Research Article

miR-204 Shifts the Epithelial to Mesenchymal Transition in Concert with the Transcription Factors RUNX2, ETS1, and cMYB in Prostate Cancer Cell Line Model

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Epithelial to mesenchymal transition is an essential step in advanced cancer development. Many master transcription factors shift their expression to drive this process, while noncoding RNAs families like miR-200 are found to restrict it. In this study we investigated how the tumor suppressor miR-204 and several transcription factors modulate main markers of mesenchymal transformation like E- and N-cadherin, SLUG, VEGF, and SOX-9 in prostate cancer cell line model (LNCaP, PC3, VCaP, and NCI-H660). We found that SLUG, E-cadherin, and N-cadherin are differentially modulated by miR-204, using miR-204 specific mimics and inhibitors and siRNA gene silencing (RUNX2, ETS-1, and cMYB). The genome perturbation associated TMPRSS2-ERG fusion coincided with shift from tumor-suppressor to tumor-promoting activity of this miRNA. The ability of miR-204 to suppress cancer cell viability and migration was lost in the fusion harboring cell lines. We found differential E-cadherin splicing corroborating to miR-204 modulatory effects. RUNX2, ETSi, and cMYB are involved in the regulation of E-cadherin, N-cadherin, and VEGFA expression. RUNX2 knockdown results in SOX9 downregulation, while ETSi and cMYB silencing result in SOX9 upregulation in VCaP cells. Their expression was found to be also methylation dependent. Our study provides means for understanding cancer heterogeneity in regard to adapted therapeutic approaches development.

1. Introduction

Prostate cancer is one of the most common malignancies and the second leading cause of death from cancer in men. Androgen receptor (AR) is paramount for the lineage-specific differentiation of the prostate, inducing the expression of prostate-specific genes, such as PSA and TMPRSS2, and maintaining the differentiated prostate epithelial phenotype [1]. Cellular dedifferentiation and epithelial to mesenchymal transition (EMT), by contrast, are a hallmark of malignant transformation and metastatic disease. In this process the reexpression of conserved developmental programs plays a key role [2]. Chromosomal rearrangements fusing the androgen-regulated gene TMPRSS2 to the oncogenic ETS transcription factor ERG occur in approximately 50% of prostate cancers and more than 90% of them overexpress ERG [3]. TMPRSS2-ERG is crucial for cancer progression by disrupting lineage-specific differentiation of the prostate and potentiating the histone methyltransferase EZH2-mediated dedifferentiation program. ERG disrupts AR signalling by inhibiting AR expression, binding to and inhibiting AR activity at gene-specific loci, and inducing repressive epigenetic programs via direct activation of the EZH2. The latter causes an epigenetic silencing of developmental regulators and tumor suppressor genes, subverting cancer cells to a stem-cell-like epigenetic state [4]. During this process, part of which is EMT, a transformation of epithelial cells into the invasive and proliferating mesenchymal cells occurs. The main event that follows is the transcriptional shift, involving ETSi, SLUG (SNAI2), and other transcription factors (TF) that suppress the epithelial markers (e.g., E-cadherin and β-catenin) and activate mesenchymal ones (e.g., N-cadherin
and vimentin), increasing the cell motility and cell migration [5]. Further under ERG control, even more invasive and proliferating phenotypes develop.

In PCa, AR can function in two opposite directions: on one side, AR signalling is crucial for prostate and PCa cell survival; however its activation can also limit cell proliferation and mediate apoptotic induction under specific circumstances [6], like in conditions of genotoxic stress, when AR is fundamental for p53 activation and for the subsequent induction of apoptosis [7]. These modalities of AR depend to a large extent on the occurrence of the ERG fusion.

