 (least differentiated)] into 9 combined grades, the so-called Gleason score or sum (ranging from 1+1 to 5+5), a feature that incorporates information from the frequent morphological heterogeneity of PCa (3). Among putative precursor lesions, the most widely acknowledged is high-grade prostatic intraepithelial neoplasia (PIN) which consists of malignant-appearing cells still confined to prostate acini, with at least partial preservation of basal layer cells (4). Interestingly, it is hypothesized that PCa may originate from either luminal or basal epithelial cells, frequently arising as multiple disease foci, thus contributing to wide diversity and heterogeneity at the molecular, cellular, and morphological levels (2).

Prostate carcinogenesis seems to require the acquisition of large-scale genomic rearrangements and copy number alterations involving multiple chromosomes. Indeed, oncogenic fusions, such as *TMPRSS2-ERG*, are found in ~50% of all PCa, as well as in smaller proportion of high-grade PIN (2). Moreover, loss of tumor suppressor genes *PTEN*, *NKX3.1*, *TP53,* and *CDKN1B* are often identified in PCa and next-generation sequencing is providing further evidence for molecular sub-classification of PCa based on *CDH1* alterations, *SPINK1* overexpression, ERG rearrangements and *SPOP* mutations (2, 5-7)*.*

Given the lack of specific therapeutic targets, high prevalence and the prolonged latency period of PCa, the role of chemoprevention has been emphasized. Currently, the most encouraging agents are 5-α reductase inhibitors, which prevent the conversion of

testosterone to dihydrotestosterone, the most active prostatic androgen. However, early development of resistance to ADT with consequent clinical progression due to the acquisition of castration-resistant phenotype is a main concern (2). The pathways involved in castration resistance are not fully elucidated, but accepted mechanisms include (a) intratumoral androgen biosynthesis, (b) androgen receptor (AR) pathway hypersensitivity via AR gene amplification, (c) expression of variant AR isoforms that are ligand-independent, (d) selection of pre-existing castration-resistant epithelial stem cells, (e) growth factor-mediated increase in AR transcription activity and (f) activation of the PI3K-AKT-mTOR pathway (7-9). Treatment of castration-resistant PCa (CRPCa) is mostly restricted to taxane-based chemotherapy and palliative care. Nevertheless, new drugs that seem to tackle CRPC with proven survival benefits, such as abiraterone (blocks androgen production) and enzalutamide (inhibition of androgen binding to AR), are able to prolong survival in men with metastatic CRPC after docetaxel treatment (7).

Owing to the importance of early diagnosis of PCa (which is clinically silent at its earliest stages) to increase disease survival, there has been an intensive search for specific PCa biomarkers. Serum PSA remains the most widely used biomarker but its usefulness has been recently questioned due to its lack of specificity and inability to accurately identify aggressive forms of PCa, causing overdiagnosis and overtreatment (10, 11). Moreover, currently used prognostic parameters, such as the Gleason score are limited in their ability to predict disease behavior, in particular for patients with Gleason score 7, which constitutes most of diagnosed PCa cases at present. Thus, the discovery of novel biomarkers that may identify clinically significant PCa, discriminating these from indolent tumors, is a major challenge, which can be met through the study of PCa epigenetics.

The term "epigenetics" was first used to explain why genetic variations occasionally did not lead to phenotypic deviations and how genes might interact with their environment to generate a phenotype (12). Currently, epigenetics refers particularly to the study of mitotically and/or meiotically heritable changes in gene expression not caused by alterations in the DNA sequence (13). In general, epigenetic mechanisms comprise DNA methylation, covalent histone modifications and noncoding RNA regulation. Epigenetic homeostasis is fundamental to a multitude of biological processes such as transcription, DNA replication, and repair. The disruption of these regulatory mechanisms affects an array of shared and specific cellular processes, influencing the genomic output, and triggering several diseases, including cancer (14, 15).

Chemical modifications of DNA have been annotated as major players for maintenance of the cellular homeostasis and memory. Such DNA modifications include 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC) and the less common 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) (16). The addition of a methyl group to a carbon 5 position (5mC) is

a common epigenetic mark in many eukaryotes and it is regularly found in the sequence context of CpG (cytosine nucleotide-phosphate-guanine nucleotide) or CpHpG (H = A/T/C) (17). This is, by far, the most widely studied epigenetic modification in humans (15). In mammals, this *cis*-regulatory alteration is primarily restricted to symmetrical CpG context. DNA methylation is dynamic and heritable: methyl groups can be added or removed and can remain stable throughout multiple cell divisions. The CpG dinucleotides are predisposed to organize in domains called CpG islands (18). These are defined as regions of more than 200 bp in length, with a GC content of at least 50% and a ratio of observed to statistically expected CpG frequencies of at least 0.6. CpG dinucleotides are relatively uncommon in mammalian genomes (~1%). There are approximately 28 million CpGs in the human genome, 60-80% of which are generally methylated. Only less than 10% of CpGs are found in CpG islands. High levels of 5mC in CpG-rich promoters are strongly associated with transcriptional repression, whereas CpG-poor regions exhibit a more intricate and context-dependent relationship between DNA methylation and transcriptional activity.

Of all human gene promoters about 60% are associated with CpG islands and these are often unmethylated in normal cells, although some of them (~6%) progress to a methylated state in a tissue-specific manner through early development or in differentiated tissues (19). Likewise, CpG nucleotides located in repetitive sequences, inserted viral sequences and retrotransposons are also methylated in normal cells (18), to avoid transcription of these elements and maintain genomic integrity by obstructing recombination events that may lead to gene disruption, oncogene activation, translocations, and chromosomal instability during development and differentiation (20). Thus, epigenomic states are established in normal cells as result of development (18). This is due to the marks present in DNA and chromatin structure and the cell state is maintained through mitosis (18).

Chemically, 5-methylcytosine is the result of the addition of a methyl group, donated by S-adenosylmethionine (SAM), to the fifth carbon of the cytosine residue ring, mediated by DNA methyltransferase (DNMT) (21, 22). There are five main DNA methyltransferases (23): *DNMT1* acts on hemi-methylated DNA substrates created during DNA synthesis and maintains the existing methylation patterns after DNA replication and mitosis. *DNMT3A* and *DNMT3B* target previously unmethylated CpGs (23) and are thought to be responsible for the establishment of methylation patterns during embryogenesis and are also able to add methyl groups at non-CpG sites. *DNMT2* shows sequence and structural characteristics of the above-mentioned DNA methyltransferases (*DNMT1, DNMT3A* and *DNMT3B)* except for a putative nucleic acid binding cleft that cannot easily accommodate duplex DNA. It is responsible for the methylation of aspartic acid transfer RNA (tRNA[ASP]), specifically at the cytosine-38 residue in the anticodon loop, beside its residual DNA methyltansferase activity (24). *DNMT3L,* is structurally similar to DNMTs but do not contain the catalytic domain

necessary for methyltransferase activity (23). However, it has the ability to recognize DNA or chromatin by specific domains and interact with the unmodified H3K4(23).

Given the dual and rather unspecific activity of DNMTs (they are able to deposit methyl groups at non-CpG sites), whole-genome maps of 5-methylcytosine have revealed interesting patterns such as cell state-dependent occurrences of 5mC in contexts other than canonical CpGs and in partially methylated domains (PMDs), and conserved regions depleted of 5mCs across mouse and human (16).

Generation and maintenance of non-CpG methylation seems to be strictly regulated, as such modifications are enriched in specific cell types (e.g. pluripotent cells and neural progenitors in adolescent and adult cortex tissues). It is still unclear if cytosine in non-CpG methylation has any functional relevance in normal development and cancer (25).

PMDs have been mostly found in non-pluripotent cells and non-cortex tissue types. These PMDs seem to associate with low transcription rates, lamina-associated domains and late-replicating domains. Different classes of methylation-depleted regions entitled unmethylated regions (UMRs), DNA methylation valleys (DMVs) and DNA methylation canyons (DMCs) have been defined, as well (26, 27). These regions tend to be conserved across cell types and across mouse and human species. Both methylation valleys and canyons tend to be marked with H3K4me3 or H3K27me3 or both, and each can lead to active, inactive or poised transcriptional states, respectively. Interestingly, these regions cover most genes important for embryonic development (16, 25, 27).

Nonetheless, there is growing evidence that CpG DNA methylation is augmented at gene bodies of actively transcribed genes in mammals (28). It has been proposed that it might be related to elongation efficiency and prevention of erroneous initiations of transcription (29).

The transcriptional repression by DNA methylation is thought to follow two different routes: a direct mode in which promoter methylation obstructs the binding of transcriptional activators to the target promoter region (30) or an indirect mode through the recruitment of Methyl-CpG-binding domain proteins (MBD) (31). MBDs can recruit chromatin-remodeling complexes to the methylated sequence, resulting in chromatin conformation changes that also inhibit gene transcription (32).

DNA methylation does not occur exclusively at CpG islands. Recently, the concept of "CpG island shores" has been defined, referring to regions of lower CpG density that stretch out in close proximity (~2 kb) of CpG islands (33). Equally, the methylation of these CpG island shores is tightly associated with transcriptional inactivation. Indeed, the majority of the tissue-specific DNA methylation appears at CpG island shores instead at CpG islands (33, 34). Differentially methylated CpG island shores are sufficient to distinguish between specific tissues and are conserved between human and mouse. Furthermore, 70% of the

differentially methylated regions in reprogramming are associated with CpG island shores (34, 35).

Alterations in DNA methylation equilibrium are hallmarks of human cancer, including Prostate Cancer (PCa). Discrimination of driver cancer alterations from those that are merely passengers and, thus, not involved in malignant transformation, is a major challenge. During oncogenic transformation, dynamic alterations in hypermethylation and hypomethylation of DNA arise. Hypomethylation appears to be a global phenomenon, but some data indicate that it may take place at specific gene promoters, eventually causing proto-oncogene activation (15, 20). Hypermethylation mostly occurs in a gene promoter-context and is one of the main mechanisms associated with gene expression disruption due to transcriptional repression (15, 20, 36).

Conventionally, DNA methylation has been associated with protein coding genes. However, advances in the postgenomic era revealed a large number of RNA families which were thought to be non-functional elements of the genome. These include a plethora of long noncoding RNAs (lncRNA) and small regulatory RNAs (37). These are all putative targets for DNA methylation, and, among them, microRNAs (miRNA) have often been considered to be regulated by DNA methylation (38). MicroRNAs are ~22 nucleotide-long RNA molecules that mediate post-transcriptional gene silencing by guiding Argonaute (AGO) proteins to RNA targets (39). Following transcription, miRNA are processed in the nucleus by Drosha and then exported to cytoplasm by Exportin-5. In the cytoplasm, DICER processes pre-miRNA before being loaded into the miRNA-induced silencing complex (miRISC), which contains AGO proteins. This complex is involved in translational repression, mRNA decay or both (39). DNA methylation disruption of miRNA transcription impairs miRNA processing and contributes to tumorigenesis (38). MicroRNAs control the expression of tumor-suppressors and oncogenes known to be involved in tumorigenesis, angiogenesis and metastasis. Given the tissue-specific expression signatures of miRNAs, they are tightly regulated and DNA methylation is a key effector in this process.

The aberrant DNA methylation landscape of prostate cancer

Altered epigenetic patterns may serve as useful PCa biomarkers for detection, diagnosis, prognosis and post-treatment surveillance, advancing on the limitations of currently available tools (40). Over the last years, the role of aberrant DNA methylation in the development and progression in PCa has been increasingly recognized. Indeed, epigenetic alterations are early events in carcinogenesis that may even precede the acquisition of well-defined genetic alterations, and persist through invasion, metastasis and life-threatening malignant progression. Because they do not directly affect the genome sequence, therapeutic

modulation through drugs that can target epigenetic modifications opens new avenues for PCa prevention and treatment.

In theory, prostate cells with anomalous DNA methylation marks arise in non-malignant lesions, and due to a "DNA methylation catastrophe", those cells with epigenetically silenced genes undergo clonal selection to form a tumor (41). Conversely, during PCa progression, there is also a global loss of DNA methylation, probably as a consequence of reduced DNA methylation maintenance fidelity, contributing to distinctive cell-to-cell and lesion-to-lesion phenotypic heterogeneity (41). Thus, both locus-specific hypermethylation as well as global hypomethylation are involved in neoplastic transformation and contribute to tumor progression.

Understanding how epigenetics contributes to the clonal evolution of PCa is also under scrutiny. Recent reports suggest that the clonal architecture of aggressive PCa is mediated by intratumor DNA methylation heterogeneity given the extensive epigenetic (and genetic) heterogeneity observed among different regions of the same tumor (42). This intratumoral DNA methylation heterogeneity predominantly occurs at prostate-specific gene regulatory elements, with androgen receptor enhancer domains constituting a good example of that intratumor variation in methylation patterns. Thus, not only DNA methylation plays a role in the regulatory activity of tumor subclones, but it may also be a key element explaining the convergent tumorigenic processes and the diversity of metastatic origin (42). The clues to understand the lethality of PCa may in fact arise from somatic DNA alterations. These aberrations, despite showing marked inter-individual heterogeneity among patients with lethal metastatic PCa, seem to be maintained across all metastases within the same individual (43). Regions that are frequently hypermethylated across individuals are markedly enriched in cancer- and development/differentiation-related genes (43). Hence, DNA methylation alterations have the potential to generate selectable driver events in carcinogenesis and disease progression (43).

As previously mentioned, age, diet and environmental factors are thought to be involved in prostate tumorigenesis (40, 44), and its effects might be mediated by changes in the epigenetic homeostasis. Indeed, some studies pointed out that age, the most important risk factor for development of PCa (44), is positively correlated with increased aberrant promoter methylation of various genes (40), of which the age-dependent methylation of estrogen receptor alpha (*ESR1*) may represent a mechanism linking aging and PCa (45). Given the refined balance between stability and plasticity of DNA methylation patterns, it has been proposed that DNA methylation may provide a lifetime record of environmental exposures and might be used as a potential source of PCa biomarkers.

Promoter hypermethylation in prostate cancer

The best characterized epigenetic alteration in PCa is promoter hypermethylation (36), and both protein coding and noncoding genes are targeted (40). This epigenetic modification is associated with silencing of classic tumor-suppressor genes as well as genes involved in different cellular pathways such as cell cycle, hormone response, DNA repair and damage prevention, signal transduction, tumor invasion and architecture, and apoptosis (15). The hypermethylation of those genes in PCa may correlate with pathological grade, clinical stage and castration resistance.

Prostate is an endocrine gland, in which normal cells maintain an appropriated balance between sex hormones (androgen, estrogen and progesterone) and their specific receptors (2). Several data show that DNA methylation participates in the transcriptional regulation of hormone receptors (40). Androgen activity is essential for the development of both normal and PCa cells. From its earliest stages, PCa is androgen-dependent, a status that is kept during disease progression, until, eventually, tumors are enriched in cells that can grow independently of androgens, mostly as a result of ADT (44). The transition to this state androgen-independent growth has been associated with genetic alterations including mutations and amplifications of androgen receptor (AR) locus (46). Such alterations alter the sensitivity of AR to androgens and are thought to play a role in the development of castration-resistant PCa (CRPCa) (46). Not surprisingly, CRPCa displays a heterogeneous loss of AR that is associated with aberrant methylation of its promoter (47). Up to 28% of CRPCa cases show AR methylation (48), whereas about 20% of primary PCa display this alteration (49), probably at lower levels that do not significantly impair AR expression. Thus, paradoxically, DNA methylation might be an alternative mechanism involved in castration resistance in a subset of patients, through abrogation of AR expression, forcing neoplastic cells to develop alternative signaling pathways, increasing their biological aggressiveness. This may explain the observation that pharmacological reversion of AR promoter methylation, using 5,6-dihydro-5-azacytidine, in CRPCa cell lines restores AR activity, turning cells (again) sensitive to androgen deprivation therapy (50), and attenuating the more aggressive phenotype.

The hormonal environment of the prostate is also dependent of estrogens and both receptors - *ESR1* and *ESR2* - display low or diminished expression in PCa (51, 52). Although an association between *ESR1* methylation and tumor progression has been hypothesized (53), *ESR2* methylation is the main inactivation mechanism accounting for loss of *ESR2* expression in primary PCa (54). However, during PCa progression, especially in metastatic PCa, cancer cells can re-express *ERS2* (55), which is accompanied by promoter demethylation (56). This dynamic reversion of *ESR2* expression is epigenetic in nature and consequently reversible.

Cell cycle is often deregulated in PCa and endows neoplastic cells with increased proliferative capabilities. Cell cycle is very strictly regulated, with multiple checkpoints, and all genes involved are putative targets for silencing through aberrant DNA methylation. Tumor suppressor genes from the cyclin-dependent kinases family are frequently altered in PCa due to several mechanisms. *CDKN2A* has been shown to be dowregulated due to DNA methylation (46), although methylation at exon 2 is more frequent than methylation at promoter region (57). However, exonic methylation of *CDKN2A* is not associated with loss of gene expression (57), and, thus, further studies are needed to clarify how this may impact in prostate carcinogenesis.

D-type cyclins play a critical role in cell cycle regulation and their abnormal expression has been associated with several human malignancies. One of the best studied cyclins is Cyclin D2. Promoter methylation levels of *CCND2* are significantly higher in PCa compared to high-grade PIN and non-tumorous prostate tissues (p<0.01), correlating with tumor stage and Gleason score (58). Moreover, *CCND2* mRNA levels are significantly lower in PCa, inversely correlating with promoter methylation, and demethylating treatment induces a substantial increase in *CCND2* mRNA, in LNCaP cells (58).

Methylation of *SFN* promoter methylation is also a frequent event in malignant prostate lesions, but it also affects non-tumorous tissue (59). *SFN* is a putative tumor suppressor gene involved in cell cycle regulation and apoptosis following DNA damage (59). Since there is a progressive accumulation of neoplastic cells with *SFN* methylation from HGPIN to PCa, it is suggested that this epigenetic event might be relevant for prostate carcinogenesis (59).

Genes involved in signal transduction pathways are also affected by aberrant promoter methylation in PCa. Two of the best examples are endothelin receptor type B (*EDNRB*) (60) and the RAS association domain family protein 1 isoform A (*RASSF1A*) (61). The protein encoded by *EDNRB is* a G protein-coupled receptor which activates a phosphatidylinositol-calcium second messenger system(60). Methylation of *EDNRB* is a frequent event in PCa and is associated with decreased mRNA expression (60, 62), being restored after treatment with 5-azacytidine (60). *EDNRB* silencing diminishes the capacity of PCa cells to clean and even block the expression the vasoconstrictor ET1, which accompanies PCa progression *in vivo.* Promoter methylation of *RASSF1A*, a well-known tumor suppressor, is also a common event in PCa as well as in high-grade PIN lesions (61, 63), associating with more advanced tumor stage (61). These data suggest that *RASSF1A* promoter methylation occurs early in prostate carcinogenesis and increases as PCa progresses, as methylation levels are higher in locally invasive tumors compared to those organ-confined (61).

The maintenance and regulation of normal prostate tissue architecture is based on cadherin-catenin adhesion systems. In PCa, loss of expression of the cell adhesion molecule E-cadherin, encoded by *CDH1*, has been reported in association with promoter methylation of

CDH1, which increases with disease progression, suggesting a role in the metastatic potential of PCa (64). Moreover, the promoters of genes involved in the cadherin-catenin axis are also frequently methylated (*APC*, *CAV1*, *LAMA3*, *TIMP3*) (40), theoretically contributing to tumor invasion and metastization.

One of the most acclaimed biomarkers in PCa is *GSTP1*, encoding the intracellular detoxification enzyme glutathione S-transferase π. *GSTP1* promoter methylation occurs at the earliest stages of tumor development and is associated with transcriptional silencing (65). Aberrant methylation of *GSTP1* is considered a hallmark of PCa, present in about 90% of all PCa and in 75% of high-grade PIN lesions (66, 67).

Disruption of apoptotic pathways is key to PCa development and progression and promoter methylation of apoptosis-related genes is common in PCa (68). Aberrant promoter hypermethylation of *TMS1*, a pro-apoptotic tumour suppressor gene, has been reported as an early event in prostate carcinogenesis, correlating with higher Gleason score (69). Treatment with a demethylating agent restored *TMS1* expression in LNCaP cells (69, 70). Paradoxically, the oncogene *BCL2* (which is anti-apoptotic) is also a common target for gene silencing through DNA methylation in PCa (71), a finding that may support a role for apoptosis-inducing therapy in PCa. Because promoter methylation prevents the anti-apoptotic role of *BCL2*, PCa cells could be more prone to endure apoptosis upon exposure to pro-apoptotic drugs.

MiRNAs are critical regulators of many pathways and are often deregulated in carcinogenesis (72). MiRNAs are differentially expressed in PCa and may be targeted for downregulation by promoter methylation (73). For instance, the ability to escape apoptosis is an important carcinogenic event facilitated by numerous miRNAs in PCa (72).

Table 1. Common microRNA inactivated by DNA methylation in Prostate Cancer

miRNA	Putative target mRNA	Phenotypic effect	References
miR-1	*FN1, LASP1* and *XPO6*	Inhibits cell proliferation and motility, represses mitosis, invasion and filipodia formation	Hudson et al. [122]
miR-31	*E2F1, E2F2, FOXM1, MCM2*	Alters androgen receptor homeostasis and controls cell cycle	Lin et al. [131]
miR-34a	*SIRT1, CDK6*	Tumor suppressive properties, mediator of apoptosis, cell cycle arrest, and senescence	Lodygin et al. [123]
miR-126	*EGFL7, VEGF-A*	May play a role in angiogenesis and cell proliferation	Saito et al. [124]
miR-132	*HBEGF, TALIN2*	Stimulates cell death by anoikis, and impedes cell migration and invasion	Formosa et al. [75]
miR-141	*BRD3, UBAP1*	Inhibit epithelial to mesenchymal transition	Vrba et al. [125]
miR-145	*IRS1, c-Myc, MUC1, Fli1, BNIP3*	Downregulation of miR-145 is associated with aggressive phenotype and poor prognosis in prostate cancer	Suh et al. [76], Zaman et al. [126]
miR-193b	*CCND1, ETS1*	Involved in prostate cancer cell proliferation and anchorage-independent growth	Ruahala et al. [127]
miR-196b	*c-myc, HOXB8*	Putative repressor of cell migration and metastasis	Hulf et al. [73]
miR-200c	*ZEB1, ZEB2*	Inhibit epithelial to mesenchymal transition	Vrba et al. [125]
miR-205	*BCL2L2, MED1*	Promote chemotherapeutic agents-induced apoptosis in prostate cancer cells; counteracts epithelial-to-mesenchymal transition and cell migration/invasion	Bhatnagar et al. [128]

Other common pathways disrupted owing to miRNA deregulation include the cell cycle, intracellular signaling, DNA repair, and adhesion and migration (74). Table 1 summarizes the most relevant information about miRNAs downregulated by promoter hypermethylation in PCa. One of the most interesting miRNAs in PCa is miR-132, that controls cellular adhesion and directly targets *HBEGF* and *TALIN2*, which has been found to be methylated in 42% of PCa, with higher methylation levels associating with higher Gleason score and more advanced tumor stage (75). Interestingly, re-expression of miR-132 in PC3 cells induced cell detachment followed by cell death (anoikis) (75). Furthermore, miR-145 was found to be significantly downregulated PCa compared to normal prostate tissues and 5-aza-2'-deoxycytidine treatment dramatically restored miR-145 expression (76).

MiRNAs may be putative targets for epigenetic therapy in PCa since they have a role in key signaling pathways and are associated with multiple layers of gene regulation. Remarkably, it has been demonstrated that about one third of downregulated miRNA loci show a matched pattern of DNA methylation and H3K9 acetylation (73). This underlines the cooperation between the different levels of epigenetic regulation to accomplish transcriptional block of miRNAs.

Hypomethylation in prostate cancer

Although DNA hypomethylation was the first epigenetic alteration described in cancer, there are few reports on this aberration in PCa. Global DNA hypomethylation occurs at both early and late stages of PCa and it might serve as biomarker for early detection and prognostication (77-79). Global hypomethylation expands the chromatin predisposing to genomic instability and promoting deleterious mutations (80, 81). Loss of DNA methylation is associated with tumor progression and chromosome instability (80), and global lower levels of methylation are more evident in metastatic PCa (82). Genome-wide hypomethylation may trigger inappropriate transcription of proviral and retrotransposon sequences, leading to disruption of neighboring genes (83, 84). The *LINE-1* retrotransposon is hypomethylated in 50% of PCa samples, especially in cases with lymph node metastases (80). In addition, genome-wide hypomethylation is associated with gains or losses of sequences on chromosome 8, in localized PCa (80). The progressive genome hypomethylation seems to be linked with deficient DNA methylation maintenance fidelity during DNA replication and it probably contributes to generate cell and lesion specific phenotypic heterogeneity (41). This theory is supported by autopsy studies which showed low levels of DNA methylation and high frequency of copy number alterations in lethal PCa(78).

Gene-specific hypomethylation in PCa has been documented for *CAGE* (85), *CYP1B1* (86), *HPSE* (87), *PLAU* (88), as well as *CRIP1*, *S100P*, and *WNT5A* (89). Increased expression of *HPSE*, encoding the extracellular matrix degradation protein heparanase which degrades

heparan sulfate, has been implicated in tumor invasion and metastasis (87). Interestingly, *PLAU* (urokynase-type plasminogen activator) is associated with the acquisition of castration resistance and increases tumorigenesis in both *in vitro* and *in vivo* models (88).

Loss of imprinting is also associated with aberrant biallelic expression of some genes in PCa, such as *IGF2* (90). Thus, local and tissue-specific patterns of gene expression might prompt neoplastic transformation over a long period of time (40). Moreover, *IGF2* hypomethylation may be a biomarker for early detection of PCa.

The impact of DNA hypomethylation in overexpression of oncogenic miRNAs in PCa is very limited. Thus far, only miR-615 has been reported as epigenetically activated in PCa cells due to DNA hypomethylation (73). However, hypomethylation seems to play a role in deregulation of the non-coding RNA transcriptome. *XIST,* a single-copy gene heavily methylated in morphologically normal prostate cells, is hypomethylated in PCa, a feature that is associated with *LINE-1* hypomethylation, suggesting a global hypomethylation rather than a promoter specific loss of methylation (91). Moreover, *XIST* hypomethylation is increased in more aggressive tumors (91). The Melanoma antigen gene protein-A11 (*MAGE-11*) is a coregulator of the AR signaling. During PCa progression and androgen deprivation therapy there is increased expression of *MAGE-11* mediated by promoter hypomethylation, providing an an alternative mechanism for increased AR signaling in castration-resistant PCa (92).

The available data highlights that hypomethylation changes may not strongly correlate with functional gene sets or with *cis* activation of gene expression. Consequently, in case that DNA hypomethylation plays a driver role in PCa, it would most likely be through promotion of genomic instability (e.g. through promotion of retrotransposition (93)), rather than through direct *cis* regulation of specific genes.

DNA Methylation-based markers for prostate cancer detection, management and risk estimation

In Western countries, PCa screening is carried out mainly by serum PSA testing. However, serum PSA is limited in the ability to specifically detect PCa and it does not discriminate between clinically aggressive and clinically indolent PCa. Thus novel and more powerful biomarkers are warranted. Because epigenetic alterations are highly prevalent and arise early in prostate tumorigenesis, DNA methylation-based biomarkers constitute promising biomarkers for PCa detection, diagnosis, assessment of prognosis and prediction of response to therapy (40, 94).

Table 2. DNA Methylation Alterations as Biomakers for Prostate Cancer Management

Gene(s)/miR(s)	Biomarker	Sample type	Method	Sensitivity/ Specificity	References
Hypermethylated					
GSTP1	Early detection, diagnosis, prognosis	Urine Plasma Serum	MSP qMSP Restriction endonuclease qPCR,	58%/na [100]	Jerónimo et al. [99] Cairns et al. [98] Gonzalgo et al. [100] Bastian et al. [112]
GSTP1/APC/MDR1	Diagnosis, prognosis	Prostatectomy	MSP	75.9%/84.1% [111] 72%/67.8% [111]	Enokida et al. [111]
p16/ARF/MGMT/GSTP1	Early detection	Urine	qMSP	87%/100% [101]	Hoque et al. [101]
GSTP1/APC/RARB2/ RASSF1A	Early detection	Urine	qMSP	86%/na [102]	Roupret et al. [102]
GSTP1/APC/RARB2	Early detection, prognosis	Prostate biopsy	qMSP	60%/80% [104]	Baden et al. [104]
GSTP1/PTGS2/RPRM/TIG1	Prognosis	Serum	Restriction endonuclease MSP	47%/92% [103]	Ellinger et al. [103]
GSTP1/APC/PTGS2/MDR1	Prognosis, diagnosis	Prostatectomy	qMSP,	100%/92% [110]	Yegnasubramanian et al. [110]
APC	Prognosis	Prostate biopsy	qMSP	na	Henrique et al. [117]
PTGS2/CD44	Prognosis	Prostatectomy	qMSP	na	Woodsoon et al. [113]
GPR7/ABHD9/Chr3-EST	Prognosis	Prostatectomy	qMSP		Cottrell et al. [114]
PITX2	Prognosis	Prostatectomy	EpiChip microarray	na	Banez et al. [129]
GABRE/miR-452/miR-224	Diagnosis, prognosis	Prostatectomy	MethyLight	95.5%/94.3%	Kristensen et al. [118]
miR-205	Prognosis	Prostatectomy	Sequenom MassArray	na	Hulf et al. [120]
Hypomethylated					
IGF2	Early detection	Prostatectomy	Pyrosequencing	na	Bhusari et al. [130]

MSP, methylation-specific PCR; qMSP, quantitative MSP; qPCR, quantitative polymerase chain reaction.

Cancer detection and diagnosis

GSTP1 is the best-characterized epigenetic biomarker for PCa. Somatic DNA methylation of *GSTP1* is nearly universally present in almost prostate cancer cells but is absent or low in normal cells (95). Indeed, more than 90% of PCa cases show aberrant promoter methylation of *GSTP1* (65) and it might be specifically detected using MSP-based approaches in a wide range of tissue samples and bodily fluids, mainly blood and urine. Testing for *GSTP1* could be used for screening or stratification for the need of prostate biopsy (95). *GSTP1* performance methylation displays high specificity (86.8-100%) but low sensitivity, both in urine (18.8-38.9%) and serum/plasma (13.0-75.5%) (96-101). This might be overcomed by a multigene promoter methylation testing, and several different gene panels have been proposed, including *GSTP1/ARF/CDNK2A/MGMT (101)* and *GSTP1/APC/RARB2/RASSF1A* (102) in urine and *GSTP1/PTGS2/RPRM/TIG1* (103) in serum. As a result, the detection rate increased significantly to 86% in urine and 42-47% in serum, retaining high specificity: 89-100% for urine and 92% for serum (101-103). The results gathered allowed for the design of a urine-based diagnostic test - the Prostate Cancer Methylation (ProCaM) assay (104) - that interrogates the methylation levels of *GSTP1*, *APC* and *RARB2*. The assay displayed 60% sensitivity and 80% specificity, with 97% informative rate. ProCaM has been validated in a

multicentre prospective study testing samples of men with serum PSA levels of 2.0–10.0 ng/ml, in which its performance was compared with existing methods based on clinical workup and serum PSA levels (104). The ProCaM predictive accuracy was higher than that of serum PSA or any of its related parameters (AUC=0.73, p=0.038). Importantly, a positive result correlated not only with positive biopsy, but it also associated with increased risk for detecting high-grade PCa (Gleason score ≥ 7) with a substantial predictive accuracy (AUC:0.79, p=0.001). Indeed, men with positive ProCaM result were 7 time more likely to be diagnosed high-grade PCa (104).

The EGF Containing Fibulin-Like Extracellular Matrix Protein 1 (*EFEMP1)* is a tumor-suppressor gene epigenetically deregulated in PCa. *EFEMP1* methylation seems to be PCa-specific and accurately discriminates PCa from nonmalignant prostate tissues (AUC=0.98; p<0.001), as well as from bladder and renal tumors (AUC=0.986, 96% sensitivity and 98% specificity) (105). The high accuracy (96%) of *EFEMP1* methylation test shows promise for its use as an ancillary tool in diagnostically challenging lesions. Moreover, when compared with other frequently methylated genes in PCa (*e.g. GSTP1, APC* and *RARbeta*), the *EFEMP1* assay displays similar performance, even when compared with multigene panels (105).

Using prostate core biopsies, Paziewska *et al* (106) compared the performance of expression and methylation markers to distinguishing cancerous from non-cancerous prostate tissues. Although *HOXC6, AMACR* and *PCA3* expression displayed the best discrimination between PCa and BPH (AUC: 0.94; 0.92; 0.955), they were not sensitive and specific enough to be considered PCa diagnostic biomarkers. However, DNA promoter methylation levels of *APC*, *TACC2*, *RARB*, *DGKZ* and *HES5* identified PCa with high sensitivity and specificity (AUCs ranging between 0.95 and 1.0)(106). Some overlap was observed for DNA methylation levels of PCa-positive and PCa-negative needle biopsies, but with minor impact. Combination of methylation levels of *RARB, HES5* and *C5Orf4* displayed the highest performance (AUC=0.909), detecting over 50% of cancer samples with 100% specificity (106). Taking into account that problematic prostate core biopsies usually contain just a small amount of neoplastic cells, these biomarkers might be of help to pathologists, especially in patients with suspected PCa following a negative initial biopsy.

Fast and accurate DNA methylation analysis of multiple loci in clinical samples with limited DNA quantities would provide a clear advantage to patient management. A MethyLight multiplex assay combining *APC*, *HOXD3* and *TGFB2* has been evaluated in patient samples and its sensitivity was sufficient for detection methylation of those genes in formalin-fixed paraffin-embedded tissue and urine samples. These encouraging results, however, require validation in larger patient cohorts (107).

Limitations of sampling during prostatic biopsy raise the concern that cancer might not be sampled in high-risk men. This leads to a high frequency of follow-up procedures that are needed to ultimately confirm the absence of disease. Partin and collaborators developed an assay (DOCUMENT, Detection Of Cancer Using Methylated Events in Negative Tissue) aimed to screen, among patients with a negative prostate biopsy, those that are at low risk of harboring cancer which were not detected through biopsy due to inaccuracy and might avoid unnecessary repeat (108). The assay is based on the quantification of methylation levels of 3 genes commonly methylated in PCa - *GSTP1*, *APC* and *RASSF1* – using multiplex methylation-specific PCR (108). In a multicenter study, this epigenetic assay was an independent predictor of PCa detection in a repeat biopsy thirty months after the initial negative results. The performance of the assay displayed 64% specificity, 88% negative predictive value and 18% false-negative rate, based on the adjusted cancer prevalence in repeat biopsies. In multivariate models corrected for factors with diagnostic potential, the assay proved to be the most significant independent predictor of patient outcome (OR 2.69, 95% CI 1.60-4.51). Owing to the high negative predictive value, the DOCUMENT assay combined with other known risk factors may assist in clinical management of PCa suspects, reducing the rate of repeat biopsies (108).

Prognosis and prediction of response to therapy

Current prognostic markers for PCa are suboptimal, contributing to overtreatment of indolent PCa patients. A DNA methylation signature that predicts biochemical recurrence after radical prostatectomy has been uncovered. Hypermethylation of *AOX1*, *C1orf114*, *GAS6*, *HAPLN3*, *KLF8*, and *MOB3B* was shown to be highly PCa-specific (AUC ranging from 0.89 to 0.98) and high *C1orf114* methylation was significantly associated with biochemical recurrence after radical prostatectomy in multivariate analysis, both in the testing and validation sets [hazard ratio (HR)=3.10; 95% CI, 1.89 to 5.09; HR=3.27; 95% CI, 1.17 to 9.12, respectively] (109). In this multi-cohort approach, a significant three-gene prognostic methylation signature (*AOX1-C1orf114-HAPLN3*) was used to classify patients into low- and high-methylation subgroups (HR=1.91; 95% CI: 1.26 to 2.90; HR=2.33; 95% CI: 1.31 to 4.13, for cohorts 1 and 2, respectively). *AOX1-C1Orf114-HAPLN3* and *C1Orf114-HAPLN3* panels were evaluated and these models successfully predicted biochemical recurrence after radical prostatectomy in multivariate analysis including standard clinicopathological variables and in two different cohorts, indicating that these methylation-based markers hold independent prognostic value. Further testing is needed to evaluate the prognostic potential of these markers in prostatic biopsies.

Hypermethylation of *PTGS2* in localized prostate cancer predicted PCa recurrence after radical prostatectomy independently of tumor stage and Gleason score (110). The univariate

Cox proportional hazards models revealed that high *PTGS2* methylation levels significantly increased the risk of recurrence (HR=2.82; 95% CI, 1.07-7.44). Moreover, in the multivariate Cox proportional hazards model, only high *PTGS2* hypermethylation predicted for increased risk of recurrence independently of Gleason score and pathological stage (HR=4.26; 95% CI, 1.36-13.36).

A hypermethylation score derived from *GSTP1*, *APC* and *MDR1* discriminated organ-confined from locally advanced disease with 72% sensitivity and 67.8% specificity (111). Moreover, considering patients with PSA levels <10 ng/mL, the M score had a sensitivity of 67.1% with 85.7% specificity. The circulating cell-free DNA carrying *GSTP1* hypermethylation was detected in 12% of men with clinically localized disease and 28% of men with metastatic cancer. Additionally, 8 men (15%) who developed PSA recurrence were positive for serum *GSTP1* hypermethylation, whereas patients who were disease-free tested negative. In multivariate analysis, serum *GSTP1* hypermethylation was the most significant predictor of PSA recurrence (HR=4.4; 95% CI, 2.2-8.8) (112).

Furthermore, hypermethylation of *CD44* and *PTGS2* is also predicitive of PSA recurrence after radical prostatectomy (HR=8.87, 95% CI, 1.85-42.56). Kaplan Meyer analysis showed that combined hypermethylation of *CD44* and *PTGS2* associated with shorter time to biochemical recurrence compared to absence of gene methylation (113). Similar findings were reported for promoter hypermethylation of *ABHD9* (114). *PITX2* hypermethylation is a strong marker for biochemical recurrence either in a univariate and multivariate analysis (HR=3.4; 95% CI 1.9–6.0; HR=2.1; 95% CI 1.2-3.9, respectively) (115). In survival analysis, the estimated 8-year probability of biochemical recurrence free was 79% in the group with high *PITX2* methylation as opposed to 94% in the low methylation group. Interestingly, *PITX2* hypermethylation seems to be associated with biochemical recurrence in PCa with intermediate Gleason score (115). On the other hand, high methylation levels of *APC* and *CCND2* are strong predictors of short time to post-radical prostatectomy recurrence in PCa with a Gleason score 3+4=7 (multivariate model, HR=4.33; 95% CI 1.52-12.33)(116), whereas in a prospective study, high *APC* promotor methylation levels predicted poor prognosis in prostate biopsy specimens, irrespective of Gleason score (OR=3.5; 95% CI 1.23-9.96) (117).

Promoter methylation levels of *GABRE/miR-452/miR-224* not only discriminate PCa from non-malignant tissues (AUC=0.98; 95.5% sensitivity and 94.3% specificity), but also high methylation levels independently predicted early biochemical recurrence after radical prostatectomy in two independent cohorts (HR=1.75; 95% CI 1.37-2.23 and HR=2.99; 95% CI 1.71-5.21) (118). Interestingly, high methylation was significantly associated with the standard clinicopathological parameters, including serum PSA, pathological stage, Gleason score and surgical margin status. In a large cohort (407 patients), high *HOXD3* promoter

methylation levels were strongly associated with shorter recurrence free-survival (high methylation = 43.1% vs. low methylation = 34.5%) (119). Moreover, *HOXD3* methylation levels were associated with higher Gleason score and more advanced tumor stage (HR=5.23; 95% CI 1.31-20.96) (119).

Because miR-205 is epigenetically down-regulated in PCa it may have a prognostic biomarker potential (120). Indeed, hypermethylation of miR-205 locus is significantly associated with biochemical recurrence in patients with localized PCa and low preoperative PSA levels (HR=2.005; 95% CI: 1.109-3.625). Furthermore, on multivariate analysis, low miR-205 methylation was a significant predictor of biochemical relapse (HR=2.2; 95% CI=0.99- 5.0) (120).

DNA methylation-based biomarkers might also be useful for prediction of response to therapy. In a recent study, detectable baseline methylated *GSTP1* in serum was associated with poorer overall survival in men with castration-resistant PCa (HR=4.2; 95% CI: 2.1–8.2) and a decrease of methylation levels after cycle 1 of chemotherapy was associated with PSA response (121). These preliminary findings were further validated in another cohort and the results were similar (HR=2.4; 95% CI: 1.0–5.6), thus confirming that assessment of *GSTP1* methylation levels in plasma is a promising predictive biomarker for a subgroup of advanced PCa patients undergoing chemotherapy.
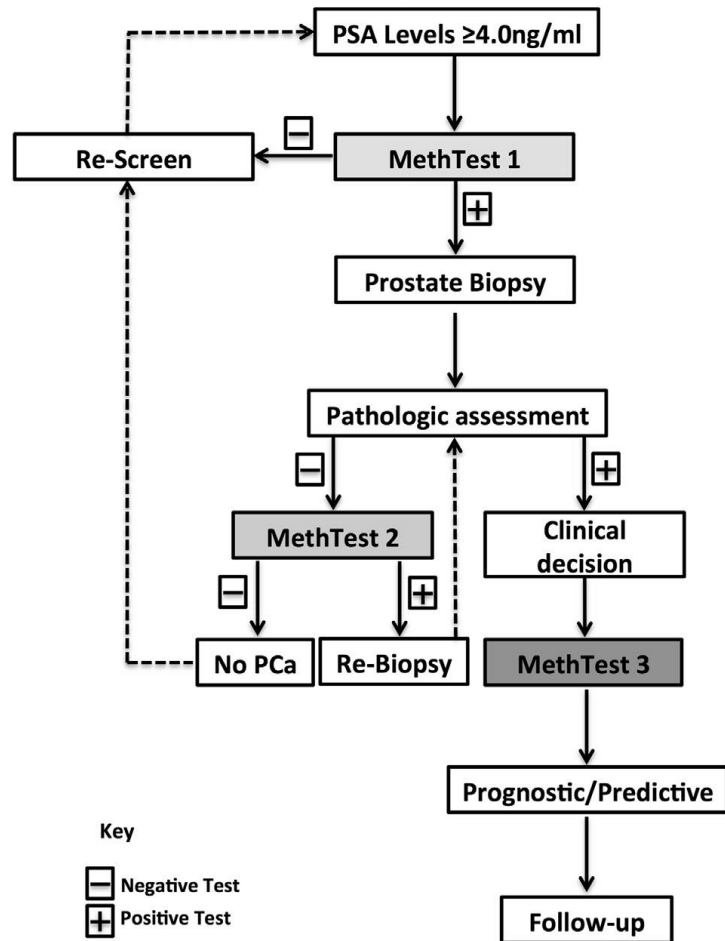
Conclusions and Perspectives

Alterations in DNA methylation patterns are frequent and early events in prostate carcinogenesis, enabling its use as tumor biomarkers. These may accurately detect PCa at its earliest stages, by means of non-invasive techniques, increasing the likelihood of curative treatment. Moreover, the diagnostic performance of several methylated genes in PCa, makes them potential ancillary tools of histopathological assessment of prostate biopsies. However, discrimination of clinically aggressive from indolent PCa is mandatory and several methylation-based biomarkers have demonstrated to be of prognostic value. Finally, the predictive value of methylation markers is just starting to be unraveled in PCa, and is likely to provide novel tools form clinical management.
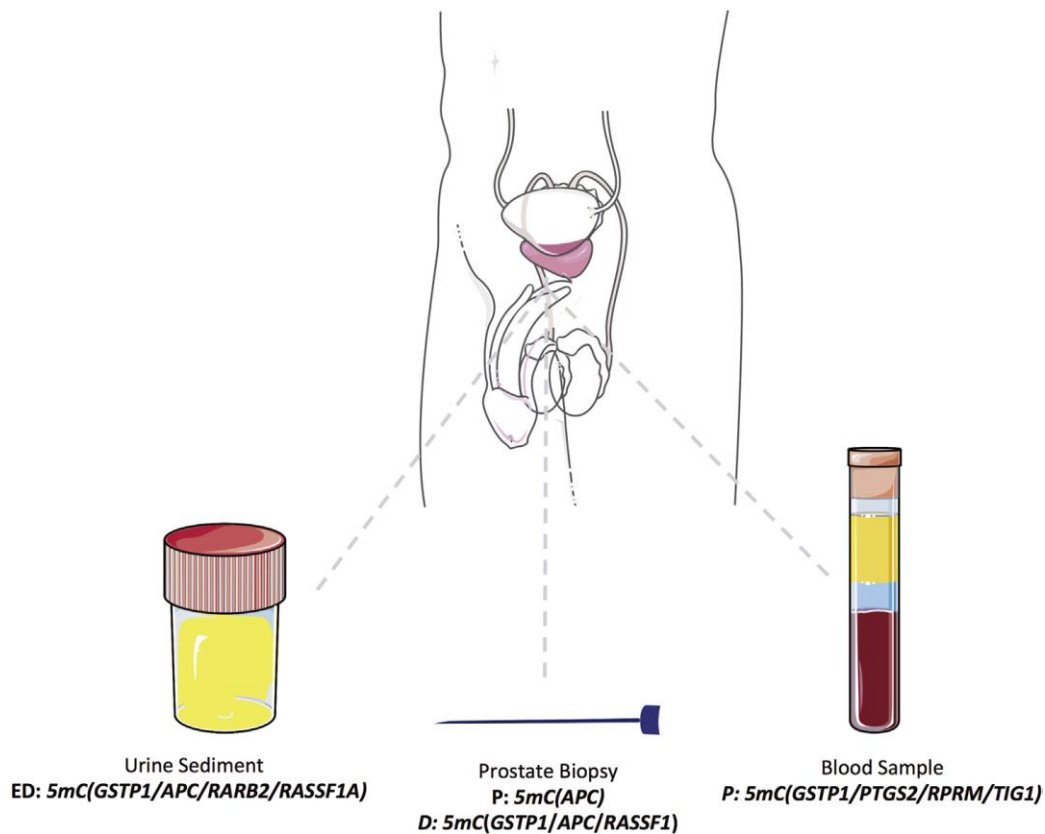
Figure 1. Clinical applications of DNA methylation analysis in prostate cancer. *Urine biomarker testing employs a noninvasive approach to detect PCa by collecting voided urine samples, isolating DNA from cells in the urine sediment, and quantifying DNA methylation levels. The DNA methylation is also an ancillary tool in samples with low cellular content such as prostate biopsy. Alternatively, isolation of circulating PCa cells/DNA from patient serum and testing for DNA methylation levels might provide predictive information for PCa. 5mC (5-methylcytosine): quantitative detection of DNA methylation levels using quantitative methylation-specific PCR (qMSP). Most accepted panels are suggested for testing in prostate samples. ED, early detection; D, diagnosis; P, prognosis.*

Interestingly, at each stage of the work-up the set of biomarkers differs whereas other remain across the algorithm. This may reflect, to the former, the biological stage at which a particular epigenetic alteration becomes more relevant to carcinogenesis, whereas for the latter its persistence or increase along tumor progression endows a particular relevance for the neoplastic process, from transformation, to invasion and dissemination. Importantly, some of the biomarkers have been already tested in a clinical-level assay (e.g., ProCaM and DOCUMENT), meaning that its translation to routine use might be foreseen for the near future. However, it must be acknowledged that a long path lies still ahead for most of the

DNA-methylation biomarkers, requiring standardization at methodological level and adequate clinical trial design to ascertain whether it represent a significant step forward in the management of PCa.

Figure 2. DNA methylation biomarkers in prostate cancer management.

Specific panels of methylated genes may be used in the clinical management of PCa patients, not only in cancer detection (MethTest 1), but also are putative ancillary tools after pathologic assessment (MethTest 2). Additionally, these biomarkers may provide prognostic/predictive

Glossary

CpG island – Regions of more than 200 bp in length, with a GC content of at least 50% and a ratio of observed to statistically expected CpG frequencies of at least 0.6. CpG dinucleotides are relatively uncommon in mammalian genomes (~1%) but are frequently found at promoters. Those CpG islands at promoters are predominantly unmethylated across cell types and are very prevalent at transcription start sites of housekeeping and developmental regulatory genes.

Partially methylated domains (PMDs) - Large contiguous regions of the genome (mean size ~153 kb) that display intermediate methylation levels (average <70%).

Unmethylated regions (UMR) - regions constitutively unmethylated that independently whether they defined or not as CpG islands. Almost all UMRs are associated with known or predicted transcription start sites, and very often packaged with nucleosomes containing H3K4me3.

DNA methylation valleys (DMV) - Large genomic domains (≥ 5kb long) that are methylation devoided. Moreover, DMV genes are poised with bivalent states (H3K4me3 andH3K27me3). DMVs are uniquely enriched for transcription factors and developmental regulatory genes. Developmental regulatory genes are preferentially located in DMVs. Strikingly genes with DMVs tend to be hypermethylated in cancer.

DNA methylation canyon (DMC) - Regions of low methylation covering conserved domains that frequently containing transcription factors and are distinct from CpG islands and shores. Approximately half of the genes in these canyons are marked with repressive histone marks.

Gleason score (GS) - Prostate Cancer's microscopic grading system ranging from 1-5 based on the glandular architecture and patterns. The combined GS (2–10) is the result of the two most prevalent Gleason grade patterns per tumor. GS is a significant prognostic indicator: high GS (≥8) is associated with a poorer outcome than an intermediate GS (7), and low GS (≤6) is globally associated with a better prognosis.

Prostate-specific antigen - serine protease produced by prostate epithelial cells implicated in seminal fluid liquefaction. Serum PSA levels are used for detection and monitoring of prostate cancer.

Biochemical relapse - when, following radical prostatectomy, PSA levels are higher that 0.2ng/ml, or when PSA consecutively rises in from the post-operative baseline. It is indicative of disease recurrence.

Castration-resistant PCa – Highly aggressive and lethal form of PCa characterized by disease progression despite androgen deprivation therapy (castrate serum levels of testosterone), presenting as one or any combination of a continuous rise in serum PSA levels, progression of pre-existing disease or appearance of new metastases.

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CHAPTER 2 – RATIONAL AND AIMS

Rational and Aims

Much progress has been made on Prostate Cancer (PCa) research over the past decade. Evidence from computational, biochemical, and genetic experiments have greatly expanded our understanding of the mechanism of action and biological properties of miRNAs as a class of regulatory molecules.

PCa is a heterogeneous disease characterized by deregulation of important pathways that control normal cellular homeostasis. Due its wide-range interactions, miRNAs are fine-tuners of those processes and, thus, "micromanagers" or "microsensors" of aggressive phenotypes of PCa. Hence, one of the aims of this research project was to understand which molecular mechanisms and interactions of miRNAs may contribute to PCa aggressiveness.

MiRNAs occupy a unique position in the hierarchy of gene regulators and represent a promising avenue for uncovering previously unknown mechanisms of cancer biology. Their mechanism of action allows them to act as fine tuners of transcriptional programs, as components of complex network motifs, and as post-transcriptional modulators to confer robustness to transcriptional programs in the face of environmental and genetic variability. MiRNAs can regulate the accumulation of DNA repair proteins at damage sites. Mammalian cells have developed an elaborate DNA damage response (DDR) system and DNA repair machinery, which play a critical role in development of resistance to DNA damaging agents. The precise regulation of DDR and DNA repair is crucial for cell survival and its abrogation often results in genetic instability. Metastatic, castration-resistant prostate cancer (CRPC) can have genomic abnormalities that affect with DNA repair. The question addressed in this Thesis is whether DNA methylation mediates the loss of function of specific miRNAs and including the control of DDR-induced senescence in PCa cells.

Although some examples are described, either functionally characterized and as biomarkers, miRNAs are decisive players in PCa development.

One of the main areas of research in PCa is the discovery of new and more accurate predictive and prognostic biomarkers. Indeed, it is clear that current PCa overdiagnosis and overtreatment is mainly due to the exhaustive Prostate-specific antigen (PSA) screening. Meticulous patient selection for screening and reducing overtreatment are important to preserve the benefits and reduce the downstream harms of PSA testing (e.g. incontinence and erectile dysfunction). Moreover, when PCa cells metastasize to bone, ¾ of the patients die within a period of 5 years. The question we addressed was whether miRNAs' promoter hypermethylation were reliable biomarkers for PCa detection and prognostication.

Consequently, the major goal of this Thesis was to understand how the deregulation of miRNAs contributes to prostate carcinogenesis and progression, providing functional evidence linking miRNAs and PCa and identifying clinically relevant alterations that might be useful for clinical management of PCa patients.

Specifically, the aims of this doctoral Thesis were:

1 – MicroRNAs downregulation contribute to Prostate Cancer
- Characterize the global expression of miRNAs in Prostate samples;
- Determine which mechanisms trigger PCa downregulation;
- Evaluate the global response of miRNA expression following DNA demethylation treatment;
- Functionally characterize the role of miR-130a in the prostate cell lines;
- Identify the molecular pathways controlled by miR-130a in prostate cells.

2 – DNA methylation signature of microRNA deregulation in Prostate Cancer
- Profile the DNA methylation dynamics underlying miRNA regulation in PCa deregulation;
- Provide evidence of how these miRNAs can contribute to tumorigenesis;
- *In vitro* dissection of the functional relevance of miRNA (miR-130b~301b) manipulation;
- Investigate how these miRNAs modulate DNA repair and cellular senescence.

3 – Integrative analysis of DNA methylation and microRNA expression in Prostate Cancer
- Identification of miRNAs downregulated in PCa targeted by DNA methylation, combining expression and methylation data generated aforementioned;
- Determine if other alterations might account for miR-152 downregulation;
- Examine the functional significance of miR-152 in PCa cell lines;
- Discover and characterize the targets of miR-152 and demonstrate their oncogenic role in PCa.

4 - Utility of promoter hypermethylation of microRNAs in Prostate Cancer clinical management
- Characterize new miRNAs regulated by promoter methylation in PCa;
- Describe their potential as PCa biomarkers in tissue samples;

- Evaluate their performance as biomarkers for PCa early detection in urine samples;
- Investigate the prognostic value in biopsy samples.

CHAPTER 3 – METHODS

Material and Methods

This section describes the methods used to obtain the results presented in this Thesis. It is not structured according to the temporal sequence of papers, but rather attempts to explain the reason of using each methodological approach, as well the variety of technics covered. The detailed procedure of each method is embedded in the respective manuscripts.

A. Sampling

Globally, different samples were used to successfully generate the data supporting this Thesis. For miRNA profiling and further validation, primary tumors from patients with clinically localized PCa (stages T1c and T2, according to TNM staging system, 7th edition) consecutively diagnosed and primarily treated at the Portuguese Oncology Institute – Porto (IPO-Porto), Portugal, were prospectively collected. For control proposes, morphological normal prostate tissue (MNPT) samples were obtained from prostates that did not harbor PCa collected from cystoprostatectomy of bladder cancer patients.

To discover and validate new biomarkers, two different cohorts were selected. Urine samples were collected from 95 patients diagnosed with PCa, before being submitted to radical prostatectomy, from 1999 to 2002. Control samples were collected from 46 healthy donors. This cohort was used to discover new biomarkers for PCa early detection. Furthermore, samples from prostatic biopsies from 74 patients were collected from individuals referred to IPO-Porto due to elevated PSA levels, from 2001 to 2003. This set, enabled us to identify miRNAs whose methylation helped to better prognostication of PCa patients.

Additionally, to further support the findings in chapters 4, 5 and 6 Prostate dataset from The Cancer Genome Atlas (TCGA) was also used.

For *in vitro* manipulation, the standard cell lines used in PCa research were also utilized.

B. microRNA methylation profiling and validation

Two different platforms were used to profile miRNAs in PCa. First, using miRNA PCR panels, the expression of 752 was evaluated in a set of 10 PCa and 5 MNPT (Chapter 4). In parallel, the same profiling was performed for PCa cell lines (DU145, LNCaP and PC3) after exposure to the demethylating agent, 5-Aza-2'-deoxycytidine (5-Aza-CdR). This strategy rendered a global increase in the expression of miRNAs. Using stringent filtering criteria, miR-130a was identified as the most promising candidate and selected for validation analyses in an independent patient series.

The Infinium HumanMethylation450 BeadChip Kit (Illumina) was used to generate a comprehensive genome wide profiling of PCa Methylome (Chapter 5). This method quantifies methylation levels at specific loci within the genome. The data obtained derived from 25 PCa and 5 MNPT samples. This approach was of particular relevance for mapping new miRNAs targeted by differential methylation in PCa.

Then, the candidates selected from the different approaches were validated. In the chapters 4 and 7, Quantitative Methylation-Specific PCR was used to the assess DNA methylation levels, while miRNA expression levels were evaluated using specific primers for each miRNA (miRNA LNA PCR primer set, Exiqon, Vedbaek, Denmark).

In the chapter 5 and 6, the DNA methylation levels were assessed by pyrosequencing. MiRNA transcript levels were assessed using TaqMan miRNA Assays specific for each miRNA.

C. *In vitro* validation of selected microRNAs.

To evaluate the contribution of the previously studied miRNAs, functional, *in vitro* assays were designed. To overexpress miRNAs, miRNA mimics were transfected into the cells to simulate naturally occurring mature miRNAs. This increased the proportion of miRISC containing the guide strand miRNA. By studying the phenotypic consequences of this increased miRNA's activity it was possible to discover miRNA functions. To silence endogenous miRNA, chemically modified, single stranded nucleic acids - miRNA Inhibitors (Anti-miR) - were designed to specifically bind to and inhibit endogenous miRNA molecules.

In Chapters 4, 5, and 6 cell viability (as measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide assay (MTT)), apoptosis (determined the translocation of phosphatidylserine from the interior to the exterior surface of the mammalian cell membrane), cell invasion assay (performed using the Matrigel® Matrix) were applied. In the Chapter 5 DNA Damage, the Single Cell Gel Electrophoresis (Comet Assay) was also assessed. Herein, the "head" corresponds to intact DNA, whereas the "tail" consists of damaged (single-strand or double-strand breaks) or broken segments of DNA.

Moreover, to support the phenotypes observed, both western blot and real time quantitative PCR (RT-qPCR) were performed to evaluate genes directly involved in the pathways altered upon *in vitro* miRNA's manipulation.

D. microRNA target determination.

One of the most critical aspects on miRNA research is target's identification. Although many approaches have been proposed for miRNA target detection, there is no consensus to set which experimental approach is the most accurate. This is, perhaps, due to the unclear definition of how the most appropriate readout of miRNA–target interactions should be. As

miRNAs repress the expression of their targets, the gain of miRNA function should lead to decreased target expression. Putative transcripts targeted by miR-130a were also assessed, using microarray analysis [Affymetrix Human Transcriptome 2.0 Arrays (Affymetrix, Santa Clara, CA, U.S.A.)] of scrambled-transfected and miR-130a-overexpressing PC3 cells. Validation of selected targets was performed using RT-qPCR and luciferase assays. As in genetic screens the identified targets are already linked to a phenotype, the next step included *in vitro* silencing of both *DEPD1C* and *SEC23B,* in order to prove their oncogenic potential.

Luciferase assays were also used to provide an alternative mechanistic link between target genes and the respective miRNAs (Chapters 4 and 6).

PART II – RESULTS

CHAPTER 4

Epigenetic Disruption of miR-130a Promotes Prostate Cancer By Targeting *SEC23B* and *DEPDC1*

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Epigenetic Disruption of miR-130a Promotes Prostate Cancer By Targeting *SEC23B* and *DEPDC1*

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Abstract

MicroRNAs (miRNAs) are small, non-coding RNAs that mediate post-transcriptional gene silencing, fine tuning gene expression.

In an initial screen, miRNAs were found to be globally down-regulated in prostate cancer (PCa) cell lines and primary tumors. Exposure of PCa cell lines to a demethylating agent, 5-Aza-CdR resulted in an increase in the expression levels of miRNAs in general. Using stringent filtering criteria miR-130a was identified as the most promising candidate and selected for validation analyses in our patient series. Down-regulation of miR-130a was associated with promoter hypermethylation. MiR-130a methylation levels discriminated PCa from non-malignant tissues (AUC=0.956), and urine samples revealed high specificity for non-invasive detection of patients with PCa (AUC=0.89). Additionally, repressive histone marks were also found in the promoter of miR-130a.

Over-expression of miR-130a in PCa cells reduced cell viability and invasion capability, and increased apoptosis. Putative targets of miR-130a were assessed by microarray expression profiling and *DEPD1C* and *SEC23B* were selected for validation. Silencing of both genes resembled the effect of over-expressing miR-130a in PCa cells.

Our data indicate that miR-130a is an epigenetically regulated miRNA involved in regulation of key molecular and phenotypic features of prostate carcinogenesis, acting as a tumor suppressor miRNA.

Key words: miR´s epigenetic regulation, miR-130a, miRNA, Prostate cancer, *SEC23B, DEPDC1*

1. Introduction

MicroRNAs (miRNAs) are small single-stranded, ~22-nucleotide-long, noncoding RNAs. The human genome encodes a large number of miRNAs, each with the potential to target hundreds of different mRNAs. By controling the rate of translation of mRNAs into proteins, miRNAs coordinate multiple biological processes such as cell proliferation and differentiation, cell death, metabolism and invasion/angiogenesis. Dysregulated miRNA expression is linked to various human diseases, including prostate cancer (PCa) (1, 2). These molecules may have either oncogenic or tumour-suppressive function, depending on the target mRNAs (3, 4). Similarly to protein coding genes, miRNA transcriptional deregulation may be due to genetic and/or epigenetic alterations and this has been implicated in neoplastic transformation (1, 2).

PCa is among the most common adult malignancies in developed countries (5). Although most patients with localized disease have slow-growing, non-lethal tumors, a relevant number of men experience disease recurrence after first-line treatment, potentially evolving to metastasis and death (6). Although locally recurrent disease might be manageable, lack of effective treatment for patients which progression to castration-resistant PCa results in a high mortality rate (7).

Epigenetic alterations such as DNA methylation and covalent histone modifications are common features of PCa pathogenesis (8, 9). Moreover, aberrant DNA methylation and activity of chromatin remodeling enzymes is also associated with altered transcriptional states, ultimately impairing miRNA expression (10). Because miRNA deregulation is a common feature of PCa, driving and coordinating tumor progression (9, 11), we hypothesized that deregulated miRNA expression might be due to epigenetic alterations. Expression profiling of primary PCa revealed widespread down-regulation of miRNAs. Following exposure of PCa cell lines to a demethylating agent the expression level of some miRNAs was restored, suggesting that DNA methylation is implicated in miRNA deregulation. We further demonstrated that miRNA-130a inhibits PCa malignant phenotype *in vitro*, partially, by

targeting two oncogenes: *DEPDC1* and *SEC23B*. These results indicate that miRNA-130a has tumor-suppressive activity in PCa.

2. Material and Methods

(See Appendix A: Supplementary materials and methods for detailed information.)

2.1. Tissue specimens and cell lines

Tissue specimens (n=101 PCa and n=15 morphological normal prostatic tissues) at Portuguese Oncology Institute-Porto, Porto, Portugal. This study was approved by the institutional review board [Comissão de Ética para a Saúde-(CES-IPOPFG-EPE 205/2013)] of Portuguese Oncology Institute of Porto, Portugal.

The Cancer Genome Atlas (TCGA) prostate cancer dataset was used for validation.

Human PCa cell lines (LNCaP, DU145 and PC3) were obtained from ATCC (Manassas, VA, USA). Culture and treatments were performed accordingly

DNA and RNA extraction were done using standard methods.

2.2. MicroRNAs global expression and validation

Expression of miRNAs was assessed in ten PCa and four MNPT using microRNA Ready-to-Use PCR Human Panel (I + II) v2.R (Exiqon, Vedbaek, Denmark), comprising of 752 miRNAs. RNA samples were submitted to cDNA synthesis using miRCURY LNA Universal RT microRNA PCR (Exiqon, Vedbaek, Denmark) following manufacturer's instructions. Then, data were analyzed using the comparative Ct method (12). Mean value of reference genes was used for normalization, and miRNAs with fold change higher than 1.5 were classified as overexpressed in PCa compared to MNPT.

For validation, cDNA was synthesized from 101 PCa, 15 MNPT and four PCa cell lines, as previously described. MiRNA expression levels were evaluated using specific primers (microRNA LNA PCR primer set, Exiqon, Vedbaek, Denmark). All samples were run in triplicates.

2.3. Bisulfite Treatment and Quantitative Methylation-Specific PCR

One microgram of DNA was use as a template for bisulfite modification using the EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA) following the manufacturer's instructions. Bisulfite-modified DNA was amplified by quantitative methylation-specific PCR (qMSP) using specific primers for miR-130a promoter (Supplementary Table S3).

2.4. MicroRNA and siRNA transfection

MiR-130a was transiently transfected in PC3 and DU145 cells with a Pre-miR™ miRNA precursor or negative control (pre-miR-130a, PM10506; miR-NC, AM17010, Applied Biosystems, Foster City, CA, USA).

Phenotypic assays (Cell viability, Apoptosis and Invasion assays) were performed according Supplementary material & methods

2.5. Gene expression profiling for identification of miR-130a target genes

To analyse the effect of miR130a over-expression, PC3 cells transfected with miR-130a and negative controls, were analyzed for gene expression genome-wide using the Affymetrix Human Transcriptome 2.0 Arrays (Affymetrix, Santa Clara, CA, U.S.A.). The microarray data have been deposited to the NCBI's Gene Expression Omnibus, with accession number GSE80750.

2.6. Quantitative Real Time-PCR assay

A total of 300 ng was reverse transcribed and amplified using TransPlex Whole Transcriptome Amplification Kit (Sigma-Aldrich, Schnelldorf, Germany) as previously described (13). Quantitative real time-PCR (qRT-PCR) reactions were performed Kapa Sybr Fast qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA) (Supplementary Table S3, primers sequences).

2.7. Western Blotting

Cell lysates were separated on 12% polyacrylamide gels and transferred onto nitrocellulose membrane using semi-dry transfer. The membrane was incubated for 1 h in blocking buffer (5% nonfat dry milk) and incubated overnight at 4 °C with CASP3 (ab90437, 1:500; Abcam, Cambridge, UK) and β-Actin (1:8,000, Sigma-Aldrich, CO., St. Louis, MO, USA). Blots were developed using Immun-Star WesternC Chemiluminescent kit (Bio-Rad, Hercules, CA, USA).

2.8. ChIP-Seq

LNCaP ChIP-seq data for the region surrounding the miR-130a locus were downloaded from the NCBI GEO website.

2.9. Luciferase assay

A reporter plasmid containing a binding site at *DEPDC1* or *SEC23B* 3′UTR for miR-130a (GeneCopoeia, Rockville, MD, USA) was transfected into HEK293Ta cells using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). Luciferase activity was assessed with the Secrete-Pair™ Dual Luminescence Assay Kit (GeneCopoeia,

Rockville, MD, USA) according to the manufacturer's instructions. Experiments were performed in triplicates at 72 h after transfection.

3. Results

3.1. Global miRNA expression in prostate tissue samples and prostate cancer cell lines

To identify differentially expressed miRNAs in PCa compared to MNPT, 752 miRNAs were profiled in ten PCa and four MNPT tissue samples. Considering only miRNAs exhibiting more than 1.5-fold difference in expression, 64 and 10 miRNAs were found to be down-regulated and up-regulated in PCa, respectively (Figure 1A, 1B, Supplementary table S4). Among downregulated miRNAs, the highest fold variations were displayed by miR-187 (~3.6 fold), miR-224, miR-100 and miR-221-222 (~2.5 fold), miR-205 and miR-145 (~2.0 fold). Consistent with previous studies, our data confirmed upregulation of miR-32, miR-141 or miR-153, and downregulation of miR-145, miR-205, miR-221-222 and miR-152.

The relative expression of the same 752 miRNAs was assessed in PCa cell lines (LNCaP, Du145 and PC3), either mock or exposed to 1 μM 5-Aza-CdR (Supplementary Figure 1A; Supplementary table S5). These results were compared with those of PCa tissues and a set of four miRNAs, including miR-130a, miR-145, miR-205 and miR-520g were found to be significantly downregulated in both PCa tissues and cell lines, and simultaneously reactivated upon 5-Aza-CdR treatment. From this panel, miR-130a had not been previously reported to be regulated by promoter methylation and, thus, it was selected for validation studies.

3.2. Alterations of the microRNA processing machinery in PCa

Given that our microRNA profiling revealed that the vast majority of miRNAs is downregulated in PCa, we analysed the expression of 4 key genes implicated in the biogenesis of microRNAs (Figure 1C). In primary tumors, *DROSHA*, *DGCR8* and *DICER* were significantly downregulated compared to MNPT, and no alterations were found for *XPO5.* As expected, *DROSHA*/*DGCR8* expression levels significantly correlated (Spearman r=0.48; p=1×10^{-8}), as well as *DGCR8*/*DICER* (Spearman r=0.57; p=1×10^{-11}), and *DROSHA*/*DICER* (Spearman r=0.33; p=0.00022) (Supplementary Figure 1B, 1C). Thus, disruption of the microRNA processing machinery might also contribute to the global microRNA dowregulation observed in PCa samples (Figure 1G). Nonetheless, these results are not so obvious in the cells lines (Supplementary Figure 1D).

3.3. Validation of miR-130a downregulation in prostate tissue samples

The expression of miR-130a was evaluated in a larger cohort of prostate tissues (101 PCa and 15 MNPT) by qRT-PCR, for validation. The overall expression of miR-130a was

significantly lower in PCa compared to MNPT (Figure 1D, p=0.0016). Similar results were found for the independent cohort from TCGA. Indeed, miR-130a expression was significantly decreased in PCa compared to adjacent normal tissues (p=0.04, Figure 1E), further supporting our observations.
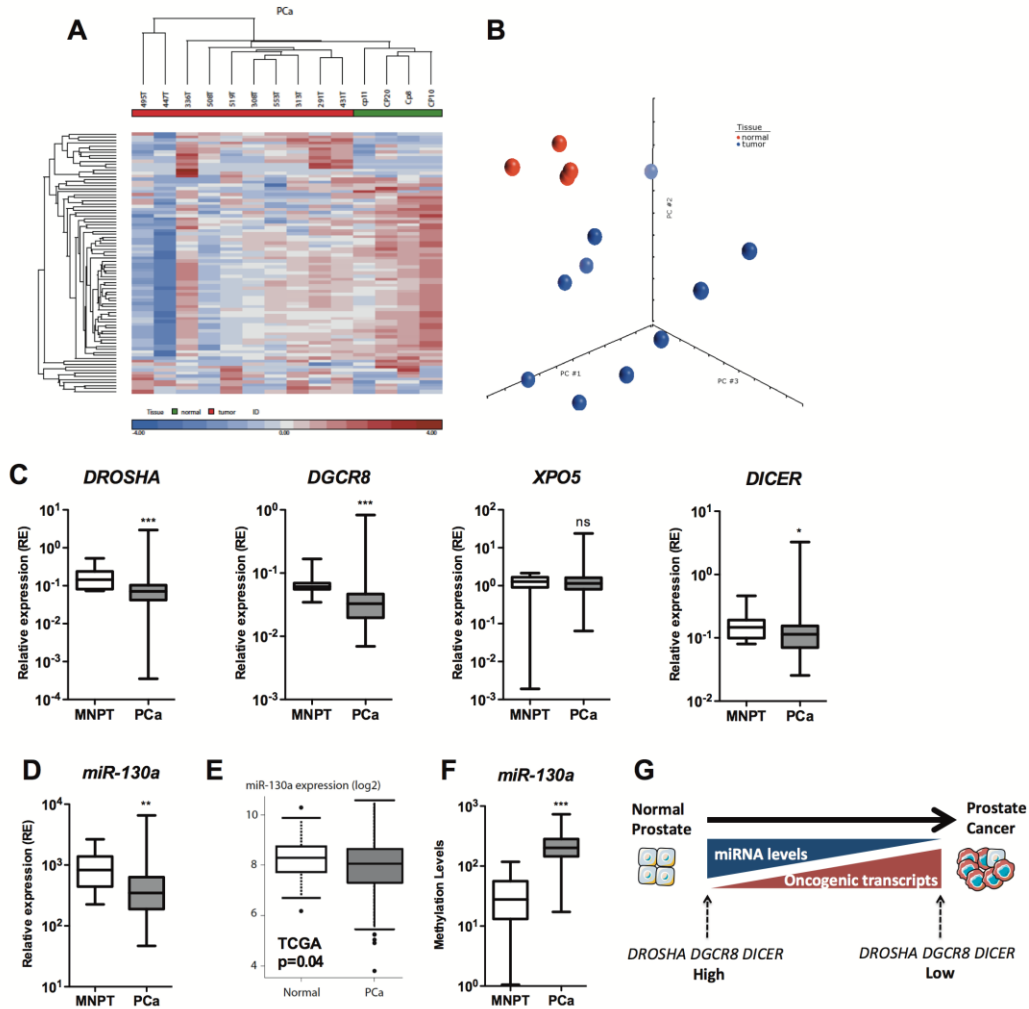
Figure 1. miR-130a deregulation in Prostate Samples

(A) heatmap of the differentially expressed miRNAs in prostate cancer (PCa) compared with morphological normal prostate tissue (MNPT). Red: high expression; blue: low expression. (B) Principal component analysis (PCA) for the samples analyzed (Red, MNPT; blue, PCa). (C) Evaluation of expression levels of the major enzymes involved in microRNA biogenesis in primary tumors samples (101 PCa) and controls (15 MNPT). MiR-130a expression determined by qRT-PCR (D) and in the TCGA patients (E). (F) MiR-130a promoter methylation levels in PCa and control tissue samples. (G) Schematic overview of how decreased miRNA levels combined with biogenesis machinery impaired activity contribute to prostate tumorigenesis. (Significance level represented as: * = p<0.05; ** = p<0.01; *** = p<0.001)

*3.4. The promoter of miR-130a is methylated in prostate cancer tissues*

Because miR-130a promoter region harbors a CpG island (CGI), we designed a specific qMSP assay to detect aberrant DNA methylation. Interestingly, PCa samples displayed significantly higher promoter methylation levels compared to MNPT ($p< 0.0001$, Figure 1F). Because 82% of PCa tissues tested positive for miR-130a methylation (Supplementary figure 2A) we assessed its diagnostic performance using ROC curve analysis of the validation cohort. MiR-130a methylation levels accurately discriminated PCa from MNPT with 95.5% sensitivity and 94.3% specificity (AUC = 0.956) (Supplementary figure 2C). Furthermore, the diagnostic performance of miR-130a promoter methylation was also tested in urine and bladder washings (Supplementary figure 2B). Although performance was not as good as in tissue samples, PCa was correctly identified with 83.5% sensitivity and 82.3 % specificity (AUC= 0.89) (Supplementary figure 2D).