During this malignant progression phenomena of non-canonical binding of AR by transcriptional factors occur, which “modify” AR signalling in cancer prosurvival manner. Thus, the EMT involved TF ETS1 was shown to physically interact with AR [8] and its binding motif was found enriched in the AR binding sites. Similarly, ERG redirects AR to other genes that are not normally androgen stimulated, like SOX9. SOX9 was found crucial for normal prostate development and also for prostate cancer initiation and further advancement [2]. Recent studies confirmed SOX9 as a critical downstream effector of ERG. It mediates androgen dependent prostate morphogenesis and AR mediated prostate cancer development. Sox9 is associated with increased cell invasiveness in VCaP.

Recently, another transcription factor RUNX2 has been implicated as the one driving bone marrow metastatic homing of prostate cancer cells. In bone marrow, these cells compete for the hematopoietic stem cells niche, a process that is RUNX2 dependent. Normally, RUNX2 stimulates osteoblast differentiation of mesenchymal stem cells [9].

On the other hand, the hematopoietic lineage differentiation is dependent on another transcription factor that has been recently implicated in cancer metastasis, the proto-oncogene c-MYB [10, 11].

MicroRNAs (miRs) are noncoding RNAs that exert a role of posttranscriptional regulators, silencing specific mRNA targets by binding specific seed sequences at their 3' UTR [12, 13]. Two major events determine the noncoding RNAs as another major player in malignant progression. First, the genome rearrangement results in gain-of-function and loss-of-function events due to the generation of new or the deletion of existing microRNA seed regions. MicroRNAs sequences could be altered as well. Second, AR mediates miR maturation in Dicer [14] in ligand dependent manner, participating in three-step pathway including miR activation, AR corepressor suppression, and DNA interaction to elicit its action. Loss of DICER results in androgen-insensitivity syndrome. miRs are mediators of AR function and the existence of a possible feedback loop between miRs, AR, and AR corepressors [14]. This implicates them directly in prostate carcinogenesis and progression as a mechanistic factor determining AR signalling specificity in androgen independent prostate cancers. It is not surprising that some novel prostate cancer specific markers, like AMACR [15] and PSMA [16, 17], were also found dependent on different micro RNAs [18].

The miR-204 family was found to inhibit the epithelial-mesenchymal transition and cancer cell migration by direct targeting some of E-cadherin transcriptional repressors [19]. Member of the same family, miR-204 has been found to target RUNX2 in mice [20] and to be highly expressed in normal renal tissue. The promoter of the gene harboring miR-204 was found to contain an ETS1 binding site [29].

We investigated how miR-204 and the master TFs (SLUG, SOX9, RUNX2, ETS1, and cMYB) are implicated in EMT (E-cadherin, N-cadherin) and in the expression of angiogenesis marker VEGFA, using cell lines model LNCaP [21], PC3 [22], VCaP [23], and NCI-H660 [24] of AR sensitive and refractive prostate cancer cell lines, being either TMPRSS2-ERG fusion harboring or fusion-free.

2. Materials and Methods

2.1. Cell Lines. LNCaP (lymph-node metastasis-derived), androgen receptor (AR) responsive (AR+), p53 enabled; PC3 (bone metastasis derived, p53 null and AR-unresponsive (AR-)); VCaP (vertebral bone metastasis derived, AR+); and NCI-H660 (lymph-node prostate small cell carcinoma, AR+) prostate cancer cell lines were purchased from the ATCC (VA, US). LNCaP cells were grown in RPMI media (ATCC, Catalog number 30-2001), PC3 were grown in ATCC-formulated F-12 K Medium (Catalog number 30-2004), and VCaP cells were grown in DMEM media (ATCC Catalog number 30-2002) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA). The H660 cells were grown in RPMI media (ATCC, Catalog number 30-2001, complete growth media contain 0.005 mg/mL insulin, 0.01 mg/mL transferrin, 30 nM sodium selenite (final conc.), 10 nM hydrocortisone (final conc.), 10 nM beta-estradiol (final conc.), extra 2 nM L-glutamine (for final conc. of 4 mM), 5% fetal bovine serum (final conc.) (Sigma, St. Louis, MO, USA)). All cell lines were cultivated in a humidified atmosphere at 5% CO₂ and 37°C.