3.5. Promoter methylation mediates silencing of miR-130a

To confirm the effect of promoter methylation on miR-130a expression, PCa cell lines were exposed to 5-Aza-CdR (Figure 2A). Interestingly, miR-130a's promoter methylation levels decreased in all cell lines (DU145, LNCaP and PC3) alongside with increased expression of miR-130a, especially in PC3 cells (Figure 2A). These results validate the previous microRNA profiling (supplementary figure 1A, supplementary table 5) and implicates DNA methylation as a regulatory mechanism for miR-130a expression.

3.6. H3K27me3 repress miR-130a expression

Considering the role of chromatin remodeling complexes in gene expression regulation, we mapped the miR-130a promoter enrichment for H3K4me2, H3K4me3, H3K36me3, H3K27Ac, H3K27me3, as well as AR and FOXA1 (Figure 2D). A marked depletion of H3K4me2, H3K4me3 and H3K27Ac was found upstream of miR-130a promoter. Conversely, a steady enrichment for H3K27me3 at miR-130a and a neighbour gene (*YPEL4*) was observed, which is consistent with both *YPEL4* (figure 2C) and miR-130a downregulation. Because trimethylation of histone H3 at lysine 27 is catalized by EZH2, we assessed *EZH2* transcript levels in PCa. Remarkably, high *EZH2* mRNA levels were found in all cell lines tested (Figure 2B).
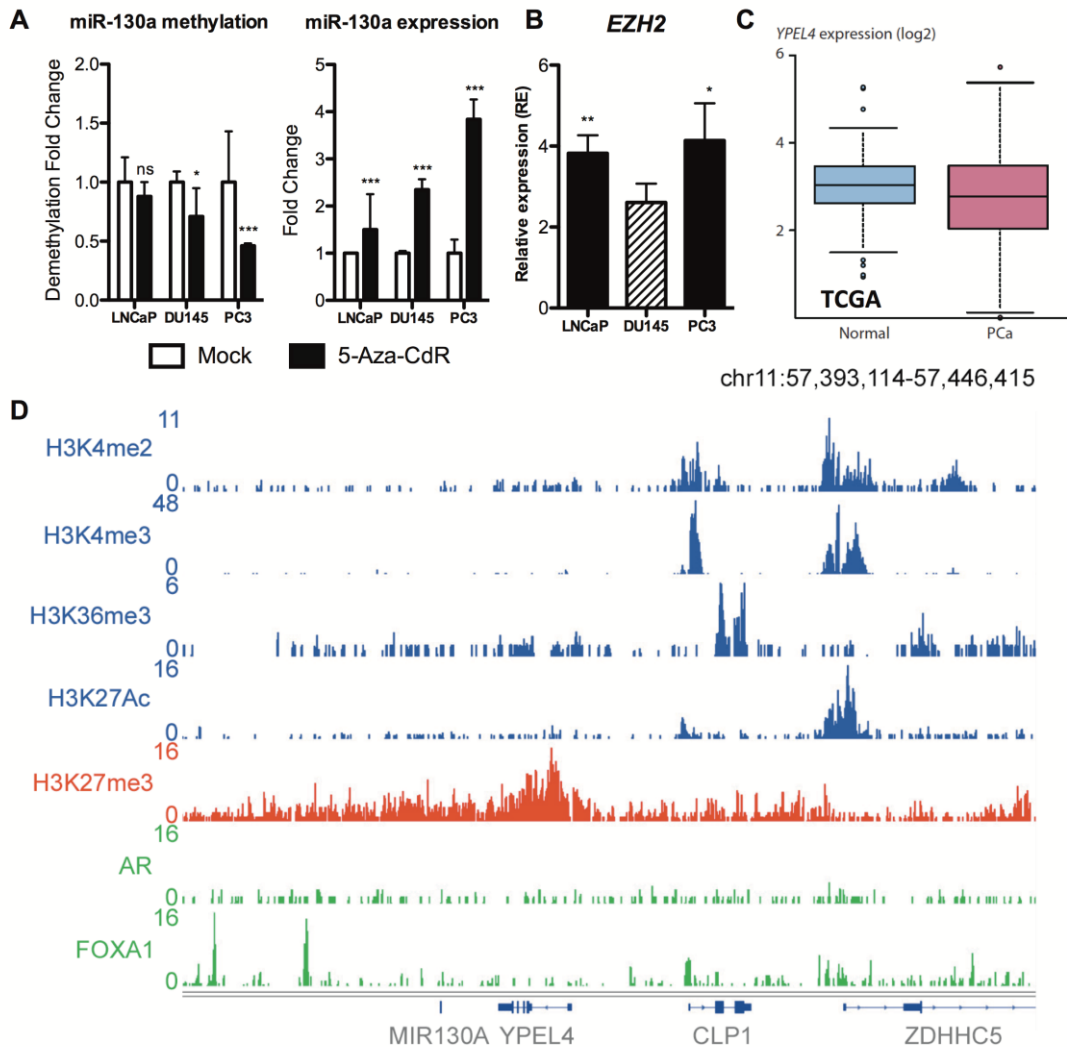
Figure 2. Epigenetic regulation of miR-130a.

*(A) Methylation levels (left panel) and re-expression of miR-130a (right panel) in PCa cells treated by vehicle [dimethyl sulfoxide (DMSO)] or 5-aza-dC. (B) Relative expression of EZH2 in PCa cell lines. (C) Expression of YPEL4 is decreased in PCa samples in the TCGA cohort (RNA-Seq). (D) A representative ChIP-seq profiling in LNCaP cells showing K3K27me3 enrichment at the miR-130a locus. (Significance level represented as: ns – not statistically significant, * = p<0.05; ** = p<0.01; *** = p<0.001)*

3.7. miR-130a overexpression attenuates the malignant phenotype of PCa cells

MiR-130a mimics were transfected into PCa cell lines (LNCaP, DU145 and PC3) and transfection efficiencies were confirmed by qRT-PCR (Supplementary Figure 3A). In miR-130a overexpressing PC3 cells, a change in cell morphology was noticed, with the acquisition of a less spindled and more polygonal (epithelial) phenotype compared to pre-miR-NC and anti-miR-130a transfected cells (Figure 3A).
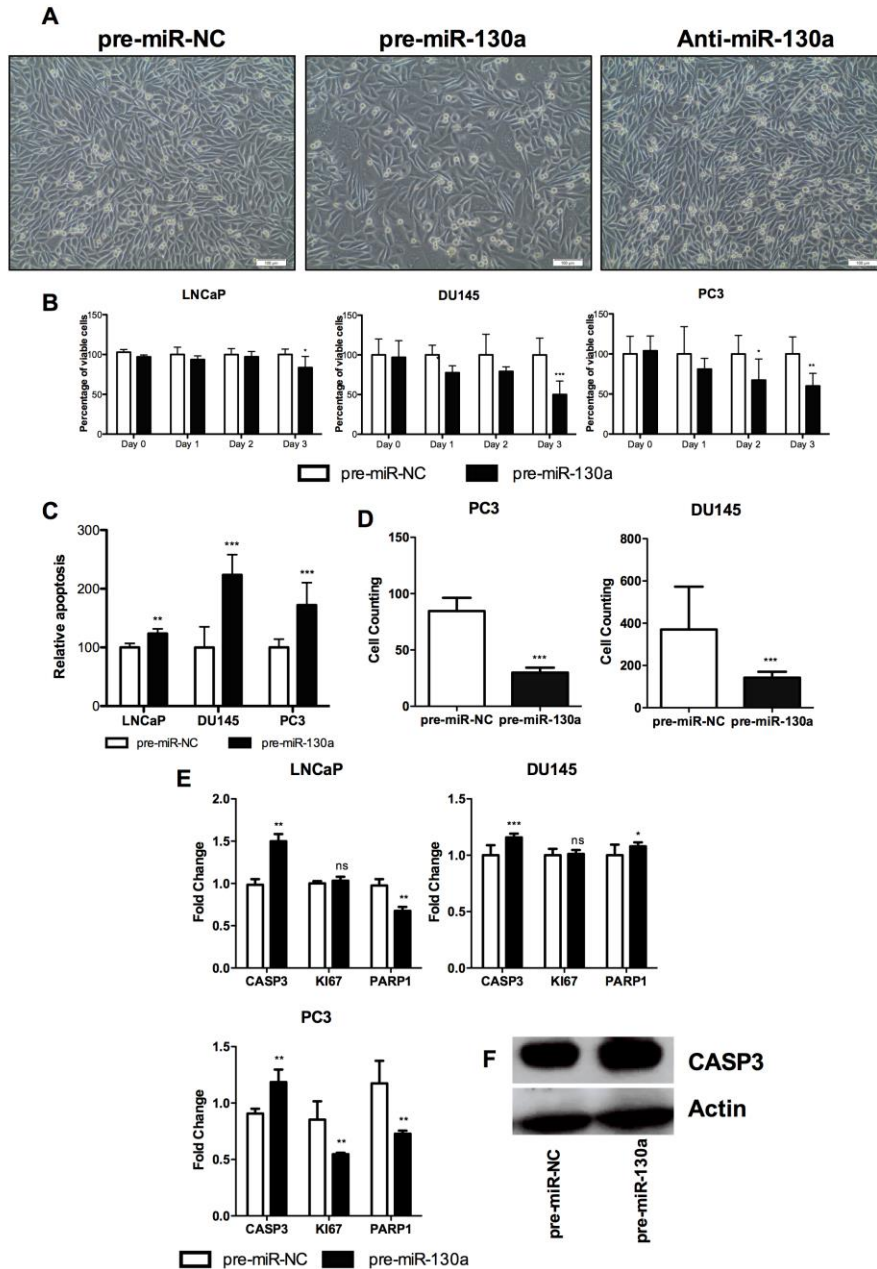
Figure 3. Ectopic expression of miR-130a attenuates malignant phenotype in PCa cell lines.

(A) Morphological features of PC3 cells upon transfection. (B) Cell viability posttransfection of miR-130a or miR-NC mimics in LNCaP, DU145 and PC3 cells determined by an MTT assay. All results are relative to pre-miR-NC miRNA transfected cells. (C) Relative apoptosis in LNCaP, DU145 and PC3 cells after miR-130a ectopic expression. (D) Matrigel invasion assays performed 48 hours posttransfection. (E) QRT-PCR analyses of genes involved in cellular homeostasis after miR-130a overexpression. (F) Western Blot of the effector caspase – CASP3. The presented results are representative of a minimum of three

experiments performed in triplicate; (Significance level represented as: * = p<0.05; ** = p<0.01; *** = p<0.001)

Moreover, in PC3 and DU145 cells, miR-130a overexpression caused an inhibitory effect on cell viability (p<0.001) (Figure 3B). Increased apoptosis in both DU145 and PC3 cells, as well (Figure 3C, p<0.001), and significantly reduced its invasive potential (Figure 3D). In LNCaP cells only a modest cell viability reduction and a mild apoptosis increase were achieved (Figure 3B, 3C).

Subsequently, we evaluated the expression of a small panel of genes comprising *CASP3* (effector caspase), *KI67* (cellular proliferation), and *PARP1* (DNA repair, differentiation) (Figure 3E). MiR-130a transfection in LNCaP cells caused an increase in *CASP3* expression and a decrease of *PARP1*, implying miR-130a as apoptosis activator and DNA damage repair blocker (Figure 3E, left panel). In PC3 cells, miR-130a forced expression significantly associated with increased *CASP3* and decreased *KI67* and *PARP1* expression*,* suggesting that miR-130a induction reprograms cell cycle and induces apoptosis (Figure 3E, right panel; Figure 3F). In DU145 results were less impressive (Figure 3E, central panel), implying that miR-130a-mediated tumor-suppressive activity might be cell-specific.

3.8. DEPDC1 and SEC23B are repressed by miR-130a

To gain insight into the cellular mechanism through which miR-130a exerts its effect, we performed genome-wide expression profile of PC3 cells overexpressing miR-130a (Figure 4A, Supplementary Table S6). Overall, 1,534 genes were found to be differentially expressed from which 667 genes were down-regulated and 867 were up-regulated in the transfected cells. The genes were ranked according to differential expression and GSEA was used to search for enrichment across the Molecular Signatures Database (MSigDB) (Supplementary Table S7). Among the highest ranked concepts in the upregulated genes, we noticed genes associated with p53 pathway, unfolded protein response, TNFa signaling via NF-KB, apoptosis, hypoxia, and EMT (Supplementary Table S7). For down-regulated genes after miR-130a transfection, the highest ranked concepts, we found genes correlated with G2/M checkpoint, E2F targets, mitotic spindle, and DNA repair (Supplementary Table S7).
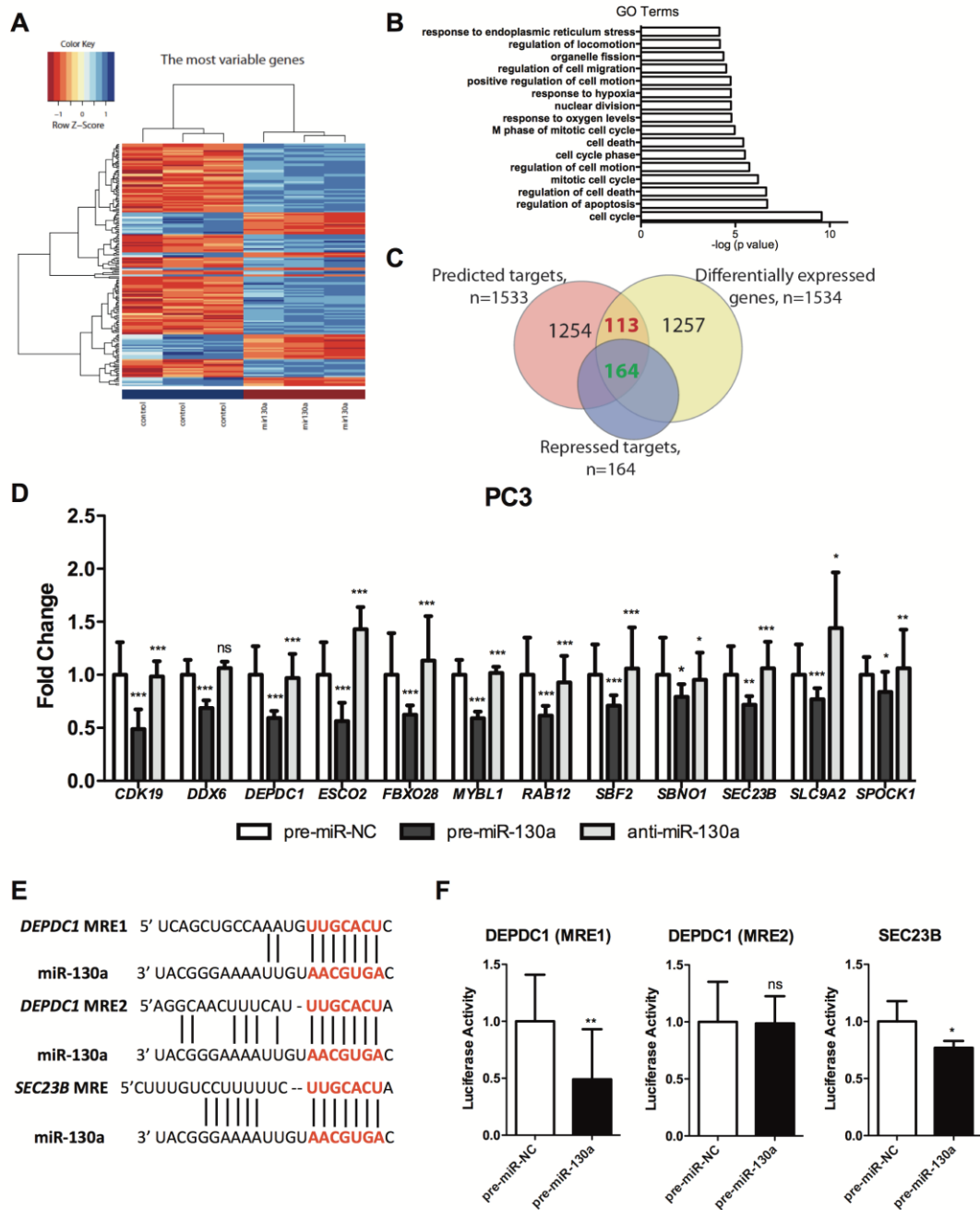
Figure 4. Identification of miR-130a putative target genes in PC3 cells.

(A) A hierarchical cluster of PC3 cells after ectopic expression of miR-130a. (B) Canonical pathways most enriched in PC3 cells overexpressing miR-130a. (C) Isolation of miR-130a targets among the deregulated genes. (D) qRT-PCR validation of putative targets derived from the array after transfection with miR-130a or Anti-miR-130a in PC3 cells. (E) Predicted miR-130a MREs in each 3'UTR (seed sequence is highlighted in red). (F) Luciferase reporter activity for miR-130a interaction of with DEPDC1 and SEC23B 3'UTR. (Significance level represented as: * = p<0.05; ** = p<0.01; *** = p<0.001)

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Moreover, gene ontology (GO) analysis disclosed that the top cellular processes included cell cycle, regulation of apoptosis, mitosis, regulation of cell motion and migration (Figure 4B, Supplementary Table S7). Then, we focused on the most variable genes to identify miR-130a targets, using the following criteria: a) containing at least one predicted miRNA response element (MRE); b) being downregulated upon transfection; c) having a known function; and d) mRNAs significantly upregulated in PCa samples. Thus, 164 genes were selected for further validation (Figure 4C, 5A-B, Supplementary figure 4A-B, Supplementary Table S8). GO processes for repressed miR-130a targets included multiple processes such as vesicle-mediated transport, protein kinase cascade, angiogenesis, and cell cycle (Supplementary table S9). We validated several candidates, involved in different cellular pathways (Figure 4D). After this initial screening, *DEPDC1* and *SEC23B* matched all criteria. Upon miR-130a transfection in PC3 cells, both microarray and qRT-PCR data revealed a significant decrease of *DEPDC1* and *SEC23B* mRNA levels (Figure 4D). Moreover, using qPCR, *DEPDC1* and *SEC23B* mRNAs were found upregulated in PCa compared to MNPT (Figure 5A and Supplementary Table S8).

These data were independently validated in the TCGA dataset, in which both *DEPDC1* and *SEC23B* were found significantly upregulated in PCa and displayed negative Spearman correlations with miR-130a expression (Figure 5C).

One putative miR-130a MRE was identified at the 3'UTR of *SEC23B*, whereas *DEPDC1* displayed two MRE (Figure 4E). Using a luciferase reporter system, a statistically significant inhibition of luciferase activity in the *SEC23B* and *DEPDC1* MRE1 constructs was observed in cells overexpressing miR-130a (Figure 4F), in accordance with our previous observations.

3.9. DEPDC1 and SEC23B are pro-tumorigenic in PCa cells

To investigate the contribution of individual miR-130a targets, we performed a loss of function assay, using RNAi to knock down *DEPDC1* and *SEC23B* in PC3 cells. PC3 cells transfected with *DEPDC1* or *SEC23B* siRNAs displayed a significant decrease in cell viability at 48h and 72h (Figure 5D; Supplementary Figure 3B), as well as a dramatic increase of apoptosis at 72h post-transfection (Figure 5E). These data suggest that the tumor suppressive role of miR-130a in PCa might be, at the least partially, mediated by *DEPDC1* and *SEC23B* downregulation.
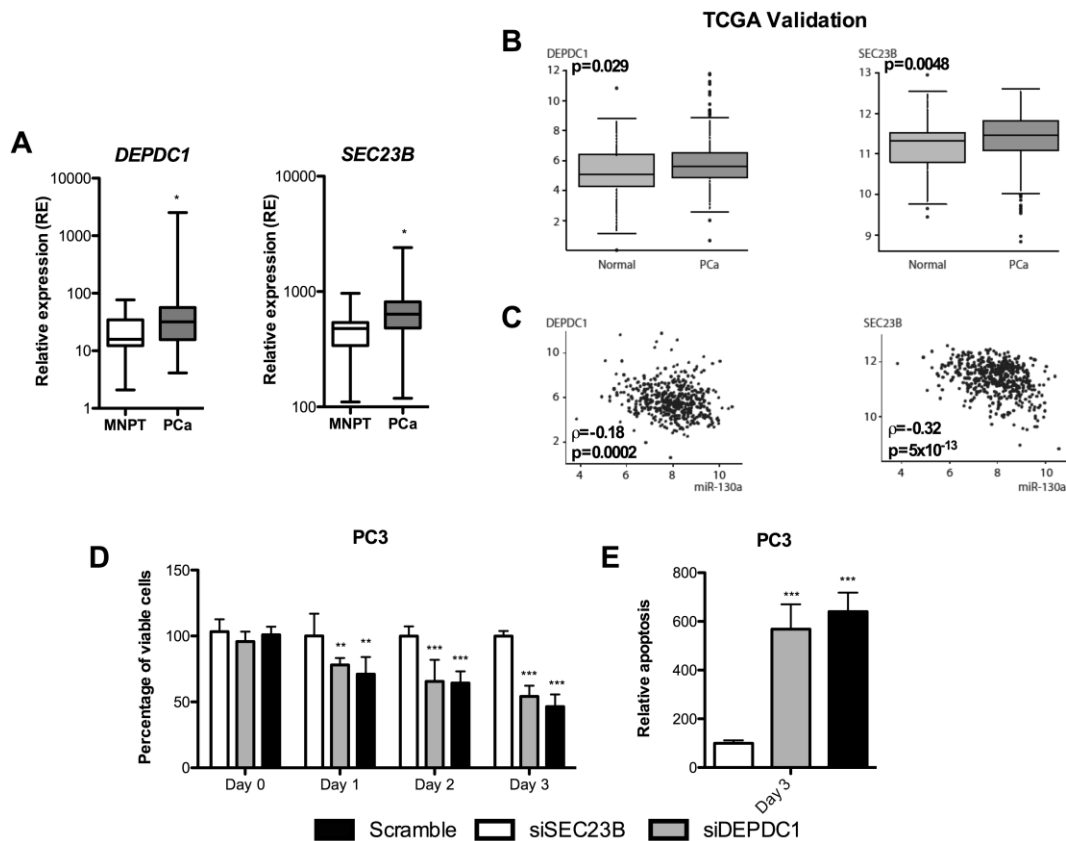
Figure 5. *DEPDC1* and *SEC23B* are physiologically important in Prostate Cancer.

*(A) DEDPC1 and SEC23B are upregulated in PCa compared to MNPT. (B) Expression of DEPDC1 and SEC23B in the TCGA cohort (RNA-Seq). (C) Correlation of miR-130a with DEPDC1 or SEC23B expression. SiRNA-mediated knock-down of DEPDC1 and SEC23B in PC3 cells decreases cell viability (D) and increases apoptosis (E). (Significance level represented as: * = p<0.05; ** = p<0.01; *** = p<0.001)*

3.10. miR-130a disrupts critical signaling pathways by overexpressing key genes

PCa cells exhibit phenotypic plasticity, allowing for adaptation to different tumour microenvironments. Much of this plasticity seems to be mediated by miRNAs that fine-tune signal transduction networks. In the microarray analysis of miR-130a overexpressing PC3 cells, several genes implicated in critical signalling pathways were found upregulated. Functional enrichment analysis revealed enrichment for critical kinases and transcription factors required for both cell cycle progression and checkpoint cascades, including activation of the DDR. These genes, including *CDKN1B*, *CDKN2B*, *PCNA*, *BIRC5*, *CCNA2*, *CCNB2*, *CCNB1*, *CDK1*, *CDK2*, *MAD2L1*, Polo-like kinase 1 (*PLK1*), topoisomerase (DNA) I (*TOP1*), and *UBE2C.* Notably, many of these proproliferative genes, including Survivin *(BIRC5)* or *PLK1* are targeted for pharmacological reduction in a diverse array of cancers (14). These

observations suggest that miR-130a may be critical for regulation of an array of proproliferative cell cycle and apoptosis genes that have been implicated in cancer (14).
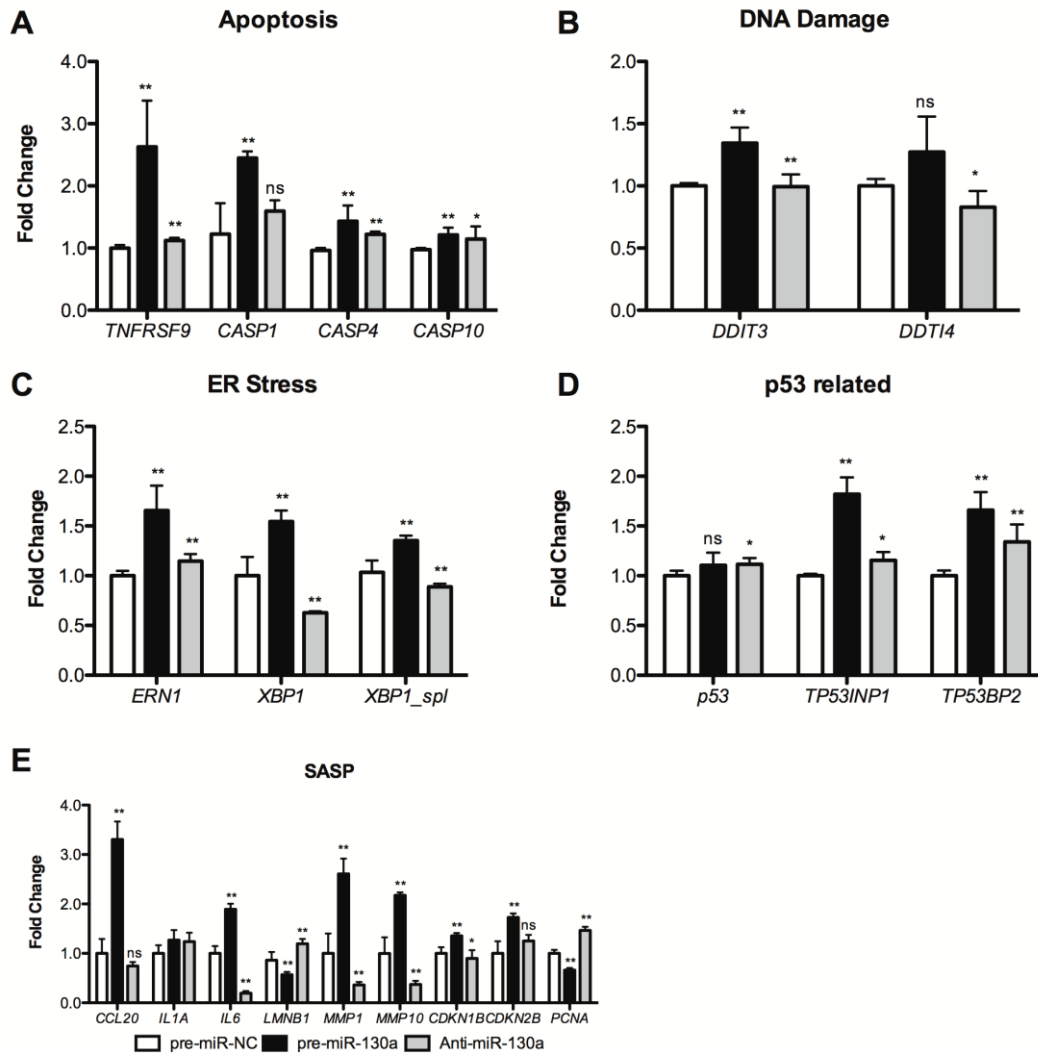
Figure 6. miR-130a expression induces associates with expression alterations of genes involved in critical cellular pathways.

*(A) Apoptosis-related. (B) TP53-related (C) DNA-Damage-Inducible Transcripts. (D) ER-stress pathway (Significance level represented as: * = p<0.05; ** = p<0.01; *** = p<0.001)*

Among the genes involved in the apoptosis cascade (Figure 6A) we detected an increase in *TNFRS9* mRNA levels, along with up-regulation of members of the receptor caspases network, *CASP1*, *CASP4* and *CASP10,* which in turn may trigger the activation of effector caspases (as previously demonstrated for CASP3). Moreover, *TP53BP2* and *TP53INP1* were also upregulated upon miR-130a transfection (Figure 6B). Both *TP53BP2* and

TP53INP1 are key regulators of apoptosis and cell growth, regulating cell cycle progression and inducing cell death by an autophagy and caspase-dependent mechanism.

In our dataset, we also found *DDIT3* and *DDIT4* upregulation upon forced expression of miR-130a, further associating miR-130a with the activation of transcripts known to be stimulated by DNA-damage (Figure 6C). As *DDIT3* induces apoptosis in response to endoplasmic reticulum (ER) stress, we investigated ER-stress pathway genes. QRT-PCR analysis of miR-130a-overexpressing PC3 cells demonstrated a statistically significant increase of *IRE1* (*ERN1*) and *XBP1spl* (a marker of ER stress (15)) transcript levels (Figure 6D). *ERN1* senses unfolded proteins in the ER lumen leading to enzyme self-activation, subsequently catalysing the splicing of X-box binding protein (XBP) 1 mRNA, converting it into a potent unfolded-protein response transcriptional activator known for triggering growth arrest and apoptosis.

Moreover, GSEA of significant upregulated in miR-130a transfected PC3 cells identified several categories associated with senescence-associated secretory phenotype (SASP), i.e "cytokine receptor interaction", "interferon alpha response", "p53 pathway". Examination of several top-ranked up-regulated SASP genes identified in this analysis demonstrated a broad and dramatic deregulation in the expression of canonical SASP genes in PC3 cells transfected with miR-130a mimics, and these results were confirmed by RT-qPCR (Figure 6E). Indeed, *CCL20*, *IL6*, *MMP1* and *MMP10* expression levels were significantly increased, whereas the opposite was observed after miR-130a endogenous inhibition. Conversely, *LMNB1* was downregulated in miR-130a overexpressing cells, but its expression was rescued by miR-130a blockade. We also confirmed the overexpression of the cell cycle inhibitors *CDKN1B* and *CDKN2B,* whereas a decreased expression of *PCNA,* a DNA replication controller, has been observed. These data implicate miR-130a in growth arrest phenotype, compatible with cellular senescence.

4. Discussion

Global miR's downregulation is a common feature of human cancer, although both oncogenic and tumor-suppressive functions have been ascribed to miRNAs. Thus, in this study, we aimed to identify and characterize the role of miR's putatively downregulated due to aberrant promoter methylation that might be implicated in prostate carcinogenesis.

Globally, the results of the miRNAs expression profiling, are in accordance with the widely accepted view that miRNAs are mostly downregulated in cancer cells (16), which has been associated with altered miRNA biogenesis (17). In our dataset, we found evidence that miRNA nuclear processing was compromised, through decreased expression of both *DGCR8* and *DROSHA,* impacting on pre-miRNA abundance and downstream processing

(18). Remarkably, *DICER* transcript levels were also decreased in PCa, which may cause low processing efficiency that coupled with decreased abundance of pre-miRNA to process might contribute to global miRNA downregulation.

Through comprehensive expression profiling analysis of miRNAs downregulated in PCa and simultaneously re-expressed after 5-Aza-CdR exposure in PCa cell lines, we were able to identify several miR's putatively regulated by promoter methylation in PCa, of which miR-130a had not been previously identified (19-21). Indeed, miR-130a methylation levels were significantly increased in PCa compared to non-malignant prostate tissues, and an inverse pattern was found for the respective expression levels, as demonstrated for other epigenetically-regulated miRs (22-24). These results are in accordance with a previous study which demonstrated that combined overexpression of miR-130a, miR-203 and miR-205 repressed oncogenic pathways in PCa (20). That study, however, was limited to the analysis of LNCaP cells and neither the molecular basis for miR-130a specific regulation nor other specific roles for miR-130a were further investigated (20), contrarily to our study.

Because quantitative promoter methylation of specific genes has been shown to accurately discriminate cancerous from non-cancerous prostate (25), we found that miR-130a methylation levels allowed for accurate identification of PCa, both in tissues and biofluids, comparing well with other methylation-based biomarkers (25). Eventually, increasing the number of urine samples of performing prostatic massage previous to collection (26, 27) may improve the biomarker performance of miR-130a methylation.

In addition to promoter methylation, post-translational histone modifications might also be involved in maintaining transcriptional repression (22). Interestingly, we found that miR-130a promoter was devoid of activating histone marks such as H3K27ac or H3K4me3, but was H3K27me3 enriched, a repressive mark catalysed by polycomb group protein EZH2, known to be up-regulated in PCa (28).

Concerning miR-130a's implication in prostate carcinogenesis, *in vitro* data indicates an inhibitory effect in cell proliferation and invasion, and promotion of apoptosis. However, earlier studies showed the involvement of miR-130a in either repression or stimulation of cell viability, apoptosis, migration, invasion and response to therapy in other cancer models, depending on the target genes (29-31). Herein, by an integrative bioinformatics and *in vitro* approach, we identified two putative targets, *DEPDC1* (a transcriptional co-repressor involved in the suppression of apoptosis) and *SEC23B* (a member of SEC23/SEC24 family involved in vesicle trafficking, particularly in ER to Golgi vesicle-mediated transport). *DEPDC1* has been found upregulated in bladder cancer (32) activating apoptosis and regulating vincristine-induced cell death by promoting JNK-dependent degradation of the BCL-2 family protein MCL1 (33) whereas *SEC23B* has been found to be deregulated in hepatocellular carcinoma (34).

Direct targeting of *SEC23B* by miR-130a suggested a link with ER-stress pathway. Indeed, disruption of SEC23B may impair vesicle trafficking from ER to Golgi, leading to the accumulation of unfolded or misfolded proteins inside the ER. This initiates a series of adaptive mechanisms known as the unfolded protein response (UPR), and if cell damage is sufficiently severe, it results in cell death (35). Interestingly, in GO analysis, we found that response to ER-stress was one of the processes in which miR-130a was involved. Indeed, other genes critical to ER-stress response would be activated, including *XBP1*, *ERN1* and *CASP4* (35). In line with those observations, we found that miR-130a induction causes a decrease in *SEC23B* expression and an increase in *ERN1* and *XBP1* expression, as well as that of *XBP1spl*, a shorter, more stable and active form *XBP1*. These data is also in agreement with a previous report that showed decreased expression of XBP1 in PCa, especially in castration-resistant tumors (36). Although current target prediction algorithms are based on full complementarity of the miRNA seed to 3'UTR of target genes, CLIP-Seq showed AGO binding occurs at sites without perfect seed matches (37). Therefore, non-canonical miRNA binding sites are prevalent, frequently contain seed-like motifs, and can regulate gene expression, generating a continuum of targeting and regulation, amplifying the effect of canonical targeting and phenotype observed upon *in vitro* modulation (37). Thus, we are tempted to speculate whether miR-130a non-canonical targets may cooperate in activation of different pathways, including ER-stress.

Remarkably, we found that *in vitro* overexpression of miR-130a promotes cellular senescence, a form of stable cell cycle exit with altered secretory pathway, typified by SASP. Senescent cells actively communicate with microenvironment through a plethora of secretory factors as part of SASP (38). Moreover, SASP activates immunity to eliminate senescent cells, thus removing potentially tumorigenic factors from the microenvironment (39). Regulation of SASP is achieved at many levels, from transcriptional regulation to autocrine feedback loops, but persistent DNA damage repair appears to be critical for its regulation (40). When irreversible cell-cycle arrest is activated by severe or irreparable DNA damage (i.e., dysfunctional telomeres or oncogenic stress), the SASP arises in senescent cells (41). We observed increase expression of *DDIT3* (and other genes related to DNA Damage response), which is activated by cellular stress conditions, inducing G1 arrest and apoptosis (42). *DDIT3* also functions as a dominant-negative inhibitor of gene transcription (43) and prevents transcription in response to DNA damage (42, 43), suggesting that miR-130a might impair DNA damage response-related genes and thus promoting SASP. We also verified a decrease in *LMNB1* upon miR-130a transfection. Lamin B1 is specifically downregulated during senescence in multiple cell types (38), and its depletion is associated with reduced cell proliferation (44), further supporting our observations on cell viability.

In summary, we demonstrated that miR-130a is significantly downregulated in PCa. Moreover, we unveiled that in addition to microRNA biogenesis disruption, miR-130 expression might be regulated through the cooperation of aberrant promoter methylation and H3K27me3 deposition. We further provided a mechanistic insight into miR-130a's role in prostate carcinogenesis, which is probably mediated through targeting of genes such as *SEC23B* and *DEPDC1* involved in cell senescence, cell cycle regulation, apoptosis and invasion. Finally, a link between miR-130a and ER-stress response was implied, through *SEC23B* targeting.

Conflict of interest: The authors declare no conflict of interest related to this work.

Appendix: Supplementary material

Author Contributions

Conceived and designed the experiments: JR-C, RAL, RH, CJ. Performed the Experiments: JR-C, JBM, LC, AS, JT-F, IG, PC-P, IAE. Analyzed the data: JR-C, LC, AS, RAL, RH, CJ. Contributed material/analysis: LA, JO.
All authors approved the final version of the manuscript to be submitted.

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CHAPTER 5

RESEARCH ARTICLE

Downregulation of miR-130b~301b cluster is mediated by aberrant promoter methylation and impairs cellular senescence in prostate cancer

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Downregulation of miR-130b~301b cluster is mediated by aberrant promoter methylation and impairs cellular senescence in prostate cancer

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Abstract

Background: Numerous DNA-damaging cellular stresses, including oncogene activation and DNA-damage response (DDR), may lead to cellular senescence. Previous observations linked microRNA deregulation with altered senescent patterns, prompting us to investigate whether epigenetic repression of microRNAs expression might disrupt senescence in prostate cancer (PCa) cells.

Methods: Differential methylation mapping in prostate tissues was carried using Infinium HumanMethylation450 BeadChip. After validation of methylation and expression analyses in a larger series of prostate tissues, functional role of the cluster miR-130b~301b was explored using *in vitro* studies testing cell viability, apoptosis, invasion and DNA damage in prostate cancer cell lines. Western blot and RT-qPCR were performed to support those observations.

Results: We found that the miR-130b~301b cluster directs epigenetic activation of cell cycle inhibitors required for DDR activation, thus stimulating the senescence-associated secretory phenotype (SASP). Furthermore, overexpression of miR-130b~301b cluster markedly reduced the malignant phenotype of PCa cells.

Conclusions: Altogether, these data demonstrate that miR-130b~301b cluster overexpression might effectively induce PCa cell growth arrest through epigenetic regulation of proliferation-blocking genes and activation of cellular senescence.

Keywords: miR-130b, miR-301b, microRNA, senescence, senescence-associated secretory phenotype, Prostate Cancer.

Background

MicroRNAs (miRNAs) are small, non-coding RNAs that act as sequence-specific guides for Argonaute (AGO) proteins, which mediate posttranscriptional silencing of target mRNA (1). MiRNAs are transcribed from individual genes containing their own promoters or are originated intragenically from spliced segments of other genes (2). They contain upstream regulatory elements and promoter regions, indicating that miRNAs might endure CpG promoter methylation via DNA methyltransferase (DMNT), histone modifications, as well as other regulatory alterations (1, 3). Importantly, whereas miRNA genes transcription-start sites (TSS) are occasionally 5–10kb away from the pre-miRNA sequence (4), promoter regions may be up to 50kb apart, which may preclude the elucidation of transcriptional regulation of particular miRNAs (1). Functional miRNAs result from sequential processing of pri-miRNAs by RNase III family enzymes DROSHA (nucleus) and DICER (cytoplasm). Unlike their protein-coding counterparts, however, miRNAs function as guides for identifying target mRNAs for repression (5).

MiRNAs are involved in development, homeostasis, cell cycle, apoptosis, as well as in diverse pathological condition in nearly all vertebrate tissues (6). Importantly, aberrant miRNA expression levels have been associated with promotion or arrest of tumorigenesis, through its ability to control the expression of a myriad of protein-coding and non-coding genes (7). Concordantly, deregulation of miRNA expression has been reported in several malignancies, including prostate cancer (PCa) (3). PCa is currently the most common non-cutaneous malignancy in developed countries and the second leading cause of death from cancer in men in the USA and in Europe, accounting for one in nine of all newly diagnosed cancers in men (8). Nonetheless, altered miRNA expression patterns in PCa have been significantly understudied compared to other cancers, despite evidence suggesting a global downregulation of miRNA expression in both tumorigenesis and treatment resistance (9, 10). Here, we examined how epigenetic alterations might contribute to miRNAs deregulation in PCa, focusing on the role of miR-130b~301b cluster. We found that miR-130b~301b cluster displays tumour-suppressive functions *in vitro*, influencing cell cycle, cell viability, apoptosis and invasion. Interestingly, an unprecedented effect of miR-130b~301b cluster on cellular senescence, which prevents cancer cell proliferation, was disclosed, suggesting that impairment of cellular senescence might underlie the deleterious effects of miR-130b~301b cluster downregulation in prostate carcinogenesis.

Methods

Patients and sample collection

Primary tumour tissues from 111 patients harbouring clinically localized PCa were prospectively collected, after diagnosis and primary treatment with radical prostatectomy at Portuguese Oncology Institute of Porto, Porto, Portugal (Supplementary Table S1). A set of 14 morphologically normal prostate tissues (MNPT) was procured from prostatic peripheral zone of bladder cancer patients submitted to cystoprostatectomy and which did not harbour concomitant PCa. All tissue specimens were promptly frozen after surgery. Upon histological confirmation of tumour or normal prostate tissue, fresh-frozen tissue fragments were trimmed to enhance yield of target cells (>70%). Histological slides from formalin-fixed paraffin-embedded tissue fragments were also routinely obtained from the surgical specimens and assessed for Gleason score and TNM stage. Relevant clinical data was collected from clinical charts and informed consent was obtained from all participants, according to institutional regulations. This study was approved by the institutional review board (Comissão de Ética para a Saúde) of Portuguese Oncology Institute of Porto, Portugal (CES-IPOPFG-EPE 205/2013).

Nuclei Acid Extractions, bisulfite conversion and cDNA synthesis

DNA from fresh frozen tissue samples and cell lines was extracted using phenol:chloroform (Sigma). RNA was obtained using TRIzol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.

Bisulfite conversion of 1000ng of genomic DNA was accomplished using EZ DNA Methylation Kit (Zymo Research), following manufacturer's instructions.

Specific-miRNA cDNA was obtained using TaqMan MicroRNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA, USA). Total cDNA synthesis was performed using high-capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Infinium HumanMethylation450 BeadChip

All DNA samples were assessed for integrity, quantity and purity by electrophoresis in a 1.3% agarose gel, picogreen quantification, and nanodrop measurements. All samples were randomly distributed into 96-well plates. Bisulfite-converted DNA (200ng) were used for hybridization on the HumanMethylation450 BeadChip (Illumina), comprising 25 PCa and 5 MNPT.

HumanMethylation450 BeadChip data were processed using Bioconductor minfi package (11). The "llumina" procedure, which mimics the method of GenomeStudio (Illumina), was performed comprising background correction and normalization taking the first array of the plate as reference. Probes with one or more single nucleotide polymorphisms (SNPs) with a minor allele frequency (MAF) >1 % (1000 Genomes) in the first 10 bp of the interrogated CpG were removed. The methylation level (β) for each of the 485,577 CpG sites was calculated as the ratio of methylated signal divided by the sum of methylated and unmethylated signals, multiplied by 100. After normalization step, probes related to X and Y chromosomes were removed. All analyses were performed in human genome version 19 (hg19) and data was deposited in GEO repository under accession number GSE52955.

Pyrosequencing

Specific sets of primers for PCR amplification and sequencing were designed using a specific software pack (PyroMark assay design version 2.0.01.15). Primer sequences were designed to hybridize, whenever possible, with CpG-free sites, ensuring methylation-independent amplification. PCR was performed under standard conditions with biotinylated primers, and the PyroMark Vacuum Prep Tool (Biotage, Uppsala, Sweden) was used to prepare single-stranded PCR products according to manufacturer's instructions. Pyrosequencing reactions and methylation quantification were performed in a PyroMark Q96 System version 2.0.6 (Qiagen) using appropriate reagents and recommended protocols.

Real Time Quantitative PCR (RT-qPCR)

MiRNA transcript levels were assessed using TaqMan MicroRNA Assays specific for each miRNA (miR-130b, assay ID: 000456; miR-301b, assay ID: 002392) and normalized with RNU48 (assay ID: 001006; Applied Biosystems).

RT-qPCR analysis was performed using gene-specific primers (supplementary table S2) and normalized to the expression of *GUSB* housekeeping gene.

PCa cell lines

LNCaP cells were grown in RPMI 1640, DU145 cells were maintained in MEM and PC3 cells were grown in 50% RPMI-50% F-12 medium (GIBCO, Invitrogen, Carlsbad, CA, USA). All basal culture media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (GIBCO, Invitrogen, Carlsbad, CA, USA). Cells were maintained in an incubator at 37ºC with 5% CO_2. All PCa cell lines were routinely tested for Mycoplasma spp. contamination (PCR Mycoplasma Detection Set, Clontech Laboratories).

To reverse DNA methylation effect in the cell lines, we used 1µM of the DNA methyltransferases inhibitor 5-aza-2-deoxycytidine (5-Aza-CdR; Sigma-Aldrich, Schnelldorf, Germany) alone or in combination 0.5µM histone deacetylase inhibitor trichostatin A (TSA; Sigma-Aldrich, Schnelldorf, Germany). After 72h, cells were harvested and RNA extracted.

Pre-miRNA and anti-miRNA Transfections

To inhibit miR-130b and miR-301b, single stranded nucleic acids designed to specifically bind and inhibit endogenous miRNA (miR-130b Inhibitor, product ID: AM10777; miR-301b Inhibitor, product ID: AM12929, Ambion) were used. Anti-miR-130b and Anti-miR-301b were transfected as follows: in LNCaP, 25nM and 50nM, respectively; DU145, each at 50nM; PC3, 50nM and 70nM, respectively.

MiR-130b and miR-301b overexpression were accomplished through commercially available synthetic precursor miRNAs (pre-miR-130b, product ID: PM10777; pre-miR-301b, product ID: PM12929, Ambion), each transfected at 20nM. Transfections were performed using Oligofectamine (Invitrogen), per manufacturer instructions.

Viability assay

Cell viability was evaluated by MTT assay. Briefly, PCa cells were seeded onto 96-well flat bottoned culture plates, allowed to adhere overnight and transfected 24h later (number of cells plated before transfection: LNCaP: 10000 cells/well; DU145: 4000 cells/well; PC3: 3000 cells/well in 96 well plates). At each time point 0.5 mg/ml of MTT reagent [3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] was added to each well, and the

plates were incubated in the dark for 1 hour at 37ºC. Formazan crystals were then dissolved in DMSO and absorbance was read at 540nm in a microplate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany), subtracting the background, at 630nm. Three replicates for each condition were performed, and at least three independent experiments were carried out. Measurements were performed 24h, 48h, and 72h post miRNA manipulation.

Apoptosis evaluation

Evaluation of apoptosis was performed using APOPercentage apoptosis assay kit (Biocolor Ltd., Belfast, Northern Ireland) according to the manufacturer's instructions. PCa cells were seeded onto 24-well plates (LNCaP: 50000 cells/well, DU145 and PC3: 30000 cells/well), and 24h later were transfected. Apoptotic cells were assessed at the end of day 3 (72h after transfection), in a FLUOstar Omega microplate reader at 550nm and the background subtracted at 620nm. The results were normalized to number of viable cell determined in MTT assay according to the following formula: OD of apoptosis assay at 72h/ OD of MTT at 72h.

Cell cycle analysis

Cell cycle distribution of PC3 cells was determined by flow cytometry. Briefly, 72h after transfection (150000 cells/well at Day 0, in 6-well plates), 5×10^5 harvested cells were fixed overnight at 4ºC with 70% cold ethanol. After washing with cold PBS, cells were re-suspended in Propidium Iodide Solution (Cytognos S.L, Salamanca, Spain) and incubated for 30 minutes at room temperature. All cells were then measured on a Cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) and analysed using Modfit LT (Verity Software House, Inc, Topshan, Maine, USA).

<u>*Single Cell Gel Electrophoresis (Comet Assay)*</u>

Seventy-two hours after transfection (150000 cells/well at Day 0, in 6-well plates), 50.000 cells were harvested by trypsinization, washed in PBS and re-suspended in 75µl of low-melting point agarose (Invitrogen, Carlsbad, CA, USA). This suspension was then applied on top of the base layer consisting of normal-melting point agarose in a slide, after which it polymerized for 10 minutes at 4ºC. The slides were then immersed in lysis solution (2.5M NaCl, 100mM Na_2EDTA, 10mM Tris Base and 1% Triton X-100) at 4°C during 2 hours in the dark. To allow DNA to unwind, slides were posteriorly incubated in an alkaline electrophoresis buffer (300mM NaOH, 1mM Na2EDTA, pH=13) for 40 minutes at 4ºC. Electrophoresis was accomplished on a horizontal electrophoresis platform at 4ºC for 20 minutes at 15V. Subsequently, they were incubated in a neutralization buffer (Tris–HCl; pH=7.5) for 10 minutes. After fixation with 100% ethanol, slides were stained with Sybr

Green® (Life Technologies, Foster City, CA, USA) and DNA damage was evaluated under a fluorescent microscope. At least three independent experiments were performed for each condition. The DNA damaging effect in terms of DNA fragmentation was determined by measuring four parameters, that included tail moment, tail length, percentage of DNA in tail of the comet, and 50 DNA-damaged cells were counted at least, for each condition.

Cell Invasion Assay

Cell invasion was determined using BD BioCoat Matrigel Invasion Chamber (BD Biosciences, Franklin Lakes, NJ, USA). Both cell lines were transfected with miRNA molecules for 72 hours. Then, $5x10^4$ cells/mL of PC3 cells were added to the upper chamber. After 44h (LNCaP) or 20h (PC3), the membrane bottom containing invading cells was fixed in methanol, washed in PBS and stained with DAPI (Vector Laboratories, Burlingame, CA). All invading cells were counted under a fluorescence microscope. Three independent experiments were performed for each condition.

Transcriptomic evaluation of altered genes following cluster miR-130b~301b manipulation

Cells (LNCaP: 400000 cells/well, DU145: 200000 cells/well, and PC3: 150000 cells/well) were plated in 6-well, in the day before transfection. Cells were collected 72h post transfection and RNA was extracted and used as template for cDNA synthesis. RT-qPCR was performed as previously described.

Western blot

150000 cells per well were plated before transfection. 72h post transfections, cell lysates were separated on 4–20% Mini-PROTEAN TGXPrecast Gel at 120V and transferred onto PVDF membrane using semi-dry transfer. The membrane was incubated for 1h in blocking buffer (5% non-fat dry milk) and incubated 2h, at room temperature, with primary antibodies (Supplementary table S3). Blots were developed using Immun-Star WesternC Chemiluminescent kit (Bio-Rad, Hercules, CA, USA).

Morphometric analysis

Cell morphology was examined 72h after transfection using a digital camera connected with Olympus phase-contrast microscope. The cell area and sphericity were determined with the Olympus cellSens Dimension software (Olympus Corporation, Shinjuku, Japan) using the freehand polygon tool.

TCGA data in prostate cancer patients

Data on mRNA expression and clinical information (when available) from PCa and matched normal patient samples, deposited in The Cancer Genome Atlas (TCGA) was retrieved. mRNA expression data from samples hybridized at University of North Carolina, Lineberger Comprehensive Cancer Center, using Illumina HiSeq 2000 mRNA Sequencing version 2, were downloaded from TCGA data matrix (http://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp), including 497 PCa and 52 matched normal (12). To prevent duplicates, when there was more than one portion per patient, median values were used. The provided value was pre-processed and normalized according to "level 3" specifications of TCGA (see https://gdc-portal.nci.nih.gov/ for details). Clinical data of each patient was provided by Biospecimen Core Resources (BCRs). Data is available for download through TCGA data matrix (http://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm).

Statistical analysis

For group comparisons analysis, non-parametric tests (Kruskal-Wallis and Mann-Whitney U-test) were used. For *in vitro* assays, comparisons between two groups were performed using the Mann-Whitney U-test. Data are shown as mean ± s.d., unless otherwise specified. Student's t-tests were used for invasion assays. All statistical tests were two-sided. Statistical analysis was carried out using Graph Pad Prism version 5. Significance level was set at $p < 0.05$.

Results

Identification of a miRNAs subset targeted by DNA methylation in prostate cancer

We sought to identify specific differentially methylated miRNA loci between PCa and MNPT (Supplementary figure 1A). The DNA methylation analysis was conducted using the Infinium HumanMethylation450 BeadChip (450k array), a high-density DNA methylation array that interrogates ≈485 000 human CpGs. A total of 439 CpG sites located in miRNA gene promoter regions were found to be differentially methylated and were clustered separately (non-parametric Mann-Whitney and Wilcoxon matched pair test were applied). For all analyses, p-values inferior to 0.05, after FDR correction, were considered statistically significant (Figure 1A; Table 1; Supplementary Figure 1A). Thus, 51 differentially methylated miRNA-promoters in PCa were identified (Figure 1A, Supplementary Figure 1B) and mapped to 19 chromosomes. Chromosomes 19 (n=6), 11 and 7 (n=5) and 2 (n=4) were the most enriched genomic locations for differential methylation (Supplementary Figure 1C). Simultaneously, we identified several hypomethylated candidates, including miR-181c~181d and miR-449a~449b clusters. In the hypermethylated branch, our dataset disclosed

previously unreported miRNA-promoters, including miR-130b~301b cluster, miR-149, miR-212, miR-10a, miR-152, miR-210 and miR-129-2. Consistent with previous observations, we confirmed hypermethylation of miR-193b, miR-9 family, and miR-34b-34c cluster (Supplementary Figure 1B). Gene Ontology (GO, Supplementary table S4) revealed that putative targets of this subset of miRNAs dynamically regulated by DNA methylation are involved in critical pathways including "sister chromatid segregation", "regulation of double-strand break repair", "posttranscriptional gene silencing by RNA", "regulation of adaptive immune response", "G1 DNA damage checkpoint" or "DNA-templated transcription". Strikingly, GO analysis also disclosed that the putative targets of this miRNA panel were also involved in "hippo signalling" and "prostate gland growth", indicating a critical role in normal prostate biology. Based on β-values for DNA methylation levels, miR-130b~301b cluster ranked first (Table 1) and was selected for subsequent validation in a larger cohort.
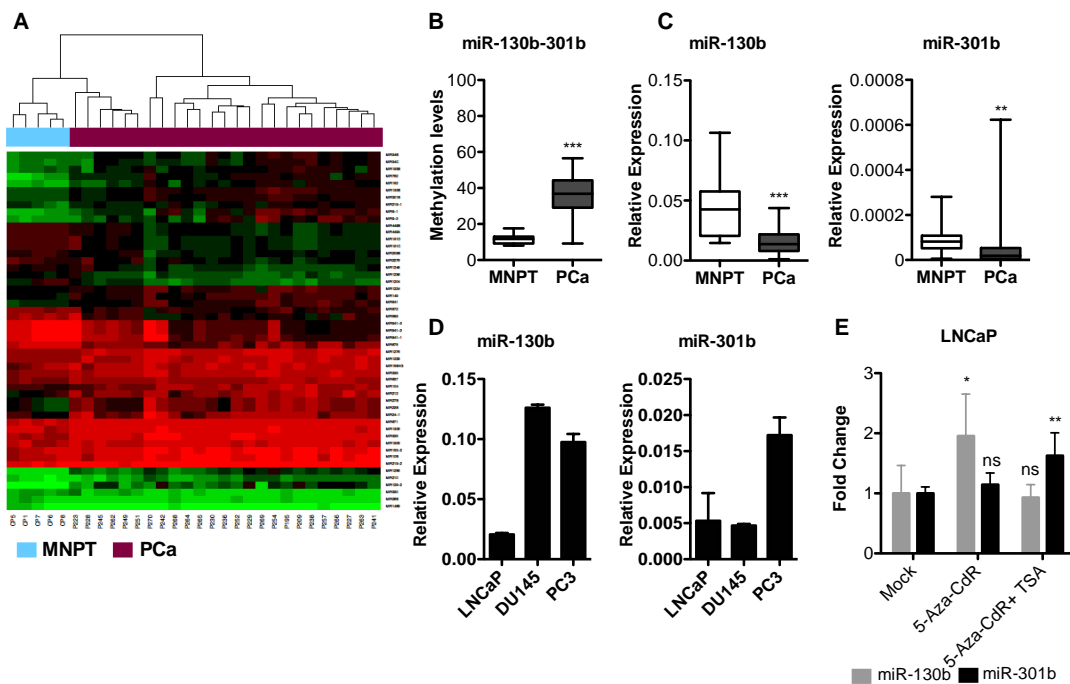
Figure 1. Differentially methylated microRNAs in prostate cancer.

(A) Unsupervised hierarchical clustering of microRNAs' promoters displaying significant alterations in DNA methylation as determined by Infinium HumanMethylation450 BeadChip in 25 prostate cancer (PCa) and 5 morphologically normal prostate tissue (MNPT) samples. Overall, 51 miRNA promoters were differentially methylated in PCa versus MNPT. (B) Validation of miR-130b~301b by pyrosequencing and (C) by RT-qPCR in 111 primary PCa and 14 MNPT cases, indicated that promoter hypermethylation was associated with miR-130b~301b downregulation. (D) LNCaP, DU145, and PC3 cell lines retain basal expression of miR-130b and miR-301b. (E) Reversal of DNA methylation in LNCaP cells using 5-aza-2-

deoxycytidine (5-AZA-CdR) increased the expression of miR-130b and, in combination, with TSA augmented miR-301b expression. Mann-Whitney U-test: *p<0.05, **p<0.01, ***p<0.001.

Validation of HumanMethylation450 BeadChip by pyrosequencing

Validation of miR-130b~301b cluster results was accomplished through pyrosequencing, which confirmed that promoter methylation levels were significantly higher in PCa compared to MNPT (Figure 1B). Likewise, PCa cell lines DU145, LNCaP and PC3 also demonstrated miR-130b~301b promoter methylation (Supplementary Figure 1D).

DNA methylation associates with miR-130b~301b cluster expression

MiR-130b~301b cluster expression levels were evaluated in a series of 125 prostate tissue samples, using RT-qPCR, and were found to be significantly downregulated in PCa ($p<0.0001$ for miR-130b; $p=0.0014$ for miR-301b, Figure 1C) compared to MNPT. Then, the effect of demethylating drugs was tested, as the PCa cell lines still displayed endogenous expression of miR-130b and miR-301b (Figure 1D). In LNCaP cells, miR-130b was significantly upregulated after exposure to 5-Aza-CdR, whereas miR-301b was only re-expressed upon combined treatment with 5-Aza-CdR and TSA (Figure 1E).

Functional impact of miR-130b~301b cluster expression manipulation in vitro

The phenotypic impact of altered miR-130b~301b cluster expression was assessed in PCa cell lines in which miR-130b~301b cluster expression was detected along with promoter methylation: LNCaP, DU145 and PC3 (Supplementary Figure 1D). The impact of endogenous miR-130b~301b blockage was firstly assessed and the efficiency of silencing was confirmed by RT-qPCR (Supplementary figure 2). In LNCaP cells, anti-miR-130b significantly enhanced growth rate at 72h (Figure 2A, $p<0.001$), whereas anti-miR-301b showed no significant effect. Conversely, at 72h post-transfection, apoptosis was only decreased in anti-miR-130b transfected LNCaP cells (Figure 2D, $p=0.0043$). Importantly, decreased *CASP3* expression levels (Figure 2F) were consistent with reduced apoptosis. Interestingly, in LNCaP cells, miR-301b knockdown significantly increased invasion capacity. However, for miR-130b silencing, no significant differences were apparent, suggesting that miR-130b is more likely implicated in invasion regulation than miR-301b. In DU145 cells, inhibition of either miRNA significantly increased cell viability (Figure 2B, $p<0.001$ for both). Interestingly, the effect of anti-miR-130b was already apparent at 48h upon transfection ($p<0.0001$). Although decreased apoptosis was depicted for both conditions, it only reached statistical significance in anti-miR-301b transfected cells (Figure 2D, $p=0.0022$). A slight increase in *Ki67* mRNA expression was found upon anti-miR-130b transfection (Figure 2G, $p=0.026$). Thus, in DU145 cells, miR-301b seems to be more critical than miR-130b,

although the latter might influence cell viability. MiR-130b or miR-301b inhibition in PC3 cells dramatically enhanced cell viability (Figure 2C, p<0.001). Moreover, increased proliferation was complemented with a significant decrease in apoptosis after anti-miR-130b or anti-miR-301b transfection (figure 2D). Remarkably, an apparent effect on cell invasion was observed for miR-130b~301b depleted PCa cells, reaching statistical significance in miR-301b-depleted LNCaP cells (Figure 2E)
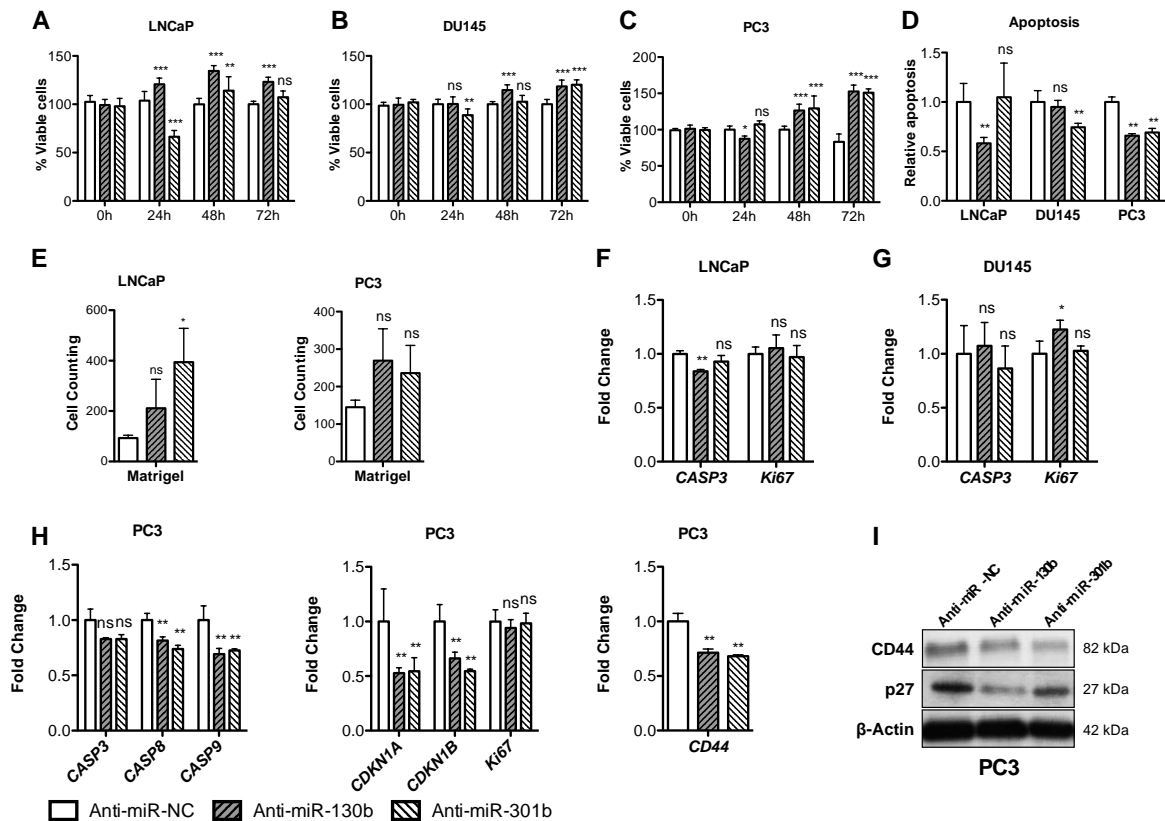
Figure 2. **Phenotypic effects induced by blocking endogenous levels of miR-130b or miR-301b in PCa cell lines.** *(A, B, C) Cell viability measured by MTT assay at different time points and (D) apoptosis evaluation 72h-post transfection for LNCaP, DU145 and PC3 cells, respectively, indicating functional specialization (LNCaP and DU145) or cooperation (PC3) among members of miR-130~301b cluster in PCa cell lines. (E) Invasion assay following anti-miR knockdown of miR-130b or miR-301b using Matrigel coated Boyden chamber assay in LNCaP and PC3 cells, 72h post-transfection. (F-G) Transcript levels of CASP3 and KI67 in LNCaP and DU145 cells, respectively, 72h after anti-miRNAs transfection. (H) mRNA expression of selected genes involved in cell cycle, apoptosis and invasion in PC3 cells transfected with anti-miRNAs, indicating that both miR-130b and miR-301b knockdown decreased the expression of Caspases (3, 8 and 9) and critical cell cycle check-point*

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regulators. (I) Representative Western blots for CD44 and p27. All data are presented as mean of three independent experiments ± s.d. (*p<0.05, **p<0.01, ***p<0.001).

Because phenotypic changes were more apparent in PC3 cells, these were selected for evaluation of expression of several genes involved in relevant signalling pathways. Thus, a significant decrease in *CASP8, CASP9, CDKN1A* and *CDKN1B* expression was depicted, whereas *CASP3* and *Ki67* mRNA levels remained unaltered (Figure 2H). Moreover a significant reduction in CD44 and p27 expression was also observed, in line with the invasive phenotype induced by anti-miR-130b and anti-miR-301b transfection in PC3 cells (Figures 2H-I).

MiR-130b~miR-301b overexpression attenuates the malignant phenotype and promotes MET

The phenotypic impact of miR-130b or miR-301b overexpression was tested in PC3 cells. A marked reduction in cell viability (Figure 3A) and increased apoptosis (Figure 3B), along with increased caspases expression, especially *CASP8* (Figure 3E), was observed. Cell cycle analysis by flow cytometry depicted a significant arrest at S phase following miR-130b or miR-301b overexpression, and at G2/M phase after miR-130b overexpression (Figure 3C). These phenotypic alterations were further confirmed by significant decrease in *Ki67* expression and increased *CDKN1A* (p21) and *CDKN1B* (p27) expression, both at mRNA and protein level (Figure 3E-F).
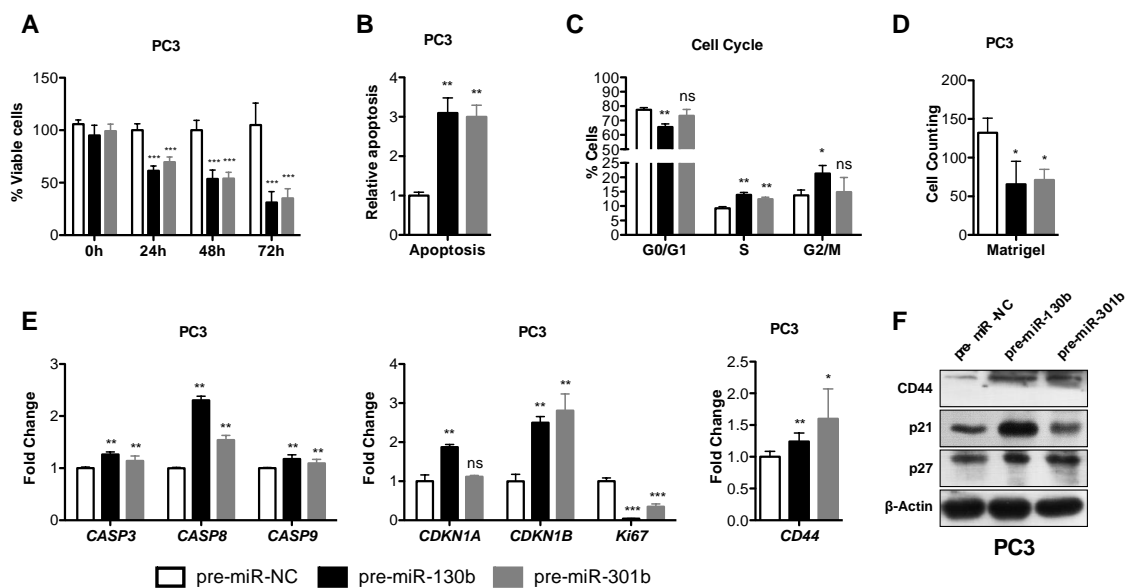
Figure 3. **miR-130b and miR-301b overexpression attenuate malignant phenotype of PC3 cells. (A)** *Cell viability measured by MTT assay indicates that transfection of pre-miR-*

130b and pre-miR-301b significantly decreased cell viability compared to pre-miR-NC transfected cells. (B) Either pre-miR-130b or pre-miR-301b significantly increased the relative apoptosis levels as determined by the phosphatidylserine-based assay. (C) Cell-cycle analysis of PC3 control cells (pre-miR-NC) and PC3 overexpressing pre-miR-130b or pre-miR-301b, indicate that both miR-130b and miR-301b significantly induce cell cycle arrest at S-phase and miR-130b also causes G2/M arrest. (D) Invasion assay in PC3 cells transfected with the pre-miRNAs 72h before plating in Matrigel-coated Boyden chambers. (E) mRNA expression levels of selected genes involved in apoptosis, cell cycle and invasion, support that miR-130b and miR-301b cooperatively reverse the acquisition of malignant features of PC3 cells. (F) Western blot for p21, p27 and CD44 in PC3 cells, depicting selected gene overexpression upon miR-130b or miR-301b overexpression. All data are presented as mean of three independent experiments ± s.d. (*p<0.05, **p<0.01, ***p<0.001).

In the TCGA dataset, these findings were confirmed at mRNA level for *CDKN1A* (p<0.01), but not for *CDKN1B*, (Supplementary Figure 3), whereas *Ki67* was strongly up-regulated in PCa samples (p<0.0001), as expected. Collectively, these observations indicate that decreased cell viability results from a combined effect of cell cycle arrest and increased apoptosis.

We then hypothesized that miR-130b~301b cluster might inhibit epithelial to mesenchymal transition (EMT) and/or facilitate mesenchymal to epithelial transition (MET) in PCa cells. PC3 cells possess a more mesenchymal-like gene expression profile (13) and phenotype. Moreover, the capacity of cancer cells to migrate and invade is an important requirement for metastasis formation, and both are EMT hallmarks. With this in mind, the effect of miR-130b~301b expression on PC3 cells migration was assessed. Restoration of miR-130b~301b impaired the invasive capacity of PC3 cells (Figure 3D), whereas the opposite was observed following miR-130b~301b depletion (Figure 2E). Moreover, miR-130b~301b overexpression was associated with increased CD44 expression, both at mRNA and protein level (Figure 3E-F), whereas inhibition of miR-130b or miR-301b decreased CD44 expression (Figure 2H-I). The expression of other genes implicated in EMT was also assessed (Supplementary Figure 4) and a differential impact of miR-130b and miR-301b was suggested.

Moreover, miR-130b or miR-301b overexpression caused a shift in PC3 cell morphology towards a more epithelial-like phenotype, compared to wild type PC3 cells or those with miR-130b or miR-301b depletion, which are more spindled (i.e., more mesenchymal-like, Supplementary Figures 4 and 5). These findings suggest that miR-130b and miR-301b facilitate MET, impairing cell migration and invasion.