2.2. MicroRNA Transfection. The miR-204 inhibitor (anti-hsa-miR204), mimic (syn-hsa-miR204), and their respective negative controls (MiScript Inhibitor Negative control and AllStars Negative siRNA) (Qiagen, Hilden, Germany) were transfected into LNCaP, PC3, VCaP, and H660 cells by HiPerFect (Qiagen, Hilden, Germany) for 24 h, according to the manufacturer's instruction. The cells were seeded a day before transfection in 24-well plate (TPP, Trasadingen, Switzerland) for RT-qPCR, in 12-well plate (TPP, Trasadingen, Switzerland) for cell migration assay and flow cytometry assay, and in 96-well plate (TPP, Trasadingen, Switzerland) for cell proliferation assay (CCK-8). Experiments were done in triplicate.

2.3. Cell Viability Assay. The cell viability assay was done for cells transfected with syn-hsa-miR204, AllStars Negative siRNA, anti-hsa-miR204, or MiScript Inhibitor negative control in 96-well plate format (100 μL cell suspension volume/well). The measurement was done using the Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich, St. Louis, MO) assay, which is based on the conversion of water-soluble tetrazolium salt, WST-8 to a water-soluble formazan dye upon reduction in the presence of an electron carrier by
dehydrogenases [25]. The assay was performed by adding 10 μL CCK-8 to each well for 4 h at 37 °C. The OD was read at 450 nm using a multiplate reader Flowstar OPTIMA (BMG LabTech, Germany). Experiments were done in triplicate.

2.4. Cell Migration Assay. Cell migration assay was done for cells transfected with syn-hsa-miR204, AllStars Negative siRNA, anti-hsa-miR204, or MiScript Inhibitor negative control in 12-well plate format. LNCaP, PC3, and VCaP cells were seeded a day before transfection. The plate was washed and replaced with cultivation medium. An invert Leica light microscope equipped with digital camera was used for time lapse study. The examination of the cell migration was done on 0, 6, 12 and 18 hours. Experiments were done in triplicate. Using ImageJ software we measured the distance traveled by the cells during the time points and calculated the relative change in the scratch width measured in arbitrary units for each treatment.

2.5. syn-hsa-miR204 and Anti-hsa-miR204 Efficiency Validated Using miR-204 Specific Luciferase Reporter Assay. LNCaP, PC3, VCaP, and H660 cell lines were seeded in a 96-well plate (Orange, Braine-l’Alleud, Belgium) and transfected for 24 h with Negative siRNA, miR-204 mimic, and miR204-inhibitor. All wells were transfected with 5 μg 3’UTR GoClone luciferase reporter construct containing 3’ UTR seed specific for miR-204 (Product ID S880168) at the end of Luciferase encoding gene. The reporter is optimized specifically for use with the LightSwitch Luciferase Assay System (SwitchGear Genomics, Carlsbad, CA, USA), and has basal high Luciferase signal, which is reduced upon miR-204 specific binding. All transfections were done with HiPerfect (Qiagen, Hilden, Germany). Experiments were performed in triplicate. After adding the assay solution to wells and 30 min incubation at RT in the dark, the plates were read in a FLOWStar Optima reader (BMG LABTECH GMBH, Ortenberg, Germany).

The reporter data was analyzed by estimating the ratio of the signal obtained by miR-204 mimic or other specific treatment and the signal obtained by nontargeted microRNA. Qiagen control resembling the latter is MiScript Inhibitor Control (Qiagen negative control for miR inhibitors, binds only AllStars Negative Control siRNA). The estimated ratios for miR-204 mimic and miR-204 inhibitor were further normalized to the reporter data for AllStars Negative Control siRNA (Qiagen control for miR mimics, same size as miRs, optimized for minimized off-target effects) and reported in arbitrary units of Luciferase reduction of microRNA mimic/inhibitor compared to nontargeting control microRNA like sequence.