Cluster miR-130b~301b induces senescence in PC3 cells

Cellular senescence is a process by which proliferation-competent cells undergo growth arrest, in response to various cellular stresses. Because miR-130b and miR-301b were able to induce cell cycle arrest and decreased cell viability, along with *CDKN1A* and *CDKN1B* overexpression and Ki67 downregulation, a link with cellular senescence was suggested.
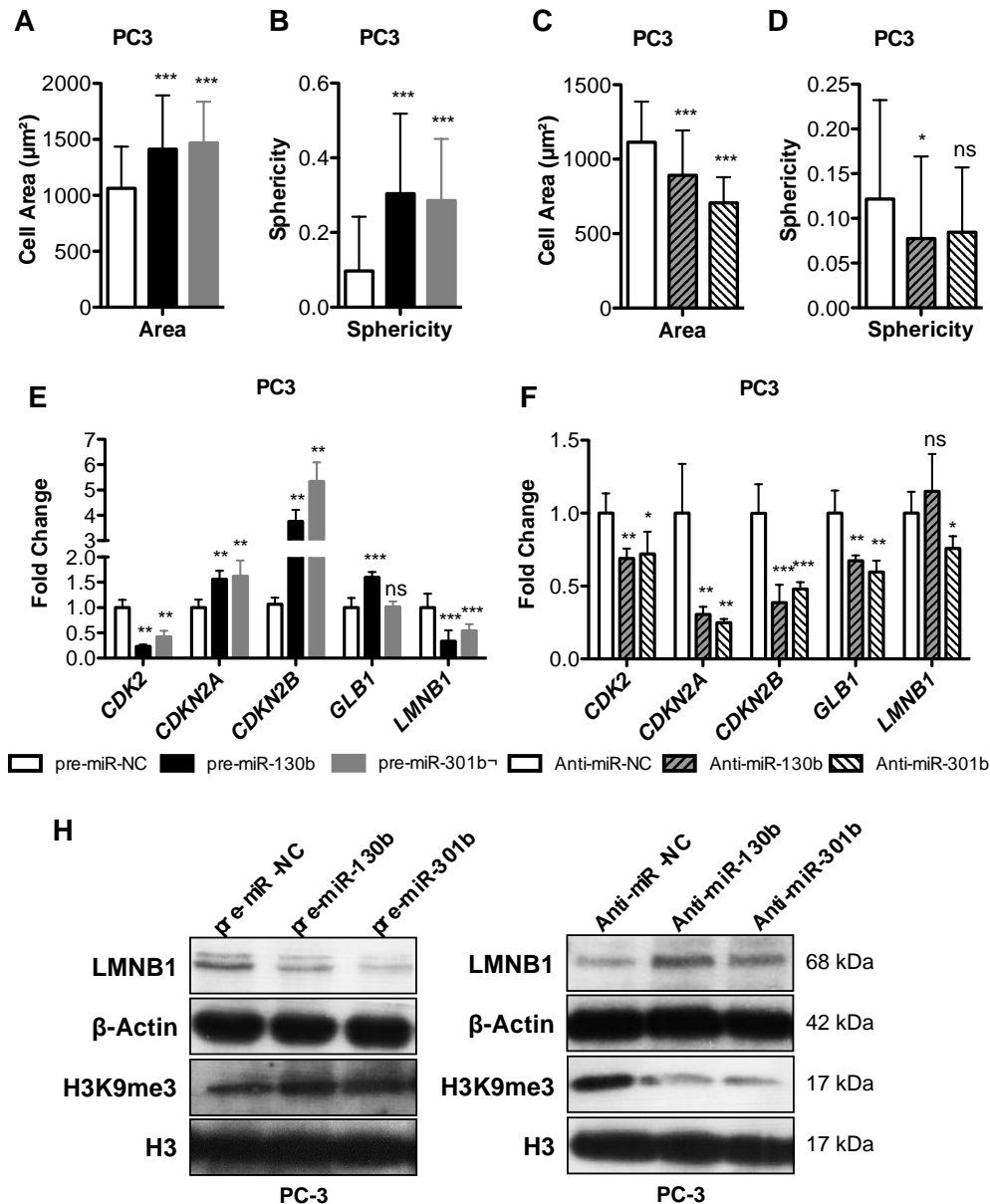
Figure 4. **Modulation of miR-130b and miR-301b induces cell senescence.**

Cell area (A) and sphericity (B), were increased upon miR-130b or miR-301b overexpression and compared to the pre-miR-NC transfected PC3 cells; Cell area (C) and sphericity (D) decreased with anti-microRNAs knockdown of miR-130b or miR-301b, indicating a more fibroblast-like phenotype. (E) RT-qPCR confirms transcriptional signature associated with promotion of cellular senescence after forced expression of miR-130b or miR-301b. (F) Attenuation of senescent phenotype following inhibition of endogenous levels of each

miRNA. (H) Western blot shows that LMNB1 levels are downregulated when miR-130b or miR-301b are overexpressed, concomitantly with locus-specific H3K9me3 increase. All data are presented as mean of three independent experiments ± s.d. (*p<0.05, **p<0.01, ***p<0.001).

Because senescent cells undergo cell size increase, this characteristic was evaluated upon miR-130b or miR-301b re-expression in PC3 cells. Morphometric analysis (Supplementary Figure 5) disclosed a significant increase in cell area (approximately 50%), compared to scramble cells (Figure 4A, p<0.0001), with a significant increase in sphericity, as well (p<0.0001, Figure 4B). Conversely, a significant decrease in cell area was apparent when endogenous miR-130b or miR-301b were depleted (Figure 4C; Supplementary Figure 6), whereas a significant decrease in sphericity was depicted for miR-130b only (Figure 4D, Supplementary Figure 6). Then, expression of other senescence-associated genes was evaluated. Transfection of miR-130b or miR-301b was associated with significant upregulation of tumour suppressor genes *CDKN2A* (p16) and, more dramatically, *CDKN2B* (p15) (Figure 4E), alongside with downregulation of *LMNB1*, a marker of cellular senescence (Figure 4E), which was confirmed at protein level (Figure 4H). Nevertheless, increased *β-galactosidase* (*GLB1*) mRNA levels were only apparent upon miR-130b expression (Figure 4E). Globally, the opposite trend was observed after endogenous miR-130b or miR-301b depletion (Figures 4F-H), although a few exceptions were apparent, including *CDK2* downregulation, at transcript level.

Formation of senescence-associated heterochromatic foci (SAHF), specifically enriched for H3K9me3, has been implicated in cellular senescence. Interestingly, following pre-miR-130b transfection, an increase in H3K9me3 was depicted, whereas anti-miR-130b and anti-miR-301b transfections were associated with H3K9me3 decrease (Figure 4H).

In TCGA dataset (Supplementary Figure 3), overexpression of *LMNB1* (p=3.32x10^{-10}) and down-regulation of *CDKN2B* (p=0.000218) was depicted in PCa tissue samples, mimicking to some extent the pattern observed following endogenous miR-130b or miR-301b depletion. Nevertheless, whether *LMNB1* reduction is caused by senescence or is promoted by a direct interaction of miR-130b or miR-301b with *LMNB1*-3'UTR (Supplementary Figure 7), remains unanswered.

miR-130b~301b induces SASP expression

The secretome of senescent cells is complex, consisting of a range of cytokines, chemokines, and proteases, among others. To further confirm our previous findings, we sought to analyse some elements of the senescence-associated secretory phenotype

(SASP), as these constitute phenotypic and molecular markers of senescence (14). Thus, *MMP1*, *MMP10*, *CCL20*, *IL1A*, *IL1B*, *IL6*, and *IL8* expression was assessed. Globally, miR-130b or miR-301b overexpression associated with increased expression of all genes tested, whereas anti-miR-130b or anti-miR-301b transfection associated with decreased *MMP1*, *MMP10* and *CCL20* expression, alongside with *IL1A*, *IL1B* and *IL6* overexpression, although at a much smaller magnitude compared to miR-130b or miR-301b overexpression (Figures 5A-B).

In TCGA dataset, PCa tissue samples displayed significantly lower *IL1A*, *IL1B*, and *IL6* expression levels compared to normal prostate tissues (Supplementary Figure 3).
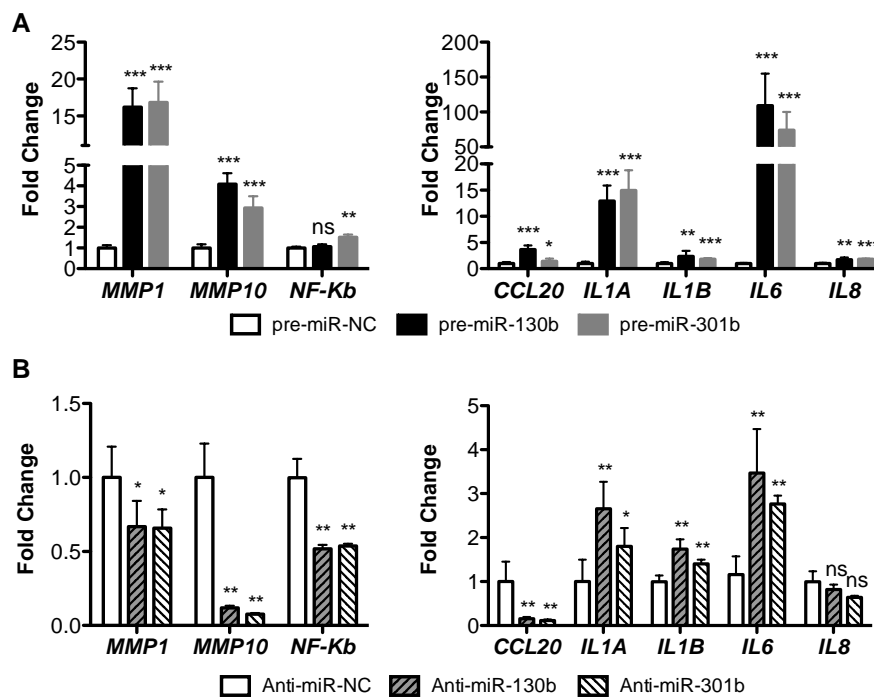
Figure 5. **miR-130b and miR-301b overexpression dramatically alters SASP expression in PC3 cells.** *(A) Comparing miR-130b or miR-301b overexpression with pre-miR-NC control, a global increase in mRNA levels of most SASP-related genes was depicted. This signature suggests that miR-130b or miR-301b-induced SASP reinforces senescence through autocrine mechanisms. (B) Comparison of miR-130b or miR-301b endogenous blockade with anti-miR-NC control, revealed a decrease (e.g. MMP10) or minimal increase (e.g. IL1A) in mRNA expression of some genes. This suggests paracrine activity of SASP when miR-130b or miR-301b are inhibited in prostate cancer cells. All data are presented as mean of three independent experiments ± s.d. (*p<0.05, **p<0.01, ***p<0.001).*

SASP activation is induced by DNA damage

In senescent cells, the control of secretome is achieved at many levels, from transcriptional regulation to autocrine feedback loops, but persistent DNA damage response (DDR) appears to be critical for regulation of SASP (15). We thus hypothesized that miR-130b and miR-301b might influence DNA damage responses and genomic instability during senescence. Upon pre-miR-130b or pre-miR-301b overexpression in PC3 cells, a significant increase in DNA damage was depicted, using the comet assay (Figure 6A-B), especially in tail moment (an index of induced DNA damage) and in the percentage of DNA in the tail. Subsequently, expression of genes involved in DDR was evaluated and a significant increase in two DNA damage inducible transcripts, *DDIT3* (that positively regulates IL6 and IL8) and *DDIT4* was found (Figure 6C). Moreover, *ATR*, a DNA-damage detector, was also upregulated. Strikingly, the growth arrest and DNA-damage-inducible proteins *GADD45A* and *GADD45B* were significantly overexpressed, as well as *RAD9A* and *RAD17* (Figure 6C). Conversely, *PCNA* (a cell proliferation marker) was among the downregulated genes.
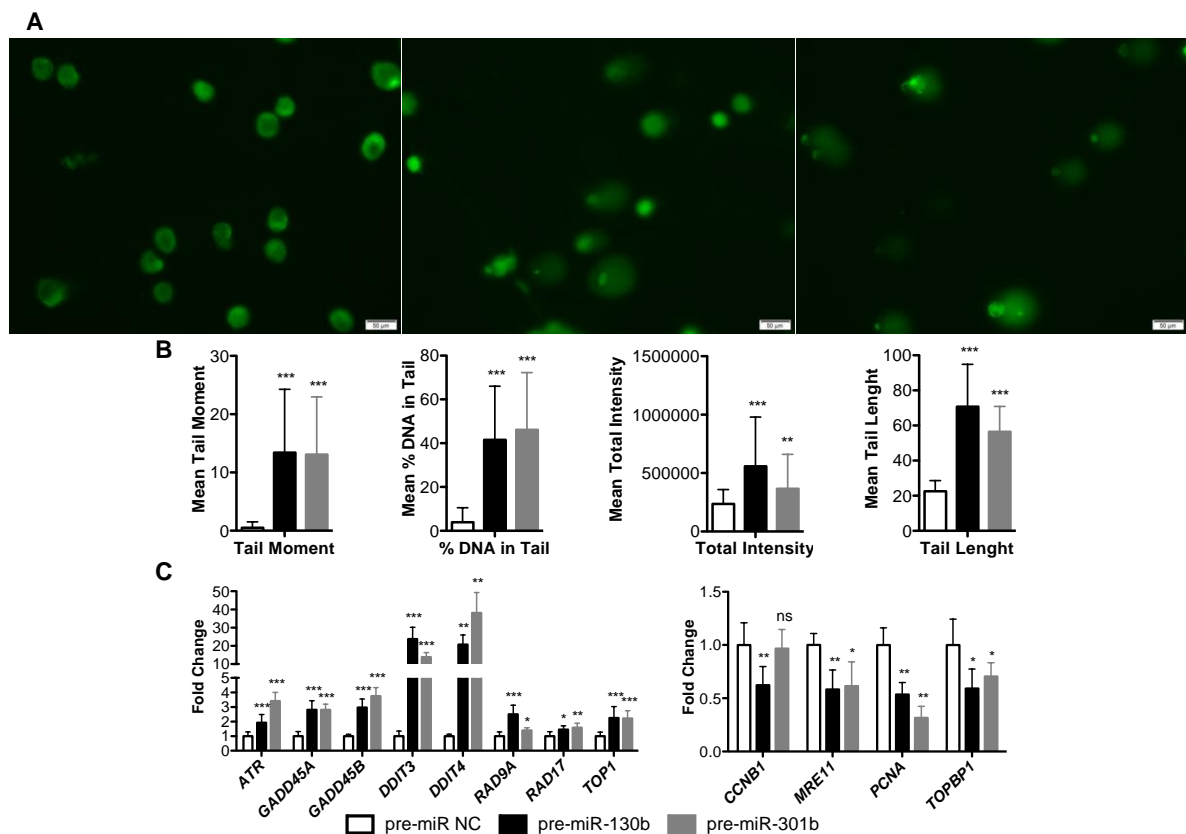
Figure 6. miR-130b and miR-301b impair DNA-damage signalling pathway.
(A) Comet assay immunofluorescence images of PC3 cells transfected with pre-miRNAs and counterstained with Syber Green, depicting DNA-damage associated morphology. (B) Graphic representation of parameters analysed in the assay, supporting that both miR-130b and miR-301b overexpression induce DNA damage in PC3 cells. (C) RT-qPCR evaluation of

multiple genes involved in DNA-damage response pathway. All data are presented as mean of three independent experiments ± s.d. (*p<0.05, **p<0.01, ***p<0.001).

Discussion

The intense research on the epigenetics field led to the discovery that genes encoding miRNAs were epigenetically silenced through DNA methylation (1).

Because the miR-130b~301b cluster ranked first among all hypermethylated miRNA promoters in our dataset and, to the best of our knowledge, had not been previously reported in PCa, it was selected for subsequent validation and functional characterization. Pyrosequencing of a large number of primary PCa and normal prostate tissues, confirmed that miR-130b~301b cluster promoter methylation levels were significantly higher in the former, whereas the opposite was apparent for expression levels of both miRNAs, thus prompting an association between aberrant promoter methylation and expression downregulation in PCa. This was further confirmed *in vitro* as PCa cell lines disclosed increased expression levels after exposure to a demethylating agent, either alone or in combination with TSA. Importantly, these findings are comparable to those reported for miR-193b, miR-34b~34c and miR-23b~27b~24-1 cluster (16-18), confirming that aberrant promoter methylation is, indeed, the mechanism underlying miR-130b~301b cluster downregulation in PCa.

Concerning the functional characterization of these findings, it should be emphasized that miR-130b and miR-301b are members of a miRNA family which is deregulated in several cancer types, acting either as onco-miRs or tumour-suppressive miRs. Indeed, a tumour-suppressive role for miR-130b in PCa has been proposed (although the mechanism underlying its downregulation was not disclosed), counteracting metastasis formation through MMP2 downregulation (19). Nevertheless, another report implicated miR-130b in tumorigenic reprogramming of adipose tissue-derived stem cells in PCa patients, acting as oncomir (20). Furthermore, the role of miR-301b in PCa remains elusive, although it appears to be induced under hypoxia and target *NDRG2* (21, 22). Interestingly, the functional assays confirmed the tumour-suppressive action of miR-130b and miR-301b. In both cases, miRNA overexpression reduced cell viability, induced apoptotic cell death and irreversibly activated the cell cycle arrest program DNA damage-induced senescence.

Phenotypic alterations were supported at molecular level, as restored expression of both miR-130b and miR-301b significantly increased the expression of genes acting as checkpoint sensors, required for effective tumour-suppression. It is not clear whether these alterations directly result from miRNA-mRNA interactions at 5'UTR or promoter (23), or from the naive output of tumour-suppression. It might be speculated that both miR-130b and miR-301b

interact with other regulatory elements and consequently enhance transcription or translation of those genes (23). Indeed, it has been hypothesized that many miRNAs have evolved to act not as genetic switches of specific pathways or individual targets but rather to modulate expression of large gene networks (24). Moreover, it should be recalled that due to the seed sequence similarity among miRNAs of the same family, targets from the same miRNAs cluster may be shared, although specific targets might also exist, as result of other base pairing determinants in addition to seed-sequence (25). This may explain why restoration of either miR-130b or miR-301b basically had the same functional impact. Nonetheless, the magnitude of the effect may be different, as demonstrated for several target genes, including *Ki67* and *CASP3*. Thus, different functional specialization of miR-130b and miR-301b is proposed.

Our data suggest that miR-130b~301b cluster might counteract malignant transformation of prostate epithelial cells through impairment of EMT, favouring MET instead. This was apparent not only morphologically, as PC3 cells exhibited a more epithelial phenotype upon miR-130b or miR-301b overexpression, but also at molecular level, through increased expression of several genes, including *CD44*. Interestingly, *CD44* downregulation was depicted following transfection with anti-miR-130b or anti-miR-301b. Decreased *CD44* expression has been associated with a more aggressive PCa phenotype, due to its association with higher grade and pathological stage, correlating with biochemical recurrence and tumour relapse (26). Our observations are in line with these findings, although the mechanism by which the miR-130b~301b cluster influences *CD44* expression requires clarification. Nevertheless, it should be emphasized that the impact of miR-130b and miR-301b on EMT-related genes seems to differ, as illustrated by the almost opposite expression patterns of *TGFB3* and *WNT5A*. Yet, because no double transfection experiments were conducted (as all were transient transfections), the net result of miR-130b~301b cluster downregulation cannot be determined.

An interesting and novel finding was the link between miR-130b~301b cluster and cellular senescence. This process induces cell cycle and cell growth arrest, and it may counteract tumour formation (27). Accumulation of DNA damage is a common basis for senescence, preventing genomic instability (28). Senescent cells display cell size increase and a more flattened shape, as well as increased p53, *CDKN2A* (p16), *CDKN1A* (p21) and *CDKN1B* (p27) expression, and *LMNB1* downregulation (29, 30). Remarkably, the same gene expression pattern was observed upon miR-130b or miR-301b overexpression, whereas miR-130b or miR-301b depletion had the opposite effect, suggesting that miR-130b or miR-301b downregulation might allow for senescence bypass. Our observations are also in line with previous reports correlating *LMNB1* reduction (particularly from H3K9me3 regions) and spatial repositioning of perinuclear heterochromatin (H3K9me3-enriched) and SAHF

formation (31). These findings are further supported by induction of SASP upon miR-130b or miR-301b overexpression. Interestingly, in oncogene-induced senescence (OIS), SASP is regulated by persistent DDR (32, 33). We found that miR-130b or miR-301b overexpression stimulated the expression of genes involved in DDR as well as in DNA repair, suggesting that miR-130b~301b cluster downregulation might impair OIS and foster malignant transformation of prostate cells.

Conclusions

In conclusion, we found novel miRNAs deregulated through aberrant promoter methylation in PCa. In particular, the miR-130b~301b cluster displays a tumour-suppressive profile and its downregulation might fuel malignant transformation and tumour progression through facilitation of EMT and bypass of cellular senescence.

Declarations

Ethics approval and consent to participate

Previously described on the "Methods section"

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the GEO repository under accession number GSE52955.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: JR-C, IG, RH, ME, CJ; Data analysis and interpretation: JR-C, IG, RH, CJ; Performed the Experiments: JR-C, IG; Performed the bioinformatics analysis: AG; Contributed materials: JO, RH, CJ; Contributed reagents: ME, CJ; Manuscript preparation: JR-C, RH and CJ; Coordination of the study: CJ. All authors read and approved the final manuscript.

Supplementary information

Accompanies the paper on the website (https://jhoonline.biomedcentral.com)

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CHAPTER 6

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A multiplatform approach identifies miR-152 as a novel epigenetically downregulated microRNA in prostate cancer

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A multiplatform approach identifies miR-152 as a novel epigenetically downregulated microRNA in prostate cancer

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Abstract

Prostate cancer (PCa) is a major cause of morbidity and mortality in men worldwide. MicroRNAs are globally downregulated in PCa, especially in poorly differentiated tumors. Nonetheless, the underlying mechanisms are still elusive.

Herein, using combined analysis of microRNAs expression and genomewide DNA methylation, we aimed to identify epigenetically downregulated microRNAs in PCa. We found that miR-152 was underexpressed in PCa and that lower expression levels were associated with promoter hypermethylation. These results were validated in our patient cohort and in TCGA dataset. Functional *in vitro* assays suggest that miR-152 suppresses cell viability and invasion potential, whereas it promotes cell cycle arrest at S and G2/M phases. Finally, *TMEM97*, which is overexpressed in PCa, was identified as a novel miR-152 target gene.

Our findings demonstrate the advantages of using a combinatory approach to identify microRNAs downregulated due to aberrant promoter methylation. MiR-152 downregulation and promoter methylation was found to be prevalent in primary PCa, which impairs its role in control of cell viability, cell cycle regulation and invasion.

Introduction

MicroRNAs (miRNAs) are a class of small (~22-nucleotide) RNAs that mediate post-transcriptional gene silencing by guiding Argonaute (AGO) proteins to target mRNAs (1, 2), either by repressing translation or by promoting destabilization (3). Target guidance and specificity is mainly determined by nucleotides at positions 2–7 of the miRNA (the 'seed') (4). Such mechanisms are critical for homeostasis maintenance, both under physiological conditions and in cell's response to environment alterations, including stress signals (5). Thus, a vast number of biological processes are subject to miRNA-dependent regulation, encompassing cell proliferation, signaling, differentiation, stress response, DNA repair, cell adhesion and motility, inflammation, cell survival, senescence, and apoptosis (1). Interestingly, miRNA's expression, processing, and functional output are also stringently controlled (6). Indeed, miRNAs' expression and activity are tightly spatially and temporally regulated, and its disruption has been extensively linked to human disease, including the development of cancer and metastasis formation (1, 7). Globally, miRNAs are mostly downregulated in cancer, including that of the prostate (1). Multiple mechanism are known to induce miRNA deregulation, including epigenetic alterations, aberrant transcription factors binding (*e.g.*, p53, MYC, REST), miRNA biogenesis machinery disruption, RNA editing, post-transcriptional RNA modifications, Argonaute loading, and RNA decay (7, 8).

Prostate cancer (PCa) is the most common non-cutaneous cancer worldwide in men, and a leading cause of cancer-related death in developed countries (9). Multiple factors, comprising age, family history, genetic susceptibility and ethnicity, contribute to the high incidence of PCa (10). Owing to its complexity and heterogeneity, and despite extensive studies, the molecular mechanisms that drive prostatic carcinogenesis are still far from complete understanding. Because miRNAs regulate a wide range of signaling pathways that are frequently deregulated in PCa, this class of noncoding RNAs might be of critical relevance for tumor development and progression. Thus, its study may provide novel insights into PCa biology and afford innovative tools for patient management, aiding in diagnosis and prognosis assessment, as well as the identification of new therapeutic targets (11).

Here, we attempted to discover new epigenetically regulated miRNA loci in PCa using a combinatory approach that compared miRNAs expression profiling with DNA methylation patterns. The candidate microRNAs were subsequently validated in two large patient cohorts, which included ours and that of TCGA; in vitro assays were performed to characterize their role in cancer cell biology, and in silico analysis, followed by in vitro validation, allowed for the identification of relevant target mRNAs. Overall, our data extends current knowledge about epigenetic deregulation and biological significance of miRNAs in prostate carcinogenesis.

Results

Identification of hypermethylated and downregulated microRNAs in prostate cancer

Global miRNAs expression was assessed using microRNA Ready-to-Use PCR Human Panel (I+II) v2.0 (Exiqon, Vedbaek, Denmark). Using a cutoff value of log fold change <-1.5, 40 miRs were found downregulated in PCa compared to morphologically normal prostate tissue (MNPT) (Supplementary Table S1 and Supplementary Figure 1). From these, 10 were selected for validation in a larger and independent dataset (miR-10a, miR-23b, miR-27b, miR-135b, miR-143, miR-152, miR-187, miR-204, miR-205, miR-221), i.e., the miRNAseq expression data from PCa patients and matched normal samples deposited in The Cancer Genome Atlas (TCGA) ($n = 326$ and $n = 52$, respectively) (Figures 1 A-C and Supplementary Figure 2).

In parallel, DNA methylation profiling by Illumina disclosed 39 hypermethylated promoter regions in known miRNA regions, three of which were further validated in TCGA dataset (Supplementary Table S1 and Supplementary Figure 3).

Because gene expression and DNA promoter methylation correlate with gene regulatory activity status, we merged the results of the two analytical platforms (expression by Exiqon platform and methylation profiling by Illumina) to identify miRNAs with decreased expression

associated with promoter hypermethylation in PCa. From this combined analysis, six miRNAs emerged as simultaneously downregulated and hypermethylated in PCa: miR-10a, miR-23b, miR-27b, miR-34c, miR-152 and miR-335 (Figure 1A; Supplementary table 1).
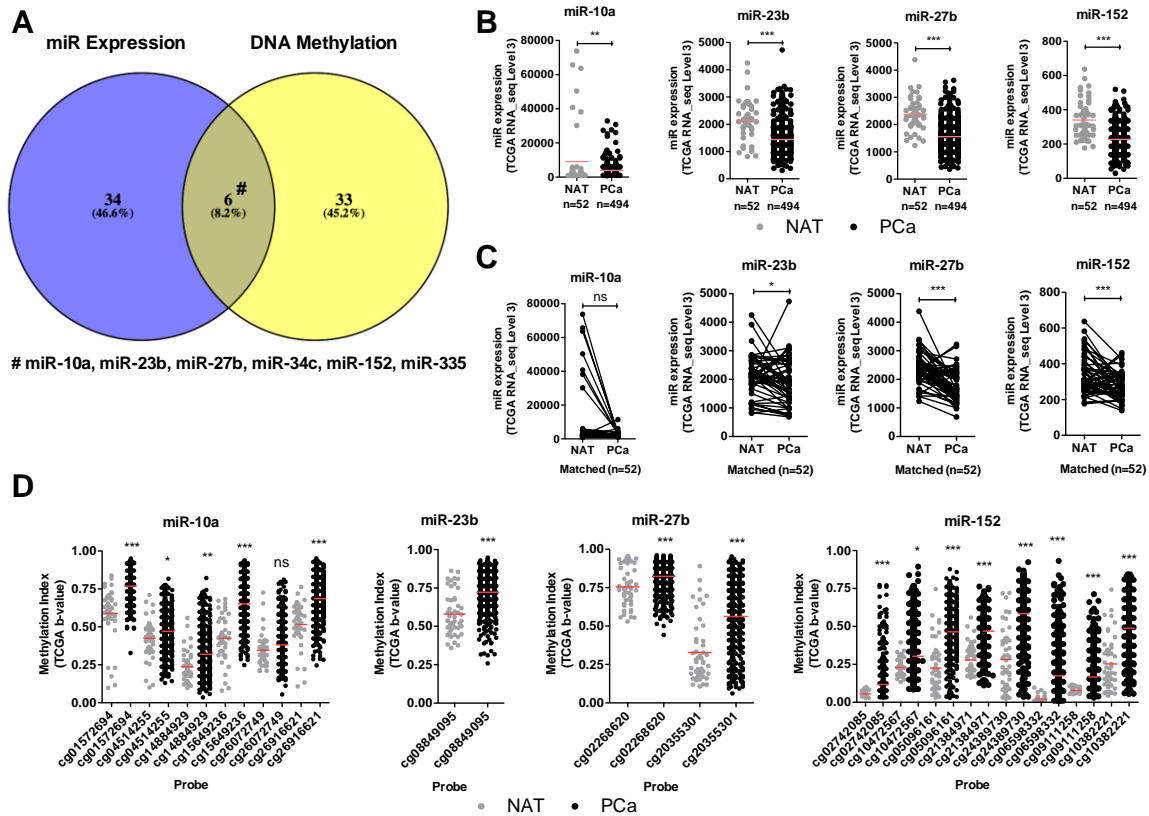
Figure 1. Identification of miRNAs downregulated by DNA methylation in prostate cancer, using a combinatorial approach.

(A) Venn diagram of the intersection of the miR expression (Exiqon) versus DNA methylation (Infinium HumanMethylation450 BeadChip) for miRNA promoters. Intersection is shown for the downregulated miRNAs and hypermethylated miRNAs. The five common miRNAs based on expression level and DNA methylation in PCa tissues are miR-10a, miR-23b, miR-27b, miR-152 and miR-335. (B) Independent validation using the TCGA Prostate RNA-seq cohort for miR-10a, miR-23b, miR-27b and miR-152 in PCa samples compared to NAT samples. (C) MiRNA expression analysis of 52 matched normal and PCa samples pairs using TCGA cohort. Except for miR-10a, all miRs were significantly downregulated in PCa. (D) DNA methylation levels (β-Values) for each probe in specific miRNA loci, comparing normal and PCa samples using TCGA Prostate 450K cohort. Overall, DNA methylation gain (hypermethylation) was found in PCa samples. NAT: Normal Adjacent Tissue; PCa: Prostate Cancer; Mann-Whitney U-test: *p<0.05, **p<0.01, ***p<0.001.

In TCGA dataset, all microRNAs, except for miR-335, were confirmed to be downregulated in primary PCa compared to noncancerous prostate tissues (Figure 1B). Moreover, and except for miR-10a, all displayed significantly lower expression in PCa in comparison with matched non-cancerous prostate tissues (Figure 1C). Because miRNA's promoter methylation status was available at TCGA database, these data were also retrieved and it confirmed our finding of increased methylation indexes in these miR's obtained with the Infinium 450K DNA methylation profiling platform (Figure 1D). Then, we focused our study on miR-152 as it fulfilled the criteria for downregulation associated with promoter hypermethylation in PCa, and it had not been previously reported in this cancer model.

MiR-152 expression and promoter methylation analysis in prostate cells

MiR-152 is located at chromosome 17q21.32, within an intronic region of *COPZ2*. In TCGA dataset, both miR-152 and *COPZ2* expression levels were inversely and significantly correlated with cg24389730 methylation levels in PCa samples ($\rho = -0.331$, $p < 0.0001$ and $\rho = -0.561$, $p < 0.0001$, respectively).

To further validate these findings, miR-152 (Figure 2A) and *COPZ2* (Figure 2B) expression levels were assessed in our cohort of prostatic tissues (PCa=100 and MNPT=14) and downregulation of both in PCa compared to MNPT was confirmed ($p < 0.0001$, and $p = 0.0022$, respectively). Pyrosequencing analysis demonstrated that the promoter shared by miR-152 and *COPZ2* was aberrantly methylated in PCa (Figure 2C, $p < 0.0001$).
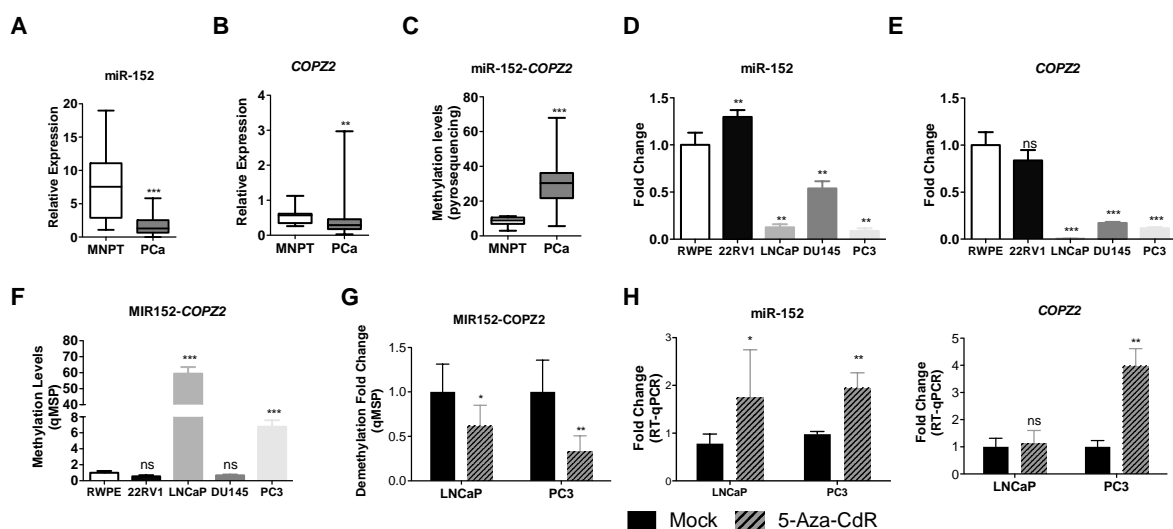
Figure 2. *COPZ2*-miR-152 transcriptional unit's DNA methylation and expression validation in IPO Porto's cohort of prostate samples.

(A) Significant miR-152 downregulation in PCa (n=100) compared to morphologically normal prostate tissues (MNPT, n=14), as determined by RT-qPCR (p<0.0001). (B) Significantly

*decreased COPZ2 transcript levels in PCa samples (p=0.0022). (C) COPZ2-miR-152 promoter hypermethylation in PCa samples determined by pyrosequencing (p=<0.0001). (D) MiR-152 expression levels in PCa cell lines compared to benign RWPE cells [expression (E):1] assessed by RT-qPCR. Expression is significantly lower in LNCaP (E:~0.13), DU145 (E:~0.54) and PC3 (E:~0.09) cells. (E) Significantly lower COPZ2 expression levels by RT-qPCR in LNCaP (E:~0.005), DU145 (E:~0.17) and PC3 (E:~0.12) cells compared to RWPE (E:1). (F) Prostate cell lines's DNA methylation profiling. LNCaP (FC of methylation levels: ~59) and PC3 (FC: ~7) cells showed increased miR152-COPZ2 promoter hypermethylation compared to RWPE (FC:1), 22RV1 and DU145 cells. (G) A 72h 5-Aza-CdR exposure, associated with significant decrease in promoter methylation levels of the transcriptional unit COPZ2-miR-152 in LNCaP (decreased ~40%) and PC3 (decreased ~68%) cells. (H) LNCaP and PC3 cells miR-152 and COPZ2's expression levels following 72 h exposure to 5-Aza-CdR associated with increased miR-152 expression levels (FC:1.75; 1.95, respectively) and COPZ2 (FC:1.12; 3.98, respectively). Error bars represent the s.d. for three biological replicates. Mann-Whitney U-test: *p<0.05, **p<0.01, ***p<0.001. MNPT: Morphologically Normal Prostate Tissue; PCa: Prostate Cancer; 5-Aza-CdR: 5-aza-2-deoxycytidine.*

In DU 145, LNCaP and PC3 cells, miR-152 and *COPZ2* expression levels were also significantly lower than those found on RWPE cells, which are benign epithelial prostate cells (Figures 2D, 2E), whereas promoter methylation levels followed the opposite trend, specifically for the latter two cell lines- LNCaP and PC3 (Figure 2F). These findings indicate that miR-152 is transcribed in parallel with its host gene, *COPZ2*. Exposure of PCa cells to demethylating agent 5-Aza-2-deoxycytidine (5-Aza-CdR), caused a 38% and 67% reduction in *COPZ2*-miR-152 promoter methylation levels, in LNCaP (p=0.0411) and PC3 cells (p=0.0043), respectively (Figure 2G). Nonetheless, the impact in gene expression differed as miR-152 re-expression was observed in both cell lines (LNCaP, FC: 1.75; and PC3, FC: 1.94) (Figure 2 H), whereas *COPZ2* transcript levels were only significantly restored in PC3 cells (FC: 3.98) (Figure 2 H).