2.6. RUNX2, ETSI, and cMYB Knockdown with Small Interfering RNA (siRNA). For siRNA knockdown of RUNX2, ETSI, and c-MYB, we used human mission esiRNAs (Sigma, St. Louis, MO, USA) against the target molecules with the following design:

| Gene | Forward Primer | Reverse Primer |
|------|----------------|----------------|
| RUNX2 | GGTACCAGATGGGACCTGTGGTACTGTCATGGCCTGTAACCGAGTAAGAAAATTTATTCTGCT | ETSI: TGGAGCTTTCAAGAGCAGCGGTGTTGGTGGACCTGTAAATTTGATTTGTCAT |
| PGK1 Fw: 5’- a t ta g cc g ag c ca g cc a aa a ta g - 3’, Re: 5’-tcaaaacccaccagcct-3’; cMYB: GGGGAGTACGCTTGGAGCGGACGAGAAAAGAAAGAGAAGAAGACCTTGGGTAGGTAGTAATTGACTATGCA. |

AllStars Negative control siRNA (5 nmol) and AllStars HsCell Death Control siRNA (5 nmol) were used as a control of transfection (Qiagen, Hilden, Germany). LNCaP, PC3, VCaP, and H660 cells were seeded a day before transfection with 80% confluence in a 24-well plate (TPP, Trasadingen, Switzerland) for RT-qPCR. Transfections were done according to the manufacturer’s instruction with HiPerFect (Qiagen, Hilden, Germany). 200 nM of siRNA was used for transfection for 72 h. Experiments were done in triplicate. siRNAs knockdown of RUNX2, ETSI, and cMYB was validated by RT-qPCR and flow cytometry assays.

2.7. Real-Time Reverse Transcription Quantitative PCR Analysis. The expression of two sets of genes—set 1: SLUG, E-cadherin, N-cadherin, VEGFA, and SOX9; set 2: RUNX2, ETSI, and cMYB—was detected by RT-qPCR. After 24 h mimic or inhibitor and 72 h esiRNAs transfection mRNA was isolated from LNCaP, PC3, and H660 cells using Midprep kit (Qiagen, Hilden, Germany). From each sample 500 ng total RNA was used to synthesize cDNA by Sensiscript Reverse Transcription kit (Qiagen, Hilden, Germany). First-strand cDNA synthesis was performed according to the manufacturer’s instruction (Qiagen, Hilden, Germany) and 500 ng cDNA was used for PCR reactions (SYBR Green Quant iTect RT-PCR MasterMix, Qiagen, Hilden, Germany). Total reaction volume was 50 μL. Primer concentrations were 0.5 μM (Fw and Re). RT-PCR Cycler (Agilent Technologies MX3005P, Stratagene, Santa Clara, CA, USA) was used in this study. PCR reactions were started at 95 °C for 15 sec, then 50 °C for 30 sec, 72 °C for 30 sec, followed by 45 cycles. The mRNA transcript expression levels of all studied genes were normalized towards transcript levels of endogenous reference gene phosphoglycerate kinase 1 (PGK1). PGK1 is a transferase enzyme that has been considered as a housekeeping gene for the cells. PGK is found in all living organisms and its sequence has been highly conserved throughout evolution [26].

The following primer sequences, designed by us and produced by Biomers (Ulm, Germany), were used:

- SLUG Fw: 5’-ata ttc gga gca aca cat tac-3’, Re: 5’-gca aat gct ctt tgg cag tga-3’; E-cadherin (CDH1) Fw: 5’-aga acg cat tgc cac ata cac tc-3’, Re: 5’-act gca ttc cgg ttg gat gac ac-3’;
- N-cadherin (CDH2) Fw: 5’-tgg gaa tcc gaa gag tgg-3’, Re: 5’-tgg aag tca tgg gag cgg agg at-3’; VEGFA Fw: 5’-cct tgc tgc tct acc tcc ac-3’, Re: 5’-gga act tca cca ctt ccg-3’;
- SOX9 Fw: 5’-gag gaa gtc ggt gaa gaa cg-3’, Re: 5’-atc gaa ggt ctc gat gtt gg-3’; PGK1 Fw: 5’-att agc cga gcc agc caa aat ag-3’, Re: 5’-tca tca aa acc cac cag cc-3’;
**MYB Fw:** 5′-aag tct gga aag cgt cac ttg-3′, Re: 5′-aca tct gttc gattcgggagata-3′