MiR-152 attenuates malignant phenotype in vitro
Using *in vitro* assays, we found that miR-152 overexpression significantly decreased cell viability in both LNCaP and PC3 cells (Figure 3A and D), and promoted a significant accumulation of cells in S and G2/M phases (Figure 3B and E). Accordingly, at transcriptional level, both cell lines displayed a significant decrease of several cell cycle regulators (Figures 3C and F). Conversely, miR-152's mimic transfection associated with increased apoptosis in LNCaP (p=0.0286) and PC3 (p=0.0286) cells (Figure 3G). These

results were further supported by the significantly reduced NF-kB expression in both PCa cell lines, as well as significantly increased *CASP3* expression levels, although only in PC3 transfected cells (Figures 3H and I). Moreover, miR-152 overexpression significantly reduced PC3 invasion ability in PC3 cells (p=0.0286; Figure 3J), and associated with specific epithelial–mesenchymal transition genes' downregulation. Indeed, along with *TWIST* and *VIM* downregulation, *MAPK1*, *SMAD4* and *STAT3* were significantly downregulated after miR-152 restored expression in both LNCaP and PC3 cells (Figures 3K and L).
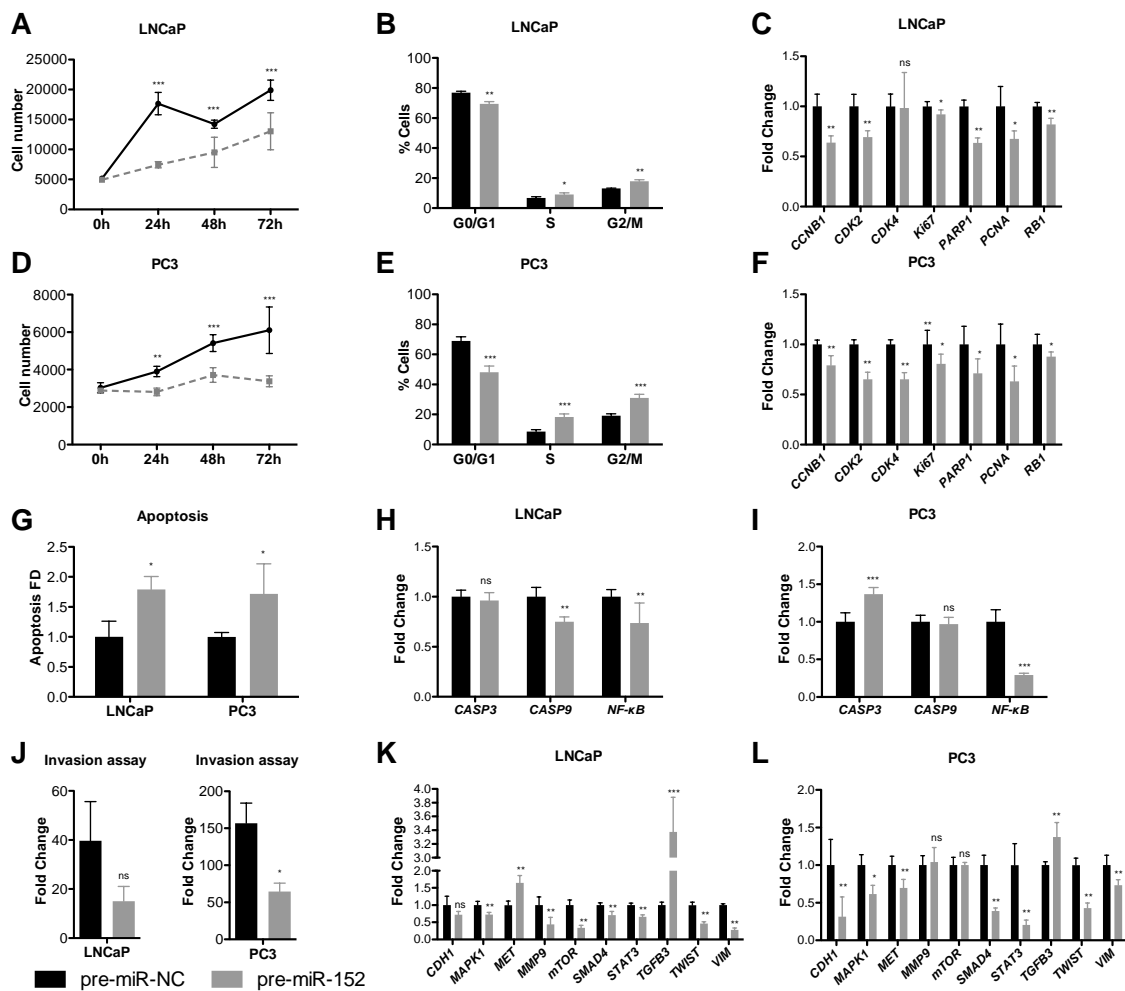
Figure 3. miR-152 overexpression associated with attenuated malignant features in LNCaP and PC3 cells. *(A and D) miR-152 overexpression in LNCaP and PC3 cells significantly decreased cell viability compared to pre-miR-NC transfected cells (MTT assay at 24h, 48h and 72h). (B and E) Cell cycle arrest at S and G2/M phases was depicted for LNCaP and PC3 cells overexpressing miR-152 (cytometry analysis was performed 72h after transfection). (C and F) reduced transcription levels of several cell cycle-promoting genes in miR-152 overexpressing LNCaP and PC3 cells. (G) MiR-152 overexpression associated with*

significant increase in apoptosis compared to cells transfected with negative control miRNA. (H-I) Apoptosis-related genes' expression levels were deregulated in PCa miR-152 overexpressing cells. Significantly reduced NF-kB levels were found in both miR-152 overexpressing cell lines. (J) MiR-152 forced expression in LNCaP and PC3 cells associated with a significant decrease number of cells invading through the Matrigel coated Boyden chamber assay. (K-L) Transcriptional deregulation of EMT and invasion-related genes in miR-152 transfected LNCaP and PC3. Differential MET, mTOR and MMP9 results suggest cell-specific gene regulation. TGFB3 overexpression and EMT markers STAT3, TWIST, or VIM decrease was shared by both miR-152 overexpressing cells. Error bars represent the s.d. for three biological replicates. Mann-Whitney U-test: *p<0.05, **p<0.01, ***p<0.001.

miR-152 targets NOL4 and TMEM97

Because the previous results suggested that miR-152 was an onco-suppressor, we sought to identify its targets for post-transcriptional regulation, using a combination of multiple *in silico* target prediction tools (putative targets must contain at least one miRNA response element – MRE) (Supplementary table S2) and a publicly available gene expression dataset.
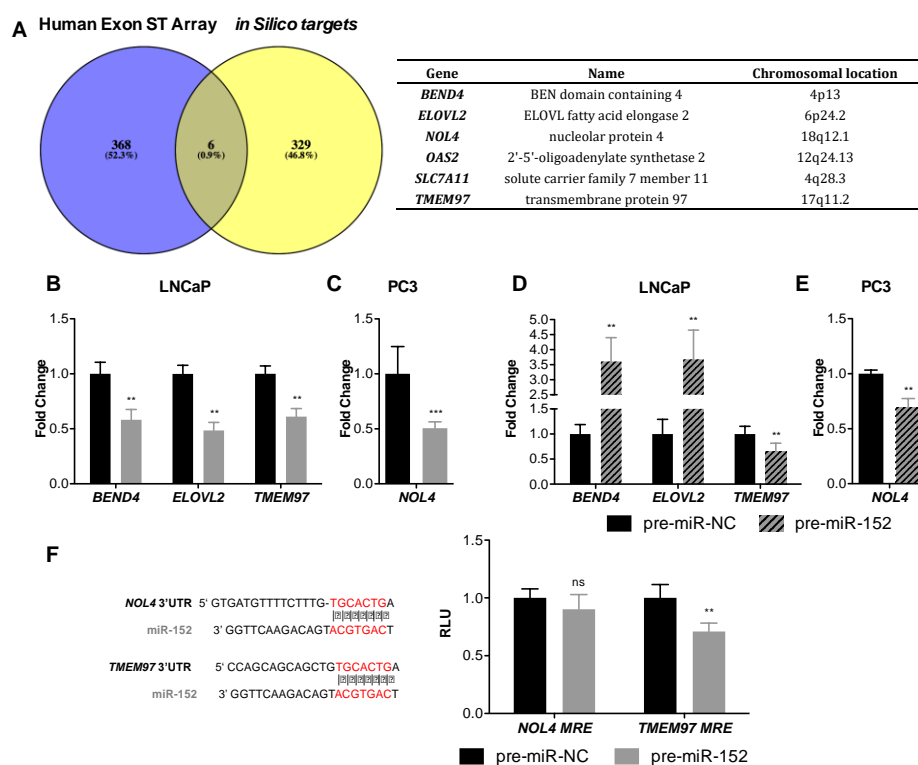
Figure 4. Identification of putative targets of miR-152 in PCa cell lines*.*

(A) Genes selected for validation in our experimental settings: combining in silico prediction targets with genome-wide expression using GeneChip® Human Exon ST Array. (B) MiR-152

overexpression in LNCaP cells associated with significant decreased levels of BEND4 (~40%), ELOVL2 (52%) and TMEM97 (40%) as determined by RT-qPCR. (C) PC3 miR-152's transfected cells displayed significantly decreased NOL4 expression (approximately 50%). (D and E) Effect of 5-Aza-CdR treatment in the selected target genes transcript levels in LNCaP revealed TMEM97 downregulation (up to 35%), whereas in PC3 cells it associated with significantly decreased NOL4 transcript levels (30%). (F) Schematic representation of the miR-152's MRE in NOL4 and TMEM97 (left panel). Luciferase activity in HEK293Ta cells co-transfected with reporter constructs containing NOL4 MRE or TMEM97 MRE and either pre-miR-152 or pre-miR-NC (Right panel). 3'UTR: 3' Untranslated Region; MRE: miRNA response element Error bars represent the s.d. for three biological replicates. Mann-Whitney U-test: *p<0.05, **p<0.01, ***p<0.001.

Among the 329 genes predicted as miR-52 targets *in silico*, only six - *BEND4*, *ELOVL2*, *NOL4*, *OAS2*, *SLC7A11* and *TMEM97* - disclosed a gene expression log fold change > 1.5 in PCa samples (n=368) analyzed by GeneChip Human Exon ST Array (Figure 4A). Moreover, forced expression of miR-152 caused a significant downregulation of *BEND4*, *ELOVL2* and *TMEM97* in LNCaP cells (Figure 4B), whereas in PC3 cells, only *NOL4* transcript levels significantly diminished following miR-152's overexpression (Figure 4C). LNCaP cells overexpressing miR-152 exposed to 5-Aza-CdR, showed significantly reduced *TMEM97* expression levels (Figure 4D) and *NOL4* was downregulated in PC3 cells (Figure 4E).

Since both *NOL4* and *TMEM97* 3'UTRs contain a MRE for miR-152 (Figure 4F, left panel), the functional interaction between miR-152 and *NOL4*, on the one hand, and *miR-152* and *TMEM97*, on the other, was investigated using luciferase assays. Interestingly, a 30% reduction (p=0.0022) in luciferase activity for the *TMEM97* MRE was found, although only a 10% decrease was depicted for the *NOL4* MRE (Figure 4F, right panel).

TMEM97 and NOL4 expression in primary PCa

To further confirm the biological significance of our previous findings, *NOL4* and *TMEM97* expression levels were assessed in two independent cohorts of PCa patients (the one from our institution and that from TCGA). Remarkably, we found that *TMEM97* and *NOL4* expression levels were significantly upregulated in PCa cases from both cohorts, compared to normal prostate tissues (p= 0.0132 and p=0.0004, respectively, in IPO Porto's cohort; p<0.0001 and p<0.0001, respectively, in TCGA cohort) (Figure 5A-5B). Moreover, no associations were found between miR-152 and *TMEM97* expression levels and patients' prognosis in IPO Porto's cohort. However, in TCGA's cohort, higher *TMEM97* expression levels (>75 percentile) independently predicted shorter disease free survival (HR= 1.805;

p=0.040), whereas miR-152 expression levels did not disclose any association with patients' outcome.
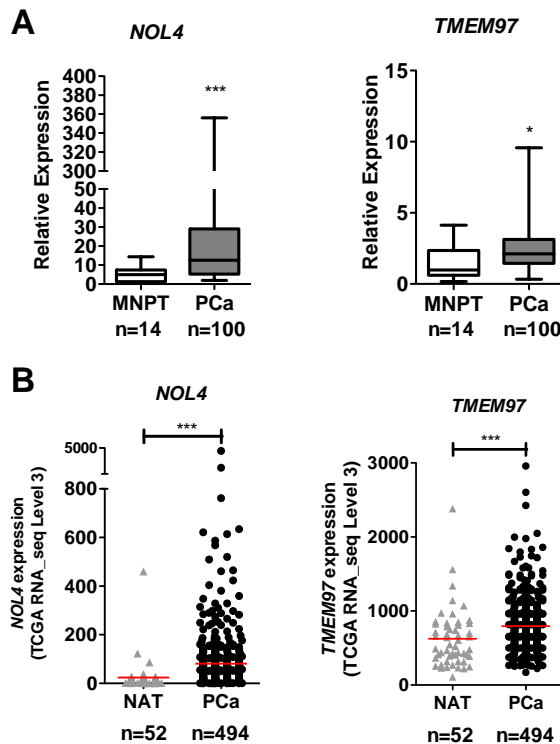
Figure 5. **NOL4 and TMEM97 upregulation in PCa tissues.** *(A and B) Significantly higher NOL4 and TMEM97 expression in PCa tissue samples (n=100) compared with morphologically normal prostate tissue (n=14), determined by RT-qPCR. (C and D) Expression levels in TCGA Prostate by RNA-seq cohort (NAT: n=52; PCa: n=497). MNPT: Morphologically Normal Prostate Tissue; NAT: Normal Adjacent Tissue; PCa: Prostate Cancer. Mann-Whitney U-test: *p<0.05, **p<0.01, ***p<0.001*

Discussion

MiRNAs are key players in cellular differentiation and homeostasis, being involved in regulation of transcriptional programs through the elimination of aberrant transcripts and suppression of random fluctuations in transcript copy number (12). Thus, its deregulation impairs cellular homeostasis and is involved in the emergence of several pathologies, including PCa.

In this study, we aimed to extend current knowledge on the impact of epigenetic deregulation of miRNAs expression in PCa. For that purpose, we used a combined analysis that allowed the identification of downregulated and aberrantly methylated microRNAs. Interestingly, only

8% of downregulated miRNAs in PCa tissues were found to be simultaneously aberrantly methylated. Thus, promoter hypermethylation does not seem to be a prevalent mechanism underlying microRNA downregulation in this cancer model, and other causes, whether genetic(13), epigenetic (14) or microenvironment-related (e.g., abnormal AR signaling (15)), are likely to be more frequent. Notwithstanding, we have recently shown that aberrant microRNA promoter methylation might constitute a clinically useful tool for PCa detection and prognostication (16). Although the number of candidate microRNAs was small, the combined approach used in this study seems to be more robust and efficient than each strategy (*i.e.*, micro-RNA expression analysis and differential methylation mapping) alone, considering the significantly higher proportion of validated candidates obtained compared to previous studies from our group (17, 18). Moreover, the results also validate this approach as it confirmed previous reports on miR-23b and miR-27b (members of the cluster miR-23b/27b/24-1) downregulation associated with promoter methylation in PCa (19-21). Remarkably, two novel microRNAs within this category were found - miR-10a and miR-152 – although only the latter was validated in two independent datasets.

Interestingly, several miRNAs, including miR-135b, miR-143, miR-187, miR-204, miR-205 and miR-221 that were commonly downregulated in the Exiqon expression dataset, were also downregulated in TCGA dataset. In contrast, the number of putatively downregulated microRNAs due to aberrant DNA methylation found in the HumanMethylation450 BeadChip and validated in TCGA dataset was much smaller. Thus, our data indicate that miRNA expression's profiling is more likely to identify *bona fide* miRNA deregulated due to promoter methylation compared to DNA methylation profiling, as aberrant DNA methylation might not indicate effective transcriptional silencing. Nevertheless, DNA methylation profiling might be particularly suitable for integrative analytic approaches (22).

Because miR-152 fulfilled the criteria for methylation-associated downregulation and it had not been previously reported in PCa, we sought to investigate its role in prostate carcinogenesis. Our study indicates that miR-152 is a sense-oriented intronic miRNA that forms a transcriptional unit (TU) with the respective host gene, *COPZ2*, being processed as part of the host gene mRNA (23, 24). Globally, aberrant promoter methylation associated with simultaneous downregulation of *COPZ2*-miR152 expression. Nonetheless, the results of 5-Aza-CdR exposure in LNCaP suggest that this is not the only mechanism regulating *COPZ2*-miR152 expression, and we are tempted to speculate whether an independent promoter might be located upstream in the host gene, as reported for other genes (25, 26).

MiR-152 promoter methylation has also been reported in endometrial cancer (27) and in MLL-rearranged infant acute lymphoblastic leukemia (28). In similarity with previous reports, our data also suggest an onco-suppressor function for miR-152 in PCa. Indeed, in non-small cell lung cancer miR-152 suppressed cell proliferation, colony formation, migration and

invasion (29), in endometrial cancer miR-152 restored expression prevented tumor cell growth both *in vitro* and *in vivo* (27), and in ovarian cancer miR-152 was suggested to contribute to cisplatin resistance *in vitro* and *in vivo* through direct *DNMT1* targeting (30). In PCa, miR-152 was shown to control cell migration and invasion (31), as well as inhibition of cell proliferation (32). Interestingly, in prostatectomy samples, lower miR-152 expression levels were significantly associated with higher risk for biochemical recurrence, although only in univariate analysis (32). We did not find miR-152 expression levels to be of prognostic value in either patient cohort assessed. However, higher *TMEM97* expression levels (>75 percentile) was an independent prognostic marker for biochemical recurrence in PCa patients, although only in TCGA cohort. These apparently contradictory results may be explained by the existence of other regulatory factors acting on TMEM97, besides miR-152. Indeed, it should be recalled that only a 30% reduction in luciferase activity was found for the *TMEM97* MRE specific for miR-152.

The gain of function *in vitro* functional assays demonstrated that miR-152 controls cell viability in PCa cells acting as S and G2/M cell-cycle transitions regulator, in both cell lines tested, but more expressively PC3 cells. However, these effects might be cell-context dependent. In line with functional assays' results, miR-152 overexpression associated with different specific transcript alterations depending on the tested cell line. Our results indicate that miR-152 fine-tunes the expression of several genes involved in the MAPK/ERK, TFG-Beta, JAK–STAT3 and EMT pathways. As these mediate biological processes that are critical for cancer progression, an important role for miR-152 in PCa progression might be anticipated.

To better understand the role of miR-152, we attempted to identify putative targets. *In silico* analysis followed by validation in two independent patient cohorts indicated NOL4 (nucleolar protein 4) and TMEM97 (transmembrane protein 97) as miR-152 targets. Although no information is available for the role of *NOL4* in cancer, *TMEM97* has been shown to be upregulated in several malignancies, including glioma (33) as well as colorectal (34) and ovarian (35) cancers. Interestingly, in glioma cells, TMEM97 depletion inhibited cancer cell growth and metastasis formation, in parallel with deregulation of EMT-related genes. Remarkably, increased *TMEM97* expression correlated with shorter survival in glioma (33), ovarian (35), non-small cell lung (36) and colorectal (34) cancer patients. Moreover, *TMEM97* cytoplasmic expression has been positively correlated with *PCNA* expression (34), which acts as a scaffold to recruit proteins involved in DNA replication or DNA repair, being required for post-replication repair (37). Remarkably, in our study, miR-152 overexpression associated with *PCNA* downregulation in both cell lines. Therefore, since *TMEM97* might be functionally associated with PCNA (38), it might suggest that both are controlled by miR-152.

Concerning other previously reported miR-152's targets, we were not able to confirm *DNMT1* as a miR-152's direct target. (30, 39-40)

In conclusion, this study uncovered novel miRNAs downregulated by aberrant DNA methylation, including a transcriptional unit formed by *COPZ2*-miR-152. Furthermore, it was demonstrated that miR-152 downregulation is a common feature of PCa, favoring the acquisition and development of the malignant traits, as *in vitro* miR-152´s restored expression attenuated PCa cell phenotype, by impairment of cell viability, cell cycle progression and invasion, through targeting of several genes involved in critical cancer-related pathways.

Methods

A flow chart depicting the different steps followed in this study is provided in Supplementary Figure 1.

Patient and samples

PCa tissue samples (n=100) from patients diagnosed and primarily treated with radical prostatectomy at Portuguese Oncology Institute of Porto, Portugal were prospectively collected. Fourteen normal prostate tissue (MNPT) samples, of peripheral zone of prostates without PCa, from patients submitted to radical cystoprostatectomy due to bladder cancer, served as controls. All specimens, promptly frozen at -80ºC, were cut for nucleic acid extraction. For routine histopathological examination, formalin-fixed and paraffin-embedded (FFPE) fragments were also collected. Relevant clinical data was retrieved from clinical charts. This study was approved by the institutional review board (IRB-CES-IPOFG-EPE 215/013). The clinical and pathological data of the patients included in this study is reported in Supplementary Table S3.

PCa cell lines and Demethylation treatment

Prostate cell lines, LNCaP, 22RV1, DU145, PC3 (malignant) and RWPE (benign) were used for *in vitro* studies. LNCaP and 22Rv1 cells were grown in RPMI 1640, whereas DU145 and PC3 cells were maintained in MEM and 50% RPMI-50% F-12 medium, while RWPE was cultured in Keratinocyte-SFM, containing human recombinant Epidermal Growth Factor 1-53 and Bovine Pituitary Extract (GIBCO, Invitrogen, Carlsbad, CA, USA), respectively. HEK293Ta were maintained in DMEM. All basal culture media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (GIBCO, Invitrogen, Carlsbad, CA, USA). Cells were maintained in an incubator at 37ºC with 5% CO_2. All cell lines were G-banding karyotyped (for validation) and routinely tested for *Mycoplasma spp.* contamination (PCR Mycoplasma Detection Set, Clontech Laboratories).

One µM of the DNA methyltransferases inhibitor 5-aza-2-deoxycytidine (5-Aza-CdR; Sigma-Aldrich, Schnelldorf, Germany) was used for DNA demethylation. Cells were harvested and RNA extracted after 72h exposure to the demethylating agent.

Nuclei acids extraction and bisulfite conversion

DNA was extracted from fresh frozen tissue samples and cell lines using phenol:chloroform (Sigma). RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according with manufacturer's instructions.

Bisulfite conversion of genomic DNA (1000 ng) was accomplished using EZ DNA Methylation Kit (Zymo Research), following manufacturer's instructions.

MicroRNA expression profiling

MiRNAs expression was assessed in ten PCa and four MNPT using microRNA Ready-to-Use PCR Human Panel (I + II) v2.R (Exiqon, Vedbaek, Denmark), comprising 752 miRNAs as previously described (17, 41). Extracted RNAs were submitted to cDNA synthesis using miRCURY LNA Universal RT microRNA PCR (Exiqon, Vedbaek, Denmark) following manufacturer's instructions. Data were analyzed using the comparative Ct method and the mean value was calculated for reference genes' expression normalization. MiRNAs with fold change of -1.5 in PCa compared with MNPT were considered downregulated.

MicroRNA's promoter methylation analysis in prostate tissues

All DNA samples were assessed for integrity, quantity and purity by electrophoresis in a 1.3 % agarose gel, picogreen quantification, and nanodrop measurements. All samples were randomly distributed into 96-well plates. Bisulfite-converted DNAs (200 ng) of 25 PCa and 5 MNPT were used for hybridization on the HumanMethylation450 BeadChip (Illumina).

HumanMethylation450 BeadChip data were processed using Bioconductor minfi package [54]. The "Illumina" procedure that mimics the method of GenomeStudio (Illumina) was performed, including background correction and normalization considering the first array of the plate as reference. Probes with one or more single nucleotide polymorphisms (SNPs) with a minor allele frequency (MAF) >1 % (1000 Genomes) in the first 10 bp of the interrogated CpG were removed. The methylation level (β) for each of the 485,577 CpG sites was calculated as the ratio of methylated signal divided by the sum of methylated and unmethylated signals, multiplied by 100. After normalization step, probes mapped within X and Y chromosomes were removed. All analyses were performed in human genome version 19 (hg19) and data was deposited in GEO repository under accession number GSE52955.

TCGA dataset analysis

Data on miRNA expression and clinical information (when available) from PCa and matched normal tissue samples was retrieved from The Cancer Genome Atlas (TCGA) database. The

mRNA expression data from samples hybridized at University of North Carolina, Lineberger Comprehensive Cancer Center, using Illumina HiSeq 2000 mRNA Sequencing version 2, were downloaded from data matrix including 494 miRNA-Seq, 496 RNA-Seq and 498 Methylation Array for PCa samples and 52 matched normal adjacent tissue samples (NAT). To prevent duplicates, when there was more than one portion per patient, median values were used. The provided value was pre-processed and normalized according with "level 3" specifications of TCGA. Clinical data of each patient was provided by Biospecimen Core Resources (BCRs). Data is available for download through https://gdc-portal.nci.nih.gov/projects/TCGA-PRAD .

Real-Time Quantitative PCR (RT-qPCR)

MiR-152 transcript levels were assessed using TaqMan MicroRNA Assay (assay ID: 000475; Applied Biosystems) and normalized with RNU48 (assay ID: 001006; Applied Biosystems).

Real-time quantitative PCR analysis was performed using gene-specific primers and normalized using GUSB housekeeping gene (Supplementary table S4). Specific-miRNA cDNA was obtained using TaqMan MicroRNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA, USA). Total cDNA synthesis was performed using high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

NOL4 and TMEM97 mRNA levels were confirmed in the same group of tissue samples previously indicated. A total of 300 ng was reverse transcribed and amplified using TransPlex® Whole Transcriptome Amplification Kit (Sigma-Aldrich®, Schnelldorf, Germany) with subsequent purification using QIAquick® PCR Purification Kit (QIAGEN, Hilden, Germany), according with manufacturer's instructions. Expression levels were evaluated using TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA), and GUSB was used as a reference gene for normalization.

The expression of each gene or small RNA was obtained using the formula: Relative expression = (Target gene mean quantity/Reference gene mean quantity). Ratios were then multiplied by 1,000 for easier tabulation. Each plate included multiple non-template controls and serial dilutions (10×) of a cDNA obtained from human prostate RNA (Carlsbad, CA, USA) were used to construct a standard curve for each plate. All experiments were run in triplicates (Supplementary Table S4.).

DNA methylation analysis

DNA methylation analysis was performed by quantitative methylation PCR (qMSP) using KAPA SYBR FAST qPCR Kit (Kapa Biosystems, MA, USA) and pyrosequencing. All reactions were run in triplicates in 384-well plates using Roche LightCycler 480 II, with *β*-

actin (*ACTB*) as internal reference gene for normalization. Primer sequences (Supplementary Table S4) were designed using Methyl Primer Express 1.0 and purchased from Sigma-Aldrich (St. louis, MO, USA).

For pyrosequencing, specific sets of primers for PCR amplification and sequencing were designed using a specific software pack (PyroMark assay design version 2.0.01.15). Primer sequences were designed, when possible, to hybridize with CpG-free sites to ensure methylation-independent amplification. PCR was performed under standard conditions with biotinylated primers, and the PyroMark Vacuum Prep Tool (Biotage, Uppsala, Sweden) was used to prepare single-stranded PCR products, according with manufacturer's instructions. Pyrosequencing reactions and methylation quantification were performed in a PyroMark Q96 System version 2.0.6 (Qiagen, Hilden, Germany) using appropriate reagents and recommended protocols (Supplementary Table S4).

Pre-miR Transfections

To overexpress miR-152, synthetic, commercially available, miRNAs' precursors (pre-miR-152, ID: PM12269; pre-miR-NC, ID: AM17110; Ambion, Carlsbad, CA, USA) were transfected at 30nM. Transfections were performed using Oligofectamine (Invitrogen, Carlsbad, CA, USA), per manufacturer's instructions.

Viability assay

Cell viability was evaluated by MTT assay. Briefly, PCa cells were seeded onto 96-well flat bottomed culture plates, allowed to adhere overnight later (number of cells plated before transfection: LNCaP: 10000 cells/well; PC3: 3000 cells/well), and transfected 24h later. At each time point, 0.5 mg/ml of MTT reagent [3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] was added to each well, and the plates were incubated in the dark for 1h at 37ºC. Formazan crystals were then dissolved in DMSO and absorbance was read at 540 nm in a microplate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany), subtracting the background, at 630 nm. The number of cells was calculated using the formula: [(OD experiment x Number of cells at day 0) / Mean OD at day 0]. Three replicates were performed for each condition and at least three independent experiments were carried out.

Apoptosis evaluation

Evaluation of apoptosis was performed using APOPercentage apoptosis assay kit (Biocolor Ltd., Belfast, Northern Ireland) according with the manufacturer's instructions. PCa cells were seeded onto 24-well plates (LNCaP: 50000 cells/well, and PC3: 30000 cells/well), and 24h

later were transfected. Apoptotic cells were assessed at the end of the day 3, in a FLUOstar Omega microplate reader at 550 nm and the background subtracted at 620 nm. The results were normalized to number of viable cell obtained in the MTT assay according to the following formula (OD of apoptosis assay at 72h/ OD of MTT at 72h).

Cell cycle analysis

Cell cycle distribution of LNCaP and PC3 cells was determined by flow cytometry. Briefly: 72h after transfections, $5x10^5$ harvested cells were fixed overnight at 4^oC with 70% cold ethanol. After cold PBS washing, cells were re-suspended in staining Propidium Iodide Solution (Cytognos S.L, Salamanca, Spain) and incubated for 30 minutes at room temperature. All cells were then measured on a Cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) and analyzed using Modfit LT (Verity Software House, Inc, Topshan, Maine, USA).

Cell Invasion Assay

Cell invasion was determined using BD BioCoat Matrigel Invasion Chamber (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, $5x10^4$ cells/mL of LNCaP or PC3 cells were added to the upper chamber. Both cell lines were transfected for 72 hours with miRNA molecules, after which, the non-invading cells were removed with cotton swabs from the upper side of the membrane. The membrane bottom containing invading cells was fixed in methanol, washed in PBS and stained with DAPI (Vector Laboratories, Burlingame, CA). All the invading cells were counted under a fluorescent microscope. Three independent experiments were performed for each condition.

Transcriptomic evaluation of altered genes following miR-152 manipulation

Cells (LNCaP: 400000 cells/well, and PC3: 150000 cells/well) were plated in 6-well, in the day before transfection. Cells were collected 72h post transfection and RNA was extracted and used as template for cDNA synthesis. RT-qPCR was performed as previously described and data analyzed according to the comparative Ct method (42).

Gene Expression Microarrays

RNA was extracted from tissue samples using TRIzol (Invitrogen by Life Technologies, Carlsbad, CA), as previously described (43, 44), and 1 µg of RNA was processed into cDNA and hybridized to Affymetrix GeneChip Human Exon 1.0 ST arrays, following the manufacturer's recommendations. The Affymetrix Expression Console v1.1 software was used to obtain exon-level robust multi-array average (RMA)-normalized expression values for

the core probe sets only. The data is freely available in GEO repository under accession number GSE42954.

Luciferase assay

A reporter plasmid containing a binding site at NOL4 or TMEM97 3'UTR for miR-152 (GeneCopoeia, Rockville, MD, USA) was co-transfected into HEK293Ta cells using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). 30nM of synthetic pre-miRNA were used. Luciferase activity was assessed with the Secrete-Pair™ Dual Luminescence Assay Kit (GeneCopoeia, Rockville, MD, USA) according with the manufacturer's instructions. The ratio of luminescence intensities (RLU, Relative Light Unit) of the GLuc (Gaussia luciferase) over SEAP (secreted Alkaline Phosphatase) was obtained as follows: GLuc/SEAP, for each triplicate.

Statistical analysis

Non-parametric tests (Kruskal-Wallis and Mann-Whitney U-test) were used for group comparisons analysis for both expression and methylation levels for the two patient cohorts (IPO's and TCGA) and for the *in vitro* assays. Correlations between expression levels and methylation were evaluated by Spearman's correlation test. Data are shown as mean ± s.d., unless otherwise specified. Student's t-test was used for invasion assays. To evaluate the prognostic value of mir152 and *TMEM97* expression in PCa patients from the TCGA dataset, univariable (Log-rank test) and multivariable (Cox regression) analyses of disease-free survival were performed, where putative confounding effects (Gleason score, patients' age, and the PSA value) were considered. Disease-free survival was calculated from the date of the radical prostatectomy to the date of biochemical relapse, or date of last follow-up or death if relapse-free. For the purposes of survival analyses, all cases were coded based on the percentile 75 value of expression levels. All statistical tests were two-sided. All experiments were run in triplicate. Statistical analysis was carried out using Graph Pad Prism version 5. Significance level was set at $p < 0.05$.

Author contributions

Conceived and designed the experiments: JR-C, RH and CJ. Performed the experiments: JR-C, IG, DB, EPS and MIG. Analyzed the data: JR-C, CSG, IG, AG, BMC, RH and CJ. Contributed reagents/material/analysis: CSG, MIG, ME, BMC, RH and CJ. All authors read and approved the final manuscript.

Competing financial interests

None of the authors have any conflict of interest to declare.

Competing interests

The authors declare that they have no competing interests.

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CHAPTER 7

MiR-193b promoter methylation accurately detects prostate cancer in urine sediments and miR-34b/c or miR-129-2 promoter methylation define subsets of clinically aggressive tumors.

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MiR-193b promoter methylation accurately detects prostate cancer in urine sediments and miR-34b/c or miR-129-2 promoter methylation define subsets of clinically aggressive tumors

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Abstract

Background: Contemporary challenges of prostate cancer (PCa) include overdiagnosis and overtreatment, entailing the need for novel clinical tools to improve risk stratification and therapy selection. PCa diagnosis and prognostication might be perfected using epigenetic biomarkers, among which aberrant DNA methylation of microRNA promoters has not been systematically explored. Herein, we identified aberrantly methylated microRNAs promoters in PCa and assessed its diagnostic and prognostic biomarker potential.

Methods: Using HumanMethylation450 BeadChip-based analysis differentially methylated CpGs in microRNA promoters were identified. Promoter methylation of six microRNAs (miR-34b/c, miR-129-2, miR-152, miR-193b, miR-663a and miR-1258) was analyzed by qMSP in three sets (180 prostatectomies, 95 urine sediments and 74 prostate biopsies). Biomarkers' diagnostic (validity estimates) and prognostic [disease-free (DFS) and disease-specific survival (DSS)] performance was assessed.

Results: Significantly higher promoter methylation levels in PCa were confirmed for six candidate microRNAs. Except for miR-152, all displayed AUC values higher than 0.90, with miR-1258 and miR-193b disclosing the best performance (AUC=0.99 and AUC=0.96, respectively). In urine samples, miR-193b showed the best performance (91.6% sensitivity, 95.7% specificity, AUC=0.96). Moreover, higher miR-129-2 independently predicted for shorter DSS and miR-34b/c methylation levels independently predicted for shorter DFS and DSS.

Conclusions: Quantitative miR-193b, miR-129-2 and miR-34b/c promoter methylation might be clinically useful PCa biomarkers for non-invasive detection/diagnosis and prognostication, both in tissue and urine samples.

Keywords: Biomarkers, aberrant miR's promoter methylation, detection, prognosis, prostate cancer.

Background

Prostate cancer (PCa) is the most incident male cancer in western countries, constituting the second most common cause of cancer and the sixth leading cause of death by cancer among men worldwide (1). For 2012, it was estimated that PCa alone accounted for 420,000 newly diagnosed cancer cases and 101,000 of all cancer-related deaths in European men (2). PCa is age-related and very heterogeneous, both molecularly and clinically, ranging from relatively indolent to highly aggressive. It is typically asymptomatic at its earliest stages, when adequate treatment is mostly curative, in contrast with its late diagnosis, which usually impairs a curative-intent therapeutic strategy (3). This led to the widespread use of serum PSA as screening tool for PCa. However, it is now commonly accepted that this entailed

overdiagnosis and overtreatment, justifying the strong recommendation against PCa screening and prompting the search for more effective biomarkers (4).

DNA methylation is a chemically stable and easily quantified alteration (5). We and others have previously reported on the use of quantitative promoter methylation of several protein-coding genes for early diagnosis and prognostication of PCa (6). Although several gene methylation panels have been then developed (7, 8), both sensitivity and specificity must be perfected to allow for clinical translation.

MicroRNAs, a class of small (19-25 nucleotides) non-coding RNA, are involved in virtually all cellular processes and frequently deregulated in cancer cells (9), although its abrogation due to aberrant promoter methylation has been seldom reported (10). Because this epigenetic alteration is likely to be highly cancer-specific, it might constitute an effective cancer biomarker. Thus, we aimed to explore the potential of microRNA-coding genes promoter methylation as diagnostic and prognostic biomarkers in PCa. Therefore, after genome-wide screening, a set of putative tumor-suppressor microRNAs (miR-34b/c, miR-129-2, miR-152, miR-193b, miR-663a and miR-1258) with increased promoter methylation levels in PCa compared to normal prostate tissues was identified and further validated in clinical samples.

Methods

Patients and samples collection

For the purposes of this study, three independent cohorts of PCa patients were defined.

PCa tissue samples were prospectively collected from 180 patients with clinically localized disease, consecutively diagnosed and submitted to radical prostatectomy (RP) from 2001 to 2006, at Portuguese Oncology Institute of Porto (Cohort #1). Fifteen control samples were obtained from cystoprostatectomy specimens with bladder cancer, not harbouring PCa nor prostatic involvement by urothelial carcinoma (morphologically normal prostate tissue, MNPT). After collection, tissue samples were fresh-frozen at -80°C and subsequently cut in a cryostat for DNA extraction. Prostate biopsy samples were collected from 74 PCa suspects (elevated serum PSA), referred to Portuguese Oncology Institute - Porto from 2001 to 2003 (Cohort #2). In addition to standard diagnostic cores, a core was collected from the most suspicious area, fresh-frozen at -80°C and subsequently cut in a cryostat for DNA extraction.

Voided urine samples from 95 PCa patients were collected from 1999 to 2002 (Cohort #3). The control set is composed of urine samples collected from 17 healthy donors and 29 patients without urological malignancy. Samples were centrifuged at 4,000 rpm for 20 minutes, washed in PBS 1X and the pellets were frozen at -80°C.

Clinical data was retrieved from clinical charts. Survival data was collected for patients of Cohort #1 and of Cohort #2. Disease-specific survival (DSS) time was calculated as the time

elapsed since diagnosis until death or the last follow-up. Disease-free survival (DFS) was calculated from the date of the radical prostatectomy or other curative treatment to the date of biochemical relapse, date of last follow-up, or death if relapse-free.

All patients enrolled (Tables 1 and 2) signed informed consent. This study was approved by institutional review board (CES-IPOPFG-EPE 019/08 and CES-IPOPFG-EPE 205/2013).

Table 1. Clinical and pathological data of tissue and urine samples used in this study

	Prostatectomies		Urine samples	
Clinicopathological data	MNPT	PCa[a]	Controls	PCa[b]
Patients, *n*	15	180	46	95
Median age, *years* (range)	63 (45–80)	65 (49–74)	61 (58–77)	64 (45–80)
Median PSA (*ng/mL*) (range)	-	8.3 (3.4-23.0)	-	8.8 (3.5-20.4)
Pathological Stage				
pT2 (%)	-	96 (53.3)	-	46 (48.4)
pT3 (%)	-	84 (46.7)	-	49 (51.6)
Gleason score				
<7 (%)	-	56 (31.1)	-	37 (39.0)
≥7 (%)	-	124 (68.9)	-	58 (61.0)

[a]Cohort #1; [b]Cohort #3

Nucleic acid isolation, bisulphite treatment, HumanMethylation 450 BeadChip and qMSP analysis

DNA extracted by phenol-chloroform as described elsewhere(11) was chemically modified using sodium bisulfite with EZ DNA Methylation-Gold™ Kit (Zymo Research, CA, USA) according to manufacturer's protocol.

HumanMethylation450 BeadChip (Illumina, USA) allowed for gene methylation profiling of tissue samples (5 controls and 25 tumors), using 500ng of bisulphite-converted DNA, according to manufacturer's instructions. DNA methylation levels were depicted as beta-values ranging from 0–1. Validation of all candidates was performed by quantitative methylation using KAPA SYBR FAST qPCR Kit (Kapa Biosystems, MA, USA**)**. All reactions were run in triplicates in 384-well plates using Roche LightCycler 480 II, with *β-actin* (*ACTB*) as internal reference gene for normalization.

Primer sequences (Supplementary Table1) were designed using Methyl Primer Express 1.0 and purchased from Sigma-Aldrich (MO, USA).

Table 2. Clinical and Pathological data of cohort #2 (prostate biopsies)

Patients, n	74
Median age, years (range)	68 (49–85)
Median PSA (ng/mL) (range)	18.22 (4.52-542)
Clinical stage	
T2 (%)	48 (64.9)
T3/T4 (%)	26 (35.1)
Gleason score	
<7 (%)	30 (40.5)
≥7 (%)	44 (59.5)
Follow up	
Median (months) (range)	114.9 (10.3–170.1)
Patients without remission (%)	3 (4)
Biochemical recurrence (%)	29 (39.2)
Death due to PCa (%)	13 (17.6)
Therapy	
Surgery (%)	17 (23.0)
ADT (%)	35 (47.3)
Radiotherapy (%)	4 (5.4)
ADT + Radiotherapy (%)	17 (23.0)
Radiotherapy + Criotherapy (%)	1 (1.3)
CAPRA Score	
Low-risk (0–2)	7 (9.5)
Intermediate-risk (3–5)	26 (35.1)
High-risk (6–10)	41 (55.4)

ADT- androgen deprivation therapy

Statistical analysis

For HumanMethylation 450 BeadChip data, a threshold intensity with P-value ≤ 0.01 was considered for further analysis. To identify consistently differentially methylated CpG sites, Wilcoxon rank sum paired test was performed for normalized beta-values. P-values were adjusted using false discovery rate, and CpGs with P-values <0.05 were selected.

In Cohort #1, pathological variables were categorized [Gleason score (GS): <7 and ≥7; pathological stage: pT2 and pT3]. Kruskall-Wallis and Mann-Whitney U tests allowed for comparisons among three or more groups and between two groups, respectively. For multiple comparisons P values were adjusted according to Bonferroni's correction. Spearman

nonparametric correlation was performed to ascertain association between methylation and PSA serum levels.

In Cohort #1 and Cohort #3, receiver operator characteristics (ROC) curves were constructed by plotting true positive rate (sensitivity) against false positive rate (1-specificity) and area under the curve (AUC) was calculated to assess diagnostic performance. Biomarker validity estimates [specificity, sensitivity, positive predictive value, negative predictive value and accuracy] were determined using as cut-off the highest value obtained through ROC curve analysis [sensitivity + (1-specificity)].

In Cohort #1 and Cohort #2, DSS and DFS curves were built using Kaplan–Meier method and the prognostic significance of clinicopathological variables (clinical stage, GS and serum PSA in both cohorts, and CAPRA Score in Cohort #2) was assessed using log-rank test. CAPRA score values were categorized as 0-2 (low-risk), 3-5 (intermediate risk) and 6-10 (high-risk) (12). To test the prognostic significance of miR-34b/c and miR-129-2 promoter methylation, samples were categorized based on methylation levels of each miR (using percentile 75 as threshold) (11). A Cox-regression model comprising all variables (multivariable analysis) was constructed. SPSS Statistics 20 (IBM, NY, USA) was used for all statistical analyses and graphics were assembled using GraphPad 5 Prism (GraphPad Software, CA, USA). P values <0.05 were considered statistically significant.

RESULTS

MicroRNA promoter hypermethylation in Radical Prostatectomy samples (Cohort #1)

Using the 450K array, we screened microRNA *loci* regulated by DNA methylation in PCa. The microarray dataset included 5 MNPT and 25 PCa tissue samples.

Candidate miRNAs were selected according to adjusted P-values and differences in the methylated fraction between MNPT and PCa tissues. CpG sites displaying statistically significant differences with adjusted P-values and mean methylation <0.3 in MNPT were further considered relevant. Among these, methylation sites located in the promoter region and in proximity to transcription start sites (TSS) [1500 and 200 base pairs upstream of TSS (TSS200; TSS1500 region)] were identified confirming the results of the array analysis.

For the validation study, we selected microRNAs in which significant differences (P<0.05) in methylation levels were observed at all CpG sites mapped and differences in methylated fractions were >0.12. Thus, six microRNAs - miR-34b/c, miR-129-2, miR-152, miR-193b, miR-663a, and miR-1258 (Figure 1; Table 3), were selected for large-scale validation in 15 MNPT and 180 PCa samples (Cohort #1). In this series, overall methylation levels remained significantly increased in PCa compared with MNPT (Figure 2).
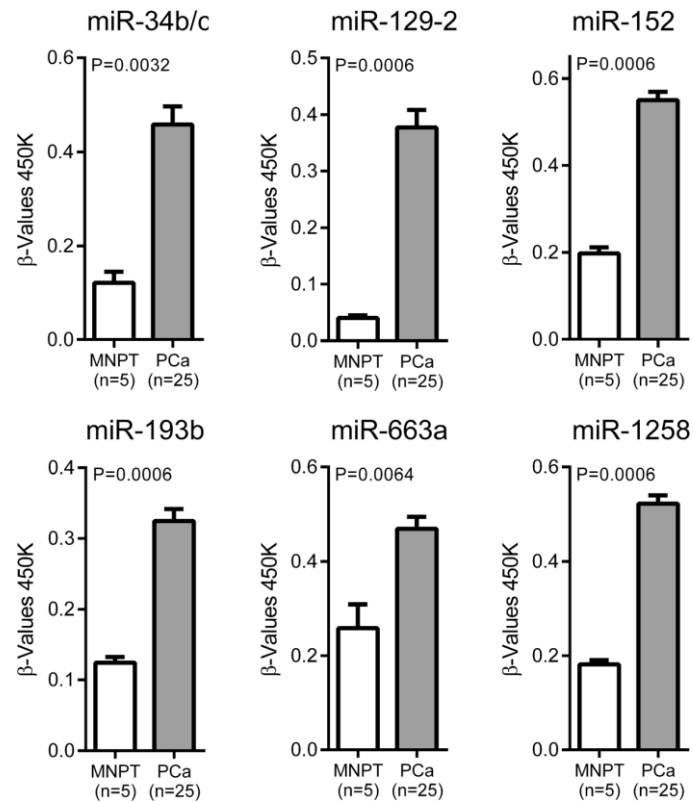
Figure 1. HumanMethylation450 BeadChip results.

The microRNAs that displayed the most significant differences between normal and neoplastic samples were selected for further analysis. All data are presented as mean + standard deviation of the samples analyzed in each group.

To assess the diagnostic potential of microRNA promoter methylation in PCa we performed ROC curve analysis (Figure 2 and Table 4), which revealed AUC values ranging from 0.89 to 0.99, with miR-1258 (AUC=0.99), miR-193b (AUC=0.96) and miR-34b/c (AUC=0.95) demonstrating the best performance. Because AUC for miR-152 was lower than 0.90, it was excluded from further analyses. Concerning validity estimates, miR-1258 promoter methylation levels displayed the highest values (97.8% sensitivity, 100% specificity) for PCa detection. Panels composed by two or more microRNAs did not improve performance (data not shown).

Table 3. MicroRNA/CpG island probe distribution derived from the Infinium HumanMethylation450 BeadChip

miR	TargetID	Probe Sequence	Chr	UCSC Refgene Name	UCSC Refgene Accession	UCSC Refgene Group	UCSC CpG Islands Name	Relation to UCSC CpG Island
miR-34b/c	cg22879515	GTCCTCCCCGGCAGCGCCGCCCGCTG GCCCAGCTACGCGTGTTGTGCGCTGCG AGGCCGG[CG]GGGGTCCCGCTGGG CCCGGGGGTGTCCTCGGGGGCC GCTTGCGCCCAGCCATGGTAGGGC	11	MIR34B; BTG4; MIR34C	NR_029839; NM_017589; NR_029840	TSS200; TSS1500; TSS1500	chr11: 111383168– 111383892	Island
miR-129-2	cg14416371	GAGACACGAGTCCAGGGGCGCGG AGGGGCGGGCAGCGCGCGGAGTG GTGAGACTGAGCCG[CG]ATGGAACG CGCTGGGGAGACCCAGCCTGTTC GGCTCCAGGGTTCGGAGACATCCTGGGCT	11	MIR129-2	NR_029697	TSS200	chr11: 43602545– 43603215	Island
miR-152	cg05687686	CAGCTTCGGCATATTTGGCGGAG CCGGGAAGGCCCGGAGCGCAAGAAGGCAT CGCAGCCT[CG]CAGCA GATCTGAAAGGGTTGTGGGCGGG GGGCTCATTTTCGCCGGATTTCTTTTCCGTGT	2	MIR1258; ZNF385B	NR_031659; NM_152520	TSS1500; TSS1500	chr2: 180725717– 180726465	S_Shore
miR-193b	cg09918657	AGTGGCGTTTCTGGTTTCTCTTTG CTTCCAATCCCCACCAAGCGGAG CGTTGGAATGCGC[CG]CTTATG TCCTCTGAGGACACATCCATATTT ATAATTTATTTTTAGGAGAAGTTGTGAAAA	16	MIR193B	NR_030177	TSS1500	chr16: 14395604– 14397075	S_Shore
miR-663a	cg08304190	GCCTCACGAGCCCCTGGTCCCGCCA CCGGGGCCCCGAAGCGACCACAG CCACAAACTCAA[CG]CCAGGGCCACA TCGCTCGTGATTCTCGTCCATCCTCCGAC CCGGTCCCGCTCCGGGAGAC	20	MIR663	NR_030386	TSS200	chr20: 26188638– 26190348	Island
miR-1258	cg05850656	ACTGCTCCAGAGCCCGAGTCGGAGTGTA TCACAGAACCTGGGCCGGGGGGGACA GCGGGC[CG]AGCCTCCTTCTTCCAGCT GATCCCTGGCCGGGCTGGACCTGCGCTA TCAGCGCGCCCCCA	17	MIR152; COPZ2	NR_029687; NM_016429	TSS200; Body	chr17: 46114573– 46115059	Island

Table 4. Validity estimates for miR's promoter methylation as markers for PCa in Cohort #1

microRNA	Sensitivity % (n positive/n total)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
miR-34b/c	94.4 (170/180)	86.7	98.8	56.5	93.8
miR-129-2	90.6 (163/180)	86.7	98.8	43.3	90.3
miR-152	79.4 (143/180)	86.7	98.6	26.0	80.0
miR-193b	90.0 (162/180)	100.0	100.0	45.5	90.8
miR-663a	84.4 (152/180)	100.0	100.0	34.9	85.6
miR-1258	97.8 (176/180)	100.0	100.0	78.9	97.9

PCa Prostate cancer; *PPV* positive predictive value; *NPV* negative predictive value

Then, we evaluated whether microRNA promoter methylation levels were associated with clinicopathological parameters. MiR-129-2 promoter methylation was associated with higher GS and pathological stage (P=0.0248 and P=0.0245, respectively), whereas, miR-34b/c, miR-663a and miR-1258 promoter methylation levels were only associated with higher pathological stage (P=0.0055, P=0.0386 and P=0.0303, respectively) (Figure 3).
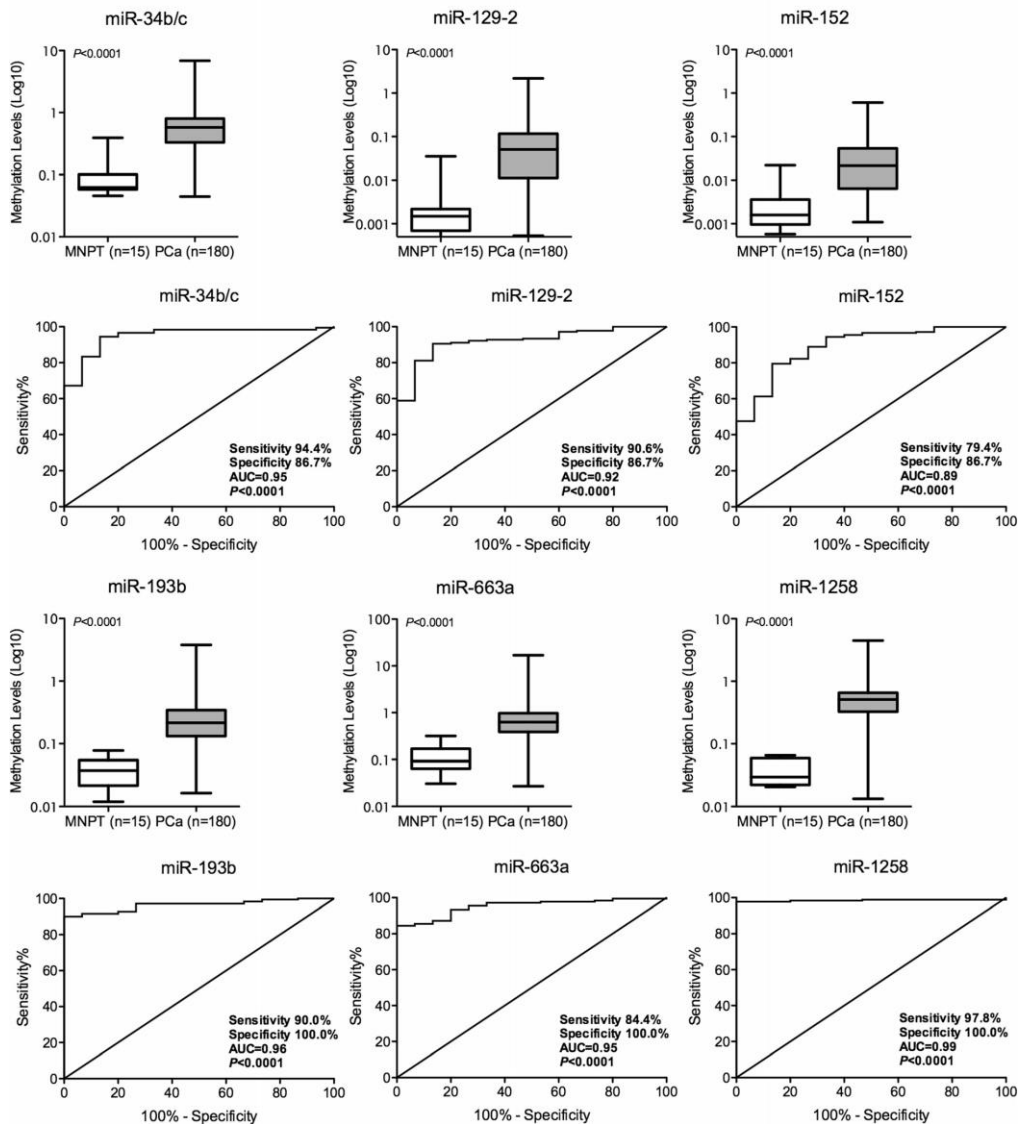
Figure 2. *Box-plots and ROC curves for miR-34b/c, miR-129-2, miR-152, miR-193b, miR-663a and miR-1258 promoter methylation levels in morphologically normal prostate tissue (MNPT) and malignant (PCa) prostatic tissues from Cohort #1*

To determine whether microRNA promoter methylation was PCa-specific, malignant and benign tissue samples from bladder (43 and 7) and kidney (50 and 9) were analyzed. MiR-34b/c, miR-193b and miR-1258 promoter methylation levels were significantly higher in PCa tissues compared to all other samples tested. Interestingly, miR-129-2 and miR-663a showed higher methylation levels in bladder cancer and were, thus, considered unsuitable for accurate detection of PCa in urine sediments (Supplementary Figure 1).
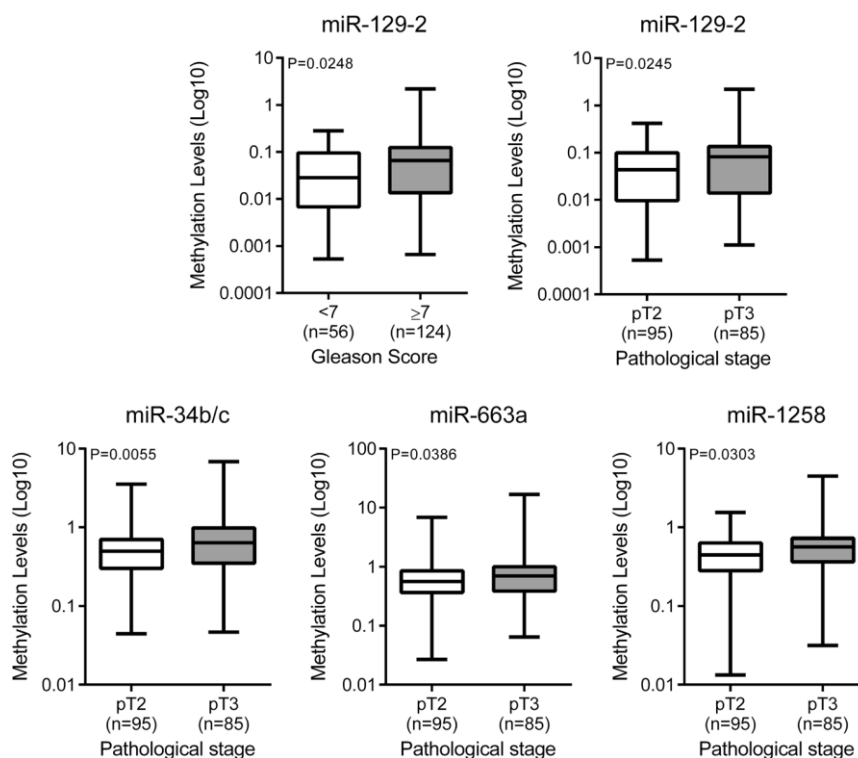
Figure 3. DNA methylation and Pathological parameters. *Distribution of methylation levels of microRNAs according to Gleason score and pathological stage in the series of patients submitted to radical prostatectomy (Cohort #1)*

MicroRNA promoter methylation in urine sediments (Cohort #3)

Best performing PCa-specific microRNAs - miR-34b/c, miR-193b and miR-1258 – were then tested in urine sediments, collected without previous prostatic massage, from PCa patients (n=95, Cohort #3) and controls (n=46). Higher miR-34b/c and miR-193b methylation levels and lower miR-1258 promoter methylation levels were depicted in PCa patients (Figure 4). MiR-193b promoter methylation displayed the best performance with high sensitivity (91.6%) and specificity (95.7%), providing an overall accuracy of 92.9% (AUC = 0.96). Moreover, the panel including both miRs (miR-34b/c, miR-193b) augmented specificity (97.8%) and positive predictive value (98.9%) (Table 5). Addition of miR-34b/c promoter methylation did not improve biomarker performance. No associations between microRNAs' promoter methylation levels and clinicopathological parameters were depicted in this series.

Urine sample set
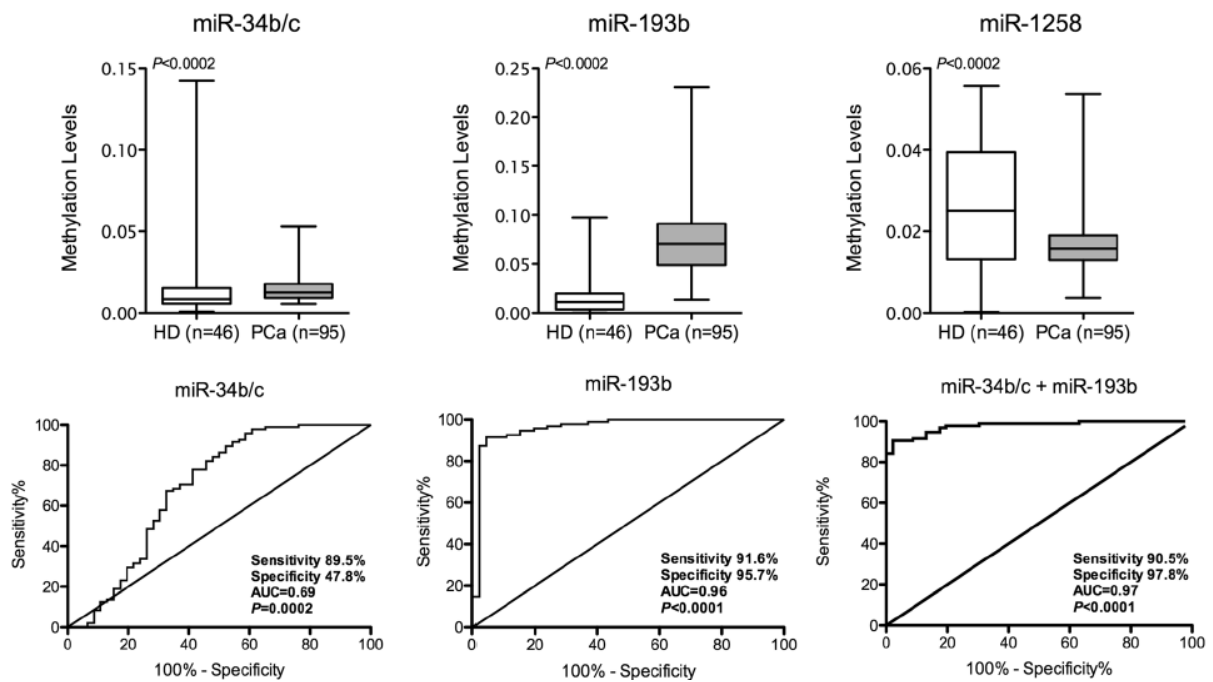
Figure 4. *Box-plots and ROC curves of miR-34b/c, miR-193b and miR-1258 promoter methylation levels across urine sediments of controls (HD) and prostate cancer patients (PCa) from Cohort #3. Receiver operator characteristic (ROC) curves were constructed to evaluate the performance of the gene promoter methylation panel (miR-34b/c+miR-193b)*

Table 5. *Validity estimates for miR's promoter methylation as markers for PCa in urine samples (Cohort #3)*

microRNA	Sensitivity % (n positive/n total)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
miR-34b/c	89.5 (85/95)	47.8	78.0	68.8	75.9
miR-193b	91.6 (87/95)	95.7	97.8	84.6	92.9
miR-34b/c+ miR-193b	90. 5% (86/95)	97.8	98.9	83.3	92.9

MicroRNA promoter methylation as prognostic biomarker (Cohorts # 1 & # 2)

Owing to its association with stage and GS, the prognostic value of miR-34b/c and miR-129-2 promoter methylation was further tested in the set of 180 radical prostatectomy (Cohort #1) and in a prospective group of 74 PCa suspects (Cohort #2).
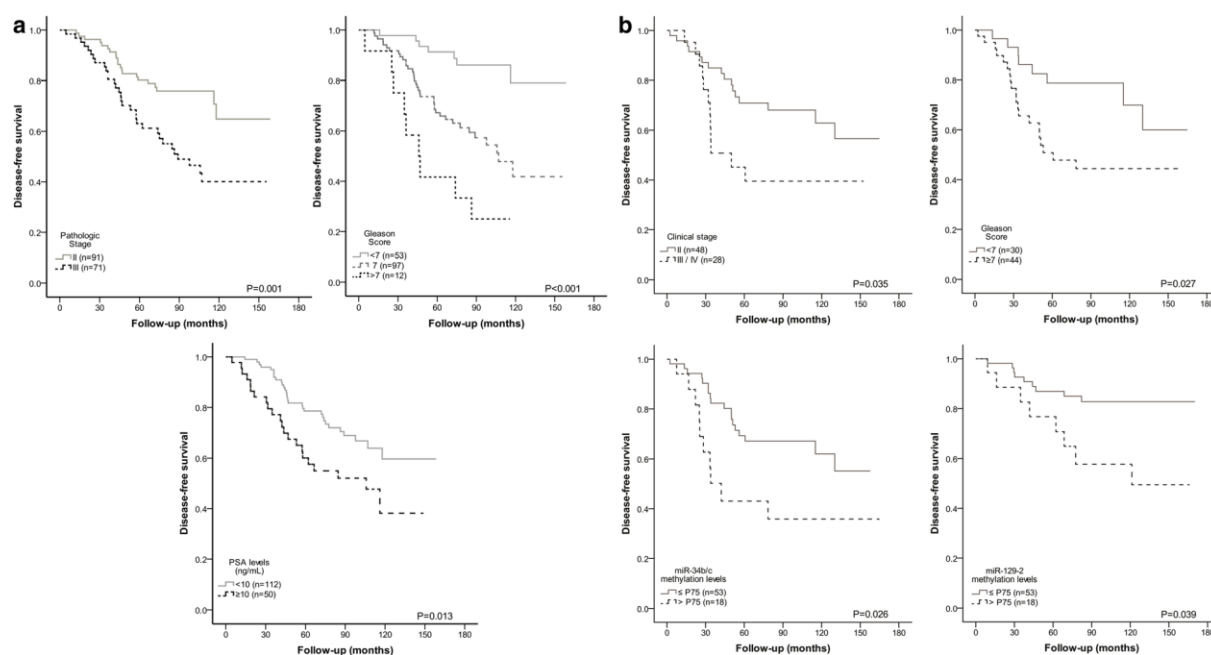
Figure 5. Survival Analysis. *Disease-free survival (DFS) curves based on pathological stage (upper left panel) and Gleason score (upper right panel), and miR-129-2 methylation levels (lower panel) in Cohort #1. b - Disease-free survival (DFS) curves based on clinical stage (upper left panel) and Gleason score (upper right panel), miR-34b/c (lower left) and miR-129-2 (lower right) methylation levels in Cohort #2*

The median follow-up in Cohort #1 was 110.1 months (range: 2.8–169.1 months). Nine patients (5%) had died from PCa and 50 (28%) developed biochemical recurrence. Eighteen were never free of disease and were excluded from DFS analysis. In this cohort, pathological stage, GS and PSA levels significantly associated with DFS (Figure 5A), whereas, only pathological stage and higher GS statistically associated with worse DSS (Figure 6A). Remarkably, high miR-129-2 methylation levels associated with shorter DSS. In multivariable analysis, only GS and PSA levels for DFS and GS and miR-129-2 methylation for DSS retained prognostic value (Table 6).

Regarding Cohort #2, the median follow-up was 114.9 months (range: 10.3–170.1 months). Thirteen patients (17.6%) had died from PCa and 29 (39.2%) developed biochemical recurrence. In 3 patients, serum PSA levels >0.2 ng/ml persisted following treatment and these were not further considered for DFS analysis. Advanced clinical stage, higher GS, higher miR-34b/c and miR-129-2 promoter methylation levels statistically associated with worse DFS (Figure 5B). In multivariable analysis only higher clinical stage and high miR-34b/c promoter methylation levels independently predicted shorter DFS (Table 6). Except for serum PSA, all clinicopathological parameters tested, as well as miR-34b/c and miR-129-2 promoter methylation levels associated with DSS in univariable analysis (Figure 6B).
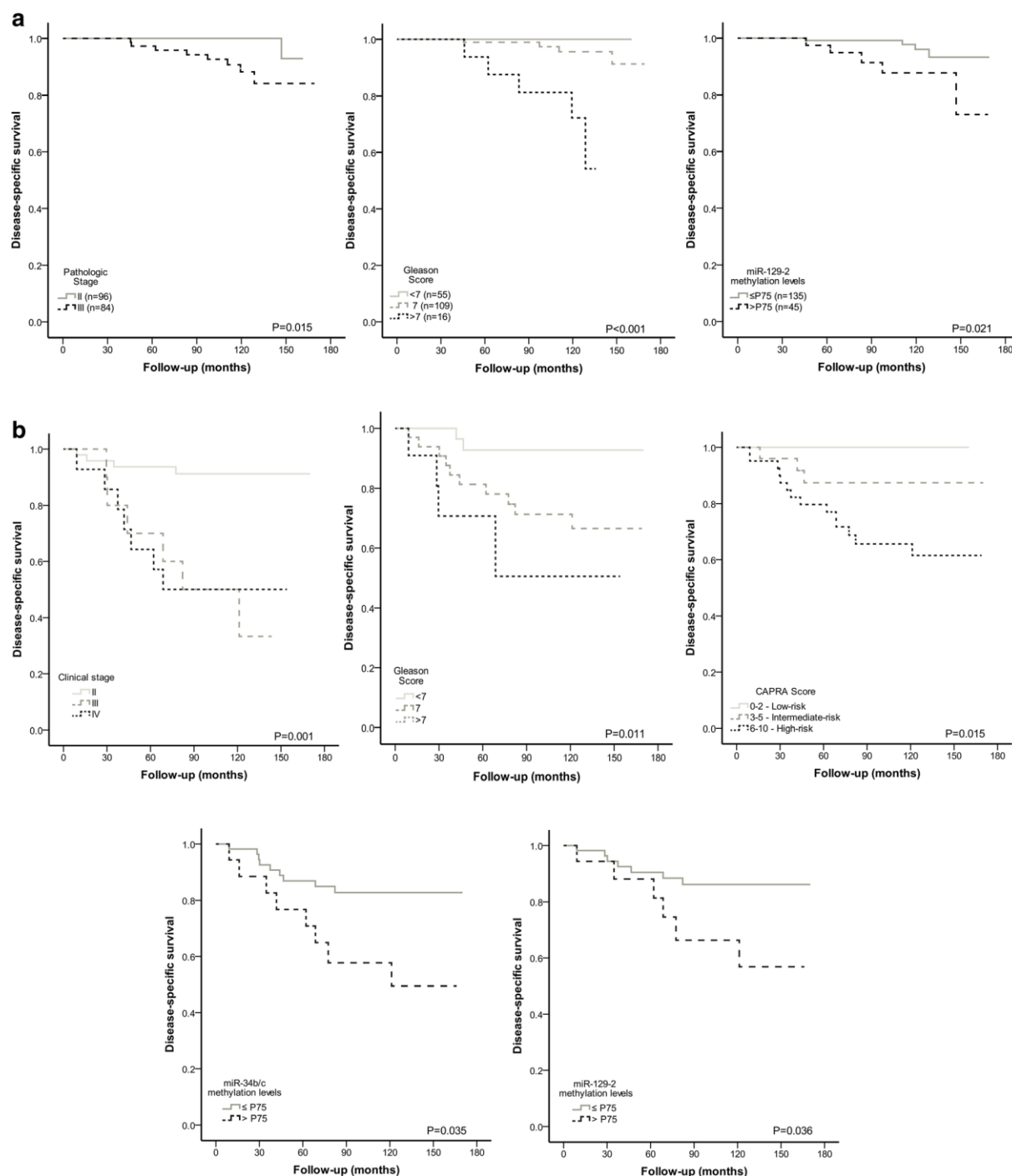
Figure 6. *Disease-specific survival (DSS) curves based on pathological stage (upper left), Gleason Score (upper right), and miR-129-2 methylation levels (lower) in Cohort #1. B - Disease-specific survival (DSS) curves based on clinical stage (upper left), Gleason Score (upper center) CAPRA Score (upper left), miR-34b/c (lower left) and miR-129-2 (lower-right) methylation levels in Cohort #2.*

Similarly to DFS, in multivariable analysis only clinical stage and high miR-34b/c promoter methylation levels independently predicted shorter DSS (Table 6).

Table 6. Cox regression analysis assessing the potential of clinical and epigenetic variables in the prediction of disease-specific survival and disease-free survival in the Cohort #1 and Cohort #2.

Cohort #1

Disease-specific survival – Cox regression analysis				Disease-free survival – Cox regression analysis			
Variable	HR	CI (95%)	P	Variable	HR	CI (95%)	P
Gleason Score				Gleason Score			
				<7	1		
≤7	1			7	3.96	1.76–8.91	0.001
>7	18.97	4.32–83.351	<0.001	>7	7.73	2.85–21.0	<0.001
miR–129-2				PSA			
≤ P75	1			<10	1		
> P75	6.12	1.56–24.07	0.009	≥10	1.87	1.07–3.26	0.027

Cohort #2

Disease-specific survival – Cox regression analysis				Disease-free survival – Cox regression analysis			
Variable	HR	CI (95%)	P	Variable	HR	CI (95%)	P
Clinical stage				Clinical stage			
II	1			II	1		
III/IV	9.64	2.60-35.8	<0.001	III/IV	2.57	1.18-5.60	0.018
miR-34b/c				miR-34b/c			
≤ P75	1			≤ P75	1		
> P75	3.84	1.27–11.6	0.017	> P75	2.76	1.24–6.15	0.013

HR hazard ratio, *CI* confidence interval

Discussion

PCa remains one of the most prevalent neoplasms and a leading cause of morbidity and mortality in men. Although PSA screening has decreased the number of men diagnosed with metastatic PCa, this was accomplished at the cost of overdiagnosis and overtreatment of a sizeable proportion of men carrying indolent/non-life threatening tumors (13). Thus, a strong recommendation against serum PSA-based PCa screening has been issued (14), prompting the search for more effective biomarkers allowing for better risk stratification of PCa suspects. Herein, we aimed to tackle this clinical quest through discovery and preliminary validation of novel biomarkers for PCa detection and prognostication, using methylation analysis of microRNAs gene promoters.

Owing to our previous experience in DNA methylation analysis of PCa (6, 11), we searched for altered methylation patterns at the promoter regions of microRNAs deregulated in PCa.

This information was then used to develop novel biomarkers, instead of microRNA expression levels, as previously attempted by other researchers (15). Indeed, DNA methylation is easier to assess than microRNA expression, it is more specific and, importantly, more stable. Moreover, because microRNAs downregulation in cancer is more common than upregulation, it seemed likely that aberrant promoter methylation might constitute an underlying mechanism, similar to protein-coding genes (16). Although several strategies might be used to identify microRNAs putatively downregulated due to promoter hypermethylation, high-throughput technologies such as methylation-array analysis are able to simultaneously pinpoint putative candidates (17) and the reliability of the results might be readily assessed through analysis of well-known hypermethylated loci. Indeed, results of the methylation array experiments confirmed the high prevalence of *GSTP1* and *APC* promoter methylation (data not shown), as we previously demonstrated in PCa (18). To increase the likelihood of finding robust candidate biomarkers, we used stringent conditions based on high fold-variation of methylation levels between cancerous and non-cancerous tissue samples. From methylation-array analysis, six candidate microRNAs, putatively deregulated by promoter hypermethylation were identified. MiR-1258, miR-193b and miR-34b/c were the most promising candidates, displaying substantial PCa-specificity compared with other urinary tract tumors, an attractive feature for testing in bodily fluids. MiR-129-2 and miR-663a showed modest results and their inability to discriminate PCa from bladder cancer rendered it unsuitable for testing in urine samples.

Association between promoter methylation levels in tumor tissue samples and standard clinicopathological variables was also assessed. Higher miR-129-2 promoter methylation levels associated with higher GS and stage, suggesting prognostic value. MiR-34b/c, miR-663a and miR-1258 methylation levels also associated with pathological stage, but higher diagnostic performance underscores the potential for detecting PCa at early stages instead of prognostication, as we previously reported for *EFEMP1* promoter methylation (19). Nevertheless, in this series of radical prostatectomies (Cohort #1) higher miR-129-2 methylation conveyed independent prognostic information, although only for DSS. Importantly, these results are in line with previous observations concerning the association of higher gene promoter methylation levels with clinicopathological features of more aggressive disease (11, 20).

Urine is a key sample to evaluate DNA methylation biomarkers for PCa, as it is readily collected and biomarkers are diluted to a smaller extent than in plasma, providing higher sensitivity (21). Nevertheless, the amount of DNA potentially deriving from prostatic cells is variable, usually low, entailing the use of a panel with limited number of biomarkers. Thus, only miR-34b/c, miR-193b and mir-1258, were tested in urine samples (Cohort #3). From these, Mir-193b was previously shown to be aberrantly methylated in PCa cell lines as well

as in primary tumors, but no data is available regarding its performance as PCa detection biomarker (22, 23). Indeed, Mir-193b performed best, with high AUC, sensitivity, specificity and PPV, whereas miR-34b/c performance was more modest.

Intriguingly, miR-1258, which showed the best performance in tissue samples (Cohort #1), displayed a strikingly different result in urines as its methylation levels were higher in controls than in PCa patients. The reason for this discrepant result is not immediately apparent, but it might be due to high miR-1258 promoter methylation in non-epithelial cells, such as leucocytes, which are relatively more abundant in urine than in tumor tissue samples. Moreover, median miR-1258 promoter methylation levels in urines from PCa were substantially inferior to those of miR-193b, impairing the robustness of the assay. It should be recalled that, contrarily to other studies, the urine samples we used were not collected following DRE or prostatic massage, which are usually employed in an attempt to increase sensitivity. Studies dealing with PCa biomarkers in urine vary in the method of urine collection and the real impact of prostatic massage has never been evaluated (24). It could be argued that the distance from the peripheral zone to the urinary tract flow may render urinary based tests less sensitive, which would be an important issue since most malignancies arise from this zone. Nevertheless, studies on *PCA3* did not find a difference in the levels of this biomarker between patients with peripheral versus transitional zone PCa (25, 26).

Currently, the performance of serum PSA and urinary *PCA3,* the only biomarkers approved for clinical use is rather limited. The reported performance of serum PSA as PCa biomarker is somewhat modest, with AUC ranging from 0.54 to 0.70 (27, 28). Even other serum PSA-derived measurements, like PSA-density, free PSA percentage and PSA-velocity have not significantly improved performance (28). Nonetheless, *PCA3*, which was reported to perform better than serum PSA both in urine and ejaculates but has not been approved for population-based screening, displays AUCs varying from 0.66 to 0.79 (27-30). Additionally, although miRs' expression has been extensively investigated in liquid biopsies, available data for urine samples is rather limited. Nevertheless, an AUC of 0.74 was reported for miR-107 (31) and simultaneous quantification of miR-107 and miR-574-3p in urine showed an AUC of 0.83, for PCa cancer detection (32). We should emphasize that in our dataset, urinary miR-193b promoter methylation (AUC = 0.96) outperformed not the only currently approved clinical biomarkers, but also the previously mentioned miRs, constituting a promising tool for non-invasive PCa detection.

Because a major goal of this study was to discriminate clinically aggressive from indolent PCa, it was critical to test the prognostic value of microRNAs in a pre-therapeutic setting, which was accomplished in series of prospectively collected prostate biopsies (Cohort #3). In univariable analysis, most standard clinicopathological parameters associated with DFS and

DSS, clinically validating this dataset. The same was demonstrated for higher miR-129-2 and miR-34b/c promoter methylation levels. The CAPRA score, however, only associated with DSS but not DFS. This was unexpected as its determination at diagnosis associated with DFS in patients with clinically localized disease submitted to RP (12). Notwithstanding, our prostate biopsy series included PCa at diverse clinical stages, submitted to different therapeutic modalities (RP, radiotherapy, androgen-deprivation therapy), which might explain the apparent flaw of CAPRA score. In multivariable analysis, only clinical stage, amongst all clinicopathological parameters, retained independent prognostic value, both for DFS and DSS. Remarkably, high miR-34b/c promoter methylation levels also predicted shorter DFS and DSS, suggesting that it might constitute a useful PCa prognostic biomarker. These results suggest that high miR-34b/c promoter methylation levels identify clinically aggressive PCa, irrespective of disease extent at diagnosis.

It should be acknowledged that in spite of the excellent diagnostic performance of miR-193b promoter methylation in urine, additional patient sets must be tested. Furthermore, a larger cohort of patients submitted to biopsy and subjected to different therapies is required to further validate our observations. Ultimately, we plan to develop a multiplex assay to simultaneously assess miR-193b, miR-129-2 and miR34b/c promoter methylation, allowing for diagnostic and prognostic assessment of PCa suspects in a single analysis.

Conclusion

Through genome-wide screening, a set of methylation-based PCa biomarkers was identified and validated. MiR-193b demonstrated high sensitivity and specificity for detection of PCa, both in tissue and urine, whereas high miR-129-2 and miR-34b/c methylation levels independently predicted for shorter DSS and DFS or DSS, respectively. If confirmed in larger and independent datasets, quantitative promoter methylation of selected miRs might provide useful tools for clinical management of PCa patients.

Authors' contributions

Conceived and designed the experiments: JR-C, RH, CJ. **Performed the Experiments:** JT-F, JR-C, AG, FDM, RF, JO, ME. **Analyzed the data:** J T-F, JR-C, LA, AG, RH, CJ. **Contributed reagents/material/analysis:** JT-F, LA, JO, RF, RH, CJ

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PART III – DISCUSSION AND CONCLUSIONS

CHAPTER 8 – GENERAL DISCUSSION

GENERAL DISCUSSION

PCa is the second most common cancer in men and the fourth most common tumor type worldwide, constituting a major cause of morbidity and mortality (53). Several features, comprising age, family history, genetic susceptibility, and race, impact the incidence of PCa (54). Due to PSA screening, approximately 90% of PCa are clinically localized at the time of diagnosis (55). This led not only to a decrease in PCa-related mortality rates but, also to an overdiagnosis and overtreatment of indolent, low grade cancers that, probably, would not be clinically apparent during a man's lifetime (56). The clinical behavior and molecular alterations of PCa are highly heterogeneous (57). Clinically, some men will have aggressive disease leading to metastasis and death, whereas many others will have indolent cancers that are cured with initial therapy or would be suitable for watchful waiting or active surveillance (58). Multiple risk stratification parameters have been developed, combining the best currently available clinical and pathological parameters (such as Gleason score, serum PSA levels, and clinical and pathological staging). These tools, however, still do not satisfactorily predict outcome in an individual basis (56) nor help distinguish indolent from aggressive PCa.

MiRNAs have been suggested as important regulators of biologic processes in PCa progression (59). An expression profile aiming to characterize each PCa subtype and stage has still not been proposed for PCa. One could argue that this task has been hindered by the molecular heterogeneity as well as differences in study design and patient selection (60). The advance of high-throughput sequencing and comparative genomics studies support that a miRNA may target hundreds of sites transversely in the transcriptome (61). Not surprisingly, most predicted miRNA targets experience small changes at the mRNA and protein levels when miRNA's expression is disrupted (28, 36, 61). MiRNAs are believed to establish thresholds in and confer coherence to the expression of its target genes, as well as reduce the cell-to-cell variability in target gene expression (28, 62). Globally, miRNAs control gene expression during developmental and pathological processes. Understanding how miRNAs become altered in PCa constituted the main focus of this work.

Thus, three different aspects have been emphasized: mechanisms driving the deregulation of miRNAs; the cellular pathways altered upon miRNA manipulation; and the translational potential of miRNAs for PCa detection and prognostication.

Globally, new concepts were exposed from this Thesis describing:

- The dynamics of miRNA regulation in PCa;
- The consequences of miRNA–target interactions;

- The role of miRNAs in regulation of cell cycle, DDR and senescence;
- MiR-193b and miR-129-2 promoter methylation potential clinical usefulness as early diagnostic and prognostic biomarkers.

In following section, the most relevant results of the manuscripts previously presented are discussed. Altogether, the results obtained disclosed the critical biological significance of miRNAs in prostate carcinogenesis and open new perspectives for diagnostic/prognostic tools in PCa.

microRNA deregulation in Prostate Cancer

MiRNA profiling enabled high-throughput analysis of the miRNAome of PCa, identifying a global downregulation. Nonetheless, due to the inherent heterogeneity of PCa, sample selection and technological platforms used, some inconsistencies are still apparent (59). MiRNAs' expression alterations can be caused by promoter methylation, chromosomal rearrangements or transcriptional deregulation (63). Indeed, 20–40% of miRNAs are located near CpG islands, confirming their possible epigenetic regulation (63). The miRNAs are frequently located within fragile chromosomal sites that exhibit DNA amplifications, deletions, or translocations during tumor progression (64). Consequently, miRNAs have been characterized either as tumor-suppressors or oncogenes, depending on the expression pattern and targeted transcripts (59). Herein, because a significant percentage of miRNAs were down-regulated (65), there was a need to characterize the mechanisms underlying the regulation of miRNAs in PCa.

Global miRNA down-regulation might also be caused by miRNA biogenesis machinery failure, in addition to genetic and epigenetic alterations. The expression of miRNA biogenesis machinery molecules has a major impact in both developmental and tumorigenic processes, since they are often deregulated in cancer (66), and specifically in PCa (67). Specifically, *DROSHA* expression levels have been shown to be downregulated in several types of cancer, being systematically correlated with invasion, metastasis (68) and reduced patients' survival (65, 69, 70). Conversely, *DGCR8* was found to be upregulated in different tumor types and correlated with tumor stage (71, 72). However in our dataset, *DGCR8* was found to be downregulated, and associated with deregulated miRNA expression. Moreover, deletion of Dgcr8 impaired tumor progression in a Pten-knockout mouse model of PCa, supporting its oncogenic function (73). These results are in sharp contrast with those presented herein. Considering that Drosha and DGCR8 regulate each other post-transcriptionally, downregulation of both DROSHA and DGCR8, depicted in Chapter 4, may suggest that a number of mRNAs may be downregulated in a microprocessor-dependent way in PCa (74).

Finally, Dicer has been considered a haploinsufficient tumor-suppressor gene. Indeed, *Dicer1* loss diminished survival in a mouse model of lung cancer (75). Therefore, decreased *DICER1* expression (Chapter 4) may result in global miRNAs downregulation in PCa, because precursor miRNA (pre-miRNA) processing is compromised. Notably, *DICER1* was reported to be upregulated in PCa, causing not only global deregulation of miRNAs but also correlating with higher clinical stage, lymph node status, and Gleason score (76).

The reason underlying deregulation of processing molecules is still not fully understood. One may argue that different cancers have different genetic or epigenetic mechanisms controlling genes expression of the biogenesis machinery, thus ensuing the abnormal expression of oncogenic or tumor-suppressive miRNAs in a given cell type. Although impaired miRNA processing enhances cellular transformation and tumorigenesis, it only partially justifies our observations.

Epigenetic alterations, such has DNA methylation and histone modifications, are also considered to be of great importance for miRNA gene regulation. They are reported as being more prevalent than genetic alterations, to be present in pre-malignant lesions and as driver events in PCa development and progression. Tumor-suppressive miRNAs are frequently hypermethylated in cancer, thus causing the epigenetic silencing of these miRNAs. However, as epigenetic alterations are reversible, pharmacological inhibition of DNA methylation with DNA-demethylating agents can reactivate the expression of tumor-suppressive miRNAs.

In Chapter 4, miRNA profiling combined with reactivation using 5-Aza-CdR, rendered a substantial increase of miRNA expression in the cell lines tested. Although this approach is an indirect evidence of miRNA repression by DNA methylation, when combined to miRNA profiling in primary PCa and *in silico* prediction of CpG islands, it enabled the identification of multiple miRNAs potentially regulated by DNA methylation. As expected, the reactivation profile of each cell line differed for expression and regulation. Nonetheless, the expression profiling enabled the discovery of new DNA methylation-regulated miRNAs (e.g., miR-130a) and validation of previous findings (e.g., miR-145 or miR-205). Regarding miR-130a, although previously implicated in several malignancies, its regulation in PCa was never dissected. Our data showed that miR-130a is a *bona fide* tumor-suppressor miRNA and its downregulation is both associated with promoter hypermethylation and a repressive histone mark enrichment (H3K27me3).

As DNA methylation is an appropriate indicator for regulatory activity, we aimed to discover new miRNA loci targeted for aberrant DNA methylation patterns in primary PCa (Chapter 5). The Infinium HumanMethylation450 bead array generated a methylome of 51 miRNA (42 hypermethylated and 9 hypomethylated). This approach had the advantage to map, in clinical prostate tissue samples, miRNAs regulated by DNA methylation, regardless of expression *status*. The validation of this approach was accomplished by the identification of

previously reported hypermethylated miRNAs: miR-34b/c, miR-193b and the cluster miR-27b~23b~24. The data analysis rendered the identification of a new cluster regulated by promoter methylation: miR-130b~301b.

Nonetheless, the intersection between the miRNAs determined as downregulated in Chapter 4 and those found as hypermethylated in Chapter 5, was rather limited. Indeed, in Chapter 6, only miR-10a, miR-23b, miR-27b, miR-34c, miR-152, and miR-335 fulfilled the criteria above mentioned. MiR-152 (and its hostgene *COPZ2*) was found to be at partially regulated by 5mC, as its expression was restored upon 5-Aza-CdR in both LNCaP and PC3 cell lines associated with significantly decreased DNA methylation levels.

The identification of miRNAs' functional alterations greatly benefited from the combination of comprehensive integration of high-resolution profiling techniques, both at expression and regulatory levels (e.g., DNA methylation). Indeed, our data demonstrates that, in PCa, miRNAs' global downregulation is a multilayered controlled process: epigenetic alterations impair primary miRNA (pri-miRNA) transcription, the decreased expression of *DROSHA* and *DGCR8* (the Microprocessor complex) apparently is associated with defective pre-miRNA generation in the nucleus, and ultimately, diminished *DICER* indicates that cytoplasmic pre-miRNA processing to generate mature miRNAs is also impaired. Overall, these findings highlight the importance of miRNA deregulation in PCa and indicate that miRNA expression profile is the most suitable method to identify deregulated miRNA, rather than interrogating alterations in regulatory elements, such as promoter DNA methylation.

The functional impact of microRNA downregulation in PCa

In addition to discover new miRNA downregulated in PCa, it was also our goal to uncover molecular pathways targeted by miRNAs deregulation that might decisively contribute for prostate tumorigenesis. In Chapter 4, miR-130a was selected for further analysis, whereas in Chapter 5, cluster miR-130b~301b's role was addressed. Strikingly, these miRNAs are members of the same highly conserved miRNA family: miR-130. Both share the same seed sequence (AGUGCAA) and are, thus, expected to target the same pathways and have affinity for common targets (28). This reinforces the network-regulating roles of these miRNAs. MiRNAs from the same family often target the same or similar subsets of genes or the same pathway or process. These observations are supported by previously reported polycistronic miRNAs and /or family-related miRNAs that control critical pathways such as epithelial-to-mesenchymal transition (EMT) (suppressed by the two polycistronic genes that encode the five members of the miR-200 family) (77, 78) or EMT and metastasis activation by the coordinated actions of miR-96, miR-182 and miR-183 (79).

Due to the capacity of miRNAs to control multiple-target genes, they are emerging as regulators of the hallmarks of cancer (33). Both miR-130a and the cluster miR-130b~301b

were found to be functionally relevant for PCa development, functioning as tumor-suppressor miRNAs. Indeed, these miRNAs proved to reduce cell viability, induce cell death by apoptosis and impair invasion ability. MiR-130a and cluster miR-130b~301b have already been implicated in multiple cancers, both as onco-miRNAs or tumor-suppressive miRNAs. MiRNAs may, indeed, have different, or even opposite, functional outcomes in different contexts. These discrepancies likely reflect the differential expression of target genes between tissues and serve both as a reminder of the complexity associated with miRNAs and as a cautionary note for the rigid assignment of any given miRNA to one specific role or function (80).

As miR-130a and cluster miR-130b~301b are parolog miRNAs, the data presented in this Thesis suggests a functional specialization and cooperation that may coexist among members of the same family. The phenotypic impact of each miRNA is very similar, however, each miRNA had the ability to trigger different signaling pathways. It is worth to mention that, due to the seed sequence similarity in a given miRNA family, it is expected that the miRNAs from the same cluster might also have additional specific targets, as a result of other base parings determinants besides seed-sequence. In a cooperative network, restoration of both any parolog miRNA will ultimately have the same functional impact (80). Nonetheless, we showed that the magnitude of fold change differs, and in common genes tested in both Chapter 4 and Chapter 5, there was a different gene expression modulation, reinforcing the functional specialization of each miRNA. Moreover, miRNA activity is cell-type specific, as described for the endogenous inhibition and functional output of each miRNA in the different cell lines tested in Chapter 5. Overall, it is clear that miRNAs exert their effects in the context of complex regulatory networks.

Although in Chapter 4, specific targets for miR-130a were found (among which *SEC23b* and *DEPDC1* were validated), the genome-wide data following pre-miR-130a overexpression, revealed a large number of genes upregulated upon transfection. In Chapter 5, some of these genes were also validated showing the same trend (e.g. *MMP1*, *MMP10*). Thus, restoration of miR-130a, miR-130b or miR-301b significantly increased the expression of genes acting as checkpoint sensors needed for tumour-suppression. Moreover, the simultaneous miRNA targeting of multiple genes may facilitate specific fine-tuning through the regulation of distinct pathways (80). Nonetheless, indirect effects caused by miRNA deregulation are also observed, as miRNAs can initiate indirect effects on gene expression through the downstream activities of miRNA-targeted transcription factors (80, 81). Most changes in mRNA level after miRNA manipulation may actually be due to altered transcription and not as direct transcript-destabilizing effects of miRNAs *per se* (81). This is well documented for miR-200 family concerning repression of the master EMT regulators *ZEB1* and *SNAIL1* (82).

Whether these alterations are a direct measure of miRNA-mRNA interactions in the 5'UTR or promoter (83), or the naive output of tumor-suppression activities, it is still unclear. Furthermore it is tempting to speculate that miRNAs might interact with other regulatory elements and, consequently, enhance transcription or translation of those genes (83). Globally, many miRNAs have evolved not acting as genetic switches of specific pathways or individual targets, but rather as modulating the expression of large gene networks (47).

One major finding of this Thesis was ability of these parolog miRNAs to induce growth and stable cell cycle arrest by cellular senescence. Senescence may counteract tumor formation in different contexts and tissues (84). This biological phenomenon causes a permanent cell proliferation arrest as a strategy to prevent genomic instability (85). Senescence is induced by multiple mechanisms, such as progressive shortening of telomeres during cellular replication and DNA damage. Senescent cells are characterized by expression of a diversity of indicators that reveal irreversible proliferation arrest, including an increase in cell size and a more flattened shape, increased levels of p53 and of the cell cycle inhibitors *CDKN2A* (p16), *CDKN1A* (p21) and *CDKN1B* (p27) or *Lamin B1* downregulation (86, 87). However, the senescent phenotype was more evident when miR-130b or miR-301b were modulated. The genome-wide study depicted in Chapter 4, demonstrated that miR-130a overexpression induced secretion of a diversity of growth factors, cytokines, and proteases, known to be part of the senescence-associated secretory phenotype (SASP). SASP seems to be a common feature of different types of cellular senescence responses that in oncogene-induced senescence (OIS) is regulated by persistent DDR (88, 89). Indeed, in Chapter 5 it was demonstrated that both senescence and SASP are related with DNA damage.

Induction of SASP markers by miR-130b or miR-301b was more impressive than that by miR-130a. SASP can act non-cell autonomously to suppress tumorigenesis by promoting an antitumor microenvironment, in part through secreted factors that modulate prostate immune response (90). Overall, it may be assumed that miR-130b and miR-301b regulate DDR and may, thus, mediate OIS. Nonetheless, other types of senescence responses present display SASP in the absence of DDR, suggesting additional mechanisms for SASP regulation (91), such as NF-KB (92) or STAT3. SASP factors have been reported to reinforce the senescence program (93) and, tumor clearance (94) in an autocrine manner and to promote aggressiveness in a paracrine mode (95). SASP factors can also promote immune surveillance of senescent cells, leading to its elimination both from tumors and normal tissues (94, 96). Nevertheless, the impact of SASP on cancer initiation and progression is still controversial, and several studies strongly suggest that, at least in some contexts, SASP may fuel inflammation and exert pro-proliferative activity, thus contributing to tumorigenesis (97). Overexpression of those parolog miRNAs induces alterations in SASP factors, such as *IL1A*, *IL6* or *MMP1*. Overall, these findings supports that miR-130a, miR-130b or miR-301b

overexpression induces autocrine SASP signaling, whereas endogenous blocking of these miRNAs is possibly involved in paracrine signaling. This observation is based on a slight increased expression of *IL1A*, *IL1B*, and *IL6.* Therefore, cellular senescence constitutes a barrier against tumor progression but, if senescent cells are not quickly removed, they may become deleterious because different SASP signaling may reinforce tumorigenesis (86). Thus, cluster miR-130b~301b seems to be intimately associated to senescence by controlling DNA damage response-related genes, but miR-130a might have a broader action. The miR-130a target *SEC23B* is involved is endoplasmic reticulum (ER)-stress response, a form of stress caused by the accumulation of unfolded proteins in the ER. The ER-stress may induce multiple adaptive mechanisms that constitute the Unfolded Protein Response (UPR). UPR activation is able to restore protein-folding homeostasis. However, in case of severe impairment, UPR signaling triggers cell death by apoptosis. Interestingly, not only miR-130a overexpression increased apoptotic levels, but also *SEC23B* knockout increased the apoptosis rate in PC3 cells. These observations suggest that inactivation of miR-130a circumvents apoptosis induction by ER-stress, due to the upregulation of *SEC23B*.

Interestingly, sustained UPR activation in response to continued ER-stress causes death of premalignant cells to prevent neoplastic progression (98). For instance, *HRAS* induces UPR-mediated cell senescence in premalignant cells (99). It would be a selective advantage for malignant PCa cells harboring miRNA alterations (e.g., miR-130a) that suppress UPR-induced apoptosis or senescence.

Lastly, it has been showed that induction of senescence by LMNB1 depletion in human diploid fibroblasts (HDFs) triggers large-scale the formation of chromatin domains enriched for H3K4me3 and H3K27me3 (termed *mesas*) and depleted for H3K27me3 (entitled *canyons*) in senescent HDFs (100). These were correlated with the expression of key senescence-associated genes (100). It was also detected a redistribution of LMNB1 along the genome: LMNB1 is specially reduced from regions enriched for H3K9me3 rather than H3K27me3, but there are small regions (~2% of the genome) where LMNB1 accumulates (101). Genes within these LMNB1-increased regions, which include some cell cycle genes, tend to be repressed during senescence, with increased H3K27me3 across the gene bodies. LMNB1 reduction (particularly from H3K9me3 regions) and spatial repositioning of perinuclear heterochromatin (H3K9me3-enriched) and SAHF formation (101). Overall, these studies suggest that alteration of LMNB1 might also contribute to senescence through gene regulation in some specific regions. Thus, the cluster miR-130b~miR-301b may act as an indirect player in the regulation of chromatin in prostate cells, as it overexpression causes LMNB1 downregulation and might trigger LMNB1-mediated chromatin reorganization.

In Chapter 6, restoration of miR-152 was shown to attenuate the malignant phenotype in both LNCaP (Androgen Receptor (AR) sensitive) and PC3 (AR castration resistant) cells.

Moreover, TCGA data analysis confirmed that this miRNA is downregulated in primary PCa. The most persistent effect of miR-152 restoration was the ability to control cell cycle progression in the two cell lines tested. Indeed, miR-152 induced cell cycle arrest in two different checkpoints: S and G2/M. This indicates that miR-152 may control same signaling pathways depending on the cellular and molecular context. As previously demonstrated, the miR-152's overexpression caused a significant attenuation of the PCa cells malignant phenotype: decreased invasion and augmented apoptosis levels. Interestingly, a specific target of miR-152 was found, *TMEM97*, a conserved integral membrane protein.

Cumulatively, there is a co- regulation of common biological processes by different miRNAs in PCa (47).

Overall, the data gathered in this Thesis demonstrates that miRNAs occupy a very particular position in the hierarchy of gene regulation. In contrast to the diversity of transcription factors and genetic *switches*, miRNAs in most cases do not seem to act as major regulators of gene expression. Their mechanism of action allows them to act as fine tuners of transcriptional networks, and as 'post-transcriptional buffers' that, ultimately, present robustness to transcriptional programs in the face of environmental and genomic variability (39).

MicroRNAs promoter methylation as potential biomarker for PCa

Finally, the last topic addressed in this Thesis was the potential of miRNA deregulation to be translated into clinical application. DNA methylation data gathered in Chapter 7 was then used for development of novel PCa biomarkers, instead of miRNA expression levels, as previously attempted by others [91-93]. Indeed, DNA methylation is easier to assess than miRNA expression, not only because it is more specific, but, importantly, because it is more stable. In fact, DNA methylation analysis uncovered valuable detection biomarkers for this disease, based on protein coding genes such as *GSTP1*, *APC*, *RARβ2* and *RASSF1A* [69]. We attempted to extend these observations for miRNAs. Thus, from methylation-array analysis, six miRNAs loci emerged as promising candidates and were further tested. MiR-34b/c, miR-193b and miR-1258 showed very high sensitivity, specificity and accuracy in tissue samples. Additionally, they demonstrated significant PCa-specificity compared with other tumors from the urinary tract, a feature that would make it the most promising candidate to be tested in bodily fluids. Indeed, urine samples analysis entails a huge potential for clinical implementation. It is minimally-invasive clinical material in which biomarkers are diluted to a smaller extent than in plasma, providing higher sensitivity. In a cohort composed of healthy donors and patients harboring PCa, miR-193b displayed the best results in this assessment, with high area under the curve (AUC), sensitivity, specificity and positive predictive value (PPV), whereas miR-34b/c performance was modest. Of note, the urine samples utilized were not collected following digital rectal examination **(**DRE) or

prostatic massage, which are usually employed in an attempt to yield a more representative sample to increase sensitivity. MiR-34b/c and miR-193b showed higher methylation levels in urine samples from PCa patients, as would be expected, showing better results than *GSTP1*, *RARβ2* and *APC*. *GSTP1*, *RARβ2* and *APC* promoter methylation levels assessed in urine samples collected after DRE showed deceptive performance as biomarkers for PCa detection (AUCs varied from 0.63 to 0.68) (102). In another study using urine samples following prostatic massage, Rouprêt *et al*. assessed methylation levels of several genes. Of these *GSTP1*, *RASSF1A*, *APC* and *RARβ2* were those that best discriminated malignant from non-malignant cases, with AUC values ranging from 0.74 to 0.86. The combination of these four genes yielded the greatest discriminatory power of 86% sensitivity and 89% overall accuracy [101].

Moreover, when compared to serum PSA and urinary *PCA3*, the performance of miR-193b is far better. The performance of serum PSA as PCa biomarker is rather modest, displaying AUC ranging from 0.54 to 0.70 [53, 100]. *PCA3*, which is currently the most widely used non-PSA based first-line test, performed better than serum PSA, both in urine and ejaculates, with AUC varying from 0.66 to 0.79 [53, 100, 102, 103]. These data emphasize that miR-193b performance compares well with those two PCa biomarkers and might constitute a promising tool for early non-invasive detection of PCa. Essentially, the diagnostic performance of miR-193b in urine compares positively with the abovementioned biomarkers, but it is mandatory to test it in larger and independent datasets.

Strikingly, miR-130a promoter methylation was also found to be a specific PCa biomarker (Chapter 4). Although performance was not as good as in tissue samples, PCa was correctly identified with 83.5% sensitivity and 82.3 % specificity (AUC= 0.89) in urine. However, this performance is rather limited when compared with miR-193b promoter methylation levels, although larger cohorts of urine samples or performing prostatic massage before collection might increase the detection potential of this miRNA.

Because miR-129-2 higher promoter methylation levels associated with higher Gleason score and stage and miR-34b/c correlated with pathological stage, a putative prognostic role was investigated. The major objective was to discriminate clinically aggressive from indolent PCa, and to test the prognostic value of the miRNAs in a pre-therapeutic setting, using prostatic biopsies.

In a cohort 180 radical prostatectomy pathological stage, Gleason score and PSA levels significantly associated with DFS, whereas, only pathological stage and higher GS statistically associated with worse DSS. Remarkably, high miR-129-2 methylation levels associated with shorter DSS. In multivariable analysis, only GS and PSA levels for DFS and GS and miR-129-2 methylation for DSS retained prognostic value.

In a prospective group of 74 PCa suspects – cohort 2 - advanced clinical stage, higher GS, higher miR-34b/c and miR-129-2 promoter methylation levels statistically associated with worse DFS. In multivariable analysis only higher clinical stage and high miR-34b/c promoter methylation levels independently predicted shorter DFS. Except for serum PSA, all clinicopathological parameters tested, as well as miR-34b/c and miR-129-2 promoter methylation levels associated with DSS in univariable analysis. Similarly to DFS, in multivariable analysis only clinical stage and high miR-34b/c promoter methylation levels independently predicted shorter DSS. Interestingly, in the radical prostatectomies series higher miR-129-2 methylation presented independent prognostic information, although only for DSS. Importantly, support previous observations concerning the association of higher gene promoter methylation levels with clinicopathological features of more aggressive disease.

Nonetheless, the major goal was to classify clinically aggressive from indolent PCa, in a pre-therapeutic set. In univariable analysis, the standard clinicopathological parameters associated with DFS and DSS, clinically validating this dataset. The same was demonstrated for higher miR-129-2 and miR-34b/c promoter methylation levels. The fact that CAPRA score failed to predict DFS might be intimately related to the heterogeneity of prostate biopsy series included PCa at diverse clinical stages, subjected to different therapeutic approaches (RP, radiotherapy, androgen-deprivation therapy).

However, in multivariable analysis, only clinical stage retained independent prognostic value, both for DFS and DSS. Curiously, high miR-34b/c promoter methylation levels also predicted shorter DFS and DSS, suggesting that it might constitute a useful PCa prognostic biomarker. These results indicate that high miR-34b/c promoter methylation levels identify clinically aggressive PCa, irrespective of disease extent at diagnosis.

However, the validation in additional independent cohorts it is still required. To ascertain the real value of miR-193b, miR-129-2 and miR-34b/c as new and powerful biomarkers for PCa. If a multiplex assay capable to simultaneously assess miR-193b, miR-129-2 and miR34b/c promoter methylation is designed and validated, this would allow for diagnostic and prognosis assessment of PCa suspects in a single analysis.

Finally, in Chapter 6 we found that *TMEM97* expression levels (>75 percentile) independently predicted shorter DFS. Although, miR-152 methylation or expression levels did not associate with patients' prognosis, this suggests *TMEM97* as a useful biomarker for PCa management, being as well a potential therapeutic target for PCa. Further studies are required to further confirm the function and the prognostic value of *TMEM97* in PCa patients.

CHAPTER 9 - MAIN CONCLUSIONS

Conclusions

In the previous sections, it was attempted to outline a coherent sequence of the rationale and the main findings of this Doctoral Thesis, integrating them with earlier and later published results in this research area. Therefore, the conclusions of this Thesis are both depicted in each manuscript and then integrated in the General Discussion. Nonetheless, and taking the risk of being redundant by enumerating the main conclusions, it seems to be important to provide a synopsis of the background behind this Doctoral Thesis.

Consequently, the main conclusions of this Doctoral Thesis are summarized in this section:

1 – Prostate Carcinomas and Morphological Normal Prostate Tissues have distinct miRNA profiling, towards a global down-regulation of miRNAs in Prostate tumours;

2 – The global miRNAs down-regulation is a multilayer process, resulting of both decreased expression of the core miRNA' biogenesis machinery – *DROSHA*, *DGCR8* and *DICER* - and aberrations in the epigenetic landscape;

3 – Prostate malignancies show distinct DNA methylation dynamics causing alterations in the expression of miRNAs;

4 – *In vitro* reversal of DNA methylation dramatically restores the global expression of the miRNAnome;

5 – DNA methylation contributes to the silencing of newly discovered PCa tumor-suppressor miRNAs: miR-130a, miR-130b~301b, and miR-152;

6 – MiR-130a targets the proto-oncogenes *SEC23B* and *DEPDC1*, whose loss of function phenotype overlaps miR-130a over-expression;

7 – MiR-130a fine-tunes multiple signaling pathways, including DNA apoptosis, cell cycle, cell migration and the response to endoplasmic reticulum stress;

8 - MiR-130a methylation levels discriminated PCa from non-malignant tissues (AUC=0.956) and its assessment urine samples showed high specificity for PCa detection (AUC=0.89);

9 - MiR-130b~301b restoration significantly reduces malignant phenotype, by arresting the cells at S phase (both miR-130b and miR-301b), or at G2/M arrest (miR-130b) and promotes mesenchymal to epithelial transition;

10 – Overexpression of cluster miR-130b~301b causes senescence bypass, repressing Ki67 and *LMNB1* or overexpressing of *CDKN1A*, *CDKN1B*, *CDKN2A*, and *CDKN2B*;

11 – The cluster miR-130b~301b dramatically fosters the secretome of senescent cells, known as SASP;

12 - The cluster miR-130b~301b induces DNA damage and DNA-Damage-Inducible Transcripts (*DDIT3*, *DDIT4*, *GADD45A*, or *ATR*);

13 – MiR-130b and miR-301b are part of a complex signaling network that in PCa causes DNA damage and, subsequently, senescence;

14 – There is a coexistence of functional cooperation and specialization among these parolog miRNAs, identifying miR-130a as multiple pathways fine tuner, whereas cluster miR-130b-301b is implicated in DNA damage and senescence;

15 – MiR-152 and its host gene *COPZ2* are downregulated and hypermethylated in Prostate tumours;

16 - MiR-152 attenuates malignant phenotype of PCa cells, mainly by triggering cell cycle arrest in S and G2/M checkpoints;

17 – MiRNAs do not only exhibit cell-specific activity but also can generate thresholds in target gene expression and mediate feed-forward and feedback loops in gene networks;

18 - MiR-193b was shown to be a very promising diagnostic biomarker in a urinary test for early detection of PCa (AUC 0.96; 91.6% sensitivity and 95.7% specificity);

19 – Higher miR-129-2 methylation levels independently predicted for shorter DSS in radical prostatectomy specimens;

20 - miR-34b/c and miR-129-2 promoter methylation levels associated with DSS in univariable analysis in prospective group of 74 PCa suspects;

21 - High miR-34b/c or miR-129-2 promoter methylation define subsets of clinically aggressive tumors.

PART IV - FUTURE PERSPECTIVES

Future perspectives

The rapid discovery of ncRNA species by high-throughput technologies has accelerated current conceptions of transcriptome complexity. MiRNAs are the most well characterized family of ncRNAs widely recognized and defined as critical actors of numerous cellular processes. The clinical translation of some miRNAs might be related with its usefulness for PCa biomarker development, and, in the future, RNA-based therapies may be a feasible alternative in clinical oncology.

Although this Thesis provides a mechanistic view of how miRNA deregulation promote PCa progression, and their eventual clinical potential, there are some interesting questions and hypothesis that derived from these results.

The role of the prostate microenvironment in microRNA homeostasis

Androgens are a male sex steroid hormone that binds to and activates the androgen receptor. In addition to epigenetic and miRNA machinery biogenesis aberrations, studies on the impact of AR in the miRNA regulation may contribute to a broader understanding of how miRNAs become deregulated in prostate carcinogenesis. Moreover, it would be of great interest to investigate the function of AR in miRNA regulation and its synergistic role in the establishment of the of CRPC phenotype. Since miRNAs might be implicated in a diverse range of resistance mechanisms in CRPC, it might present new options for disease management. Thus, combining miRNA-based therapeutics with classical drugs such as abiraterone and enzalutamide might be envisaged.

Use of miRNAs in the clinic as diagnostic/prognostic tools for PCa

DNA methylation is easier to assess than miRNA expression: it is more specific and, importantly, more stable. This Thesis revealed that quantitative assays for specific miRNA promoter methylation (miR-193b and miR-129-2) might constitute important diagnostic and prognostic ancillary tools for clinical decision making. To further confirm these results, additional studies, preferably involving multi-institutional cohorts of patients, are required.

Additionally, liquid biopsies might be used for detection, monitoring or prognostication of PCa, using extracellular nucleic acids, primarily by exosomes isolation from patients' peripheral blood or/and urine. MiRNAs in extracellular vesicles or stably bound to proteins in patient biofluids opens unique possibilities for using such circulating RNAs as easily accessible molecular biomarkers. The main question is whether DNA or RNA is the most suitable template for clinical use. The development of such tests requires an extensive characterization of the miRNA and DNA methylation profile in these liquid biopsies coupled with high-throughput next-generation sequencing techniques to unravel the complete exosomal small RNA landscape.

Opportunities and challenges for microRNA-based therapeutics

Deregulation of miRNAs' activity has been frequently implicated in the development and progression of PCa, thus constituting attractive molecules for drug discovery. Conventional PCa therapy is generally unsuccessful at advanced stages of disease. An alternative strategy would be miRNA modulation by using oligonucleotides that can either mimic a miRNA (thus, inducing gene silencing), or bind to a target miRNA (thus, blocking it intrinsic activity). An example of this strategy to restore the tumor-suppressor miRNA-34 is using the liposome-based miR-34a mimic (MRX34) (103). This MRX34 is currently in Phase I clinical trials to test its safety for patients with primary liver cancer or liver metastasis (NCT01829971), as well as pharmacodynamics and pharmacokinetics of the liposomal injection in Melanoma patients (NCT02862145). In this Doctoral Thesis some miRNAs emerged as having growth suppressive activities. It would be interesting to test *in vivo* whether any of these miRNAs retain such tumor-suppressor capabilities. However, delivery of oligonucleotides to patients or animal models is an obstacle. Nonetheless, this can be partially overcome by encapsulation in liposomes or polymer-based nanoparticles and through addition of chemical modifications. Following determination of which alteration is more likely to render full activity for these miRNAs, *in vivo* experiments may be conducted. Moreover, combining any of these miRNAs with other chemical compounds may prove greater efficacy to target PCa.

A major drawback of miRNA-based therapeutics is hybridization-associated off-target effects. The lack of target specificity and the potential off-target effects of miRNA therapeutics are a major concern, as they may cause toxic phenotypes. Thus, it will be critical to evaluate the full range of effects of miRNA manipulation at the transcriptomic and proteomic level *in vivo* to limit the eventual damaging effects of miRNA-based therapeutics.

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