**RUNX2 Fw:** 5′-ggg ccc tct ctg aac ttg-3′, Re: 5′-tgct gcc cgt cac ttg a-3′

**ETS1 Fw:** 5′-agtgctcaaggacatcgagacg-3′, Re: 5′-agccacaagcagacgctg-3′

2.8. Flow Cytometry Validation of siRNA Knockdown. Flow cytometry was used instead of western blotting as protein detection method following an already established approach [27,28]. Transfected (negative siRNAs and esiRNAs: RUNX2, ETS1, and cMYB) prostate cancer cells (LNCaP, PC3, VCaP, and H660) were detached with Accutase (eBioscience, Franklin, Germany) and washed with cold 1% BSA-PBS. Specific antibodies (Abs) (Santa Cruz Biotechnology, Dallas, Texas, USA) were used for detection of RUNX2, ETS1, and cMYB: RUNX2 (27-K) is a mouse monoclonal antibody raised against recombinant RUNX2 of human origin; ETS1 (C-4) is a mouse monoclonal antibody raised against amino acids 131–280 of ETS1 of human origin; c-MYB (D-7) is a mouse monoclonal antibody raised against amino acids 500–640 of c-MYB of human origin. After IC Fixation/Permeabilization solution (eBioscience, Frankfurt, Germany) wash and flow cytometry staining buffer (eBioscience, Frankfurt, Germany), the specific primary Abs or the appropriate isotype control Abs were used at concentration of 0.5 μg/106 cells for 60 min on ice, followed by BSA-PBS wash and secondary antibody (rabbit anti-mouse FITC conjugated IgG, Santa Cruz Biotechnology, Dallas, Texas, USA) incubation at 0.25 μg/106 cells for 30 min on ice (in the dark). Cells were gated using forward versus side scatter to exclude dead cells and debris. After washing, cells were analyzed with a BD FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Fluorescence of 10⁶ cells per sample was acquired in logarithmic mode for visual inspection of the distributions and for quantifying the expression of the relevant molecules by calculating the median fluorescence intensity (referred to as MFI) in histogram overlay graphics.

2.9. Total Demethylation. LNCaP, PC3, VCaP, and H660 cells were seeded in a 24-well plate (TPP, Trasadingen, Switzerland) for RT-qPCR. The cells were treated with 6 μM 5-Azacytidine (Sigma, St. Louis, MO, USA) (5-AzaC) for four days. The media were changed every day with fresh 5-AzaC solution. Briefly, total RNA was isolated from the cells and cDNA was synthesized following the same protocol described above. RT-qPCR was performed to measure the expression of SLUG, E-cadherin, N-cadherin, VEGFA, and SOX9 genes. Experiments were done in triplicate.

2.10. Statistical Analysis. The data were generated from three independent experiments, each performed in triplicate. One-way ANOVA test with respective multiple comparison posttests (Greenhouse-Geisser correction for nondata sphericity, Tukey correction posttest, adjusted P value, and family-wise significance, confidence level of 0.05) was used to analyze the data (GraphPad Prism 6, La Jolla, CA, USA). P < 0.05 was considered significant.

**3. Results and Discussion**

3.1. E-Cadherin and N-Cadherin Are Differentially Affected by mir-204 Overexpression Depending on the Level of Genome Perturbation of the Prostate Cancer Cell Line Model. The mir-204 was found only slightly expressed in intact human prostate tissue [29], but overexpressed in PC3 and DU145 prostate cancer cell lines [30]. While it is reported as tumor suppressor in some cancers [31,32], its role in prostate cancer is rather oncogenic [30]. Due to the limited amount of studies and the dualistic behavior of miR-204, we decided to follow the miR-204 modulatory effect on molecules important for EMT such as SLUG, E-cadherin, and N-cadherin.

We induced miR-204 overexpression using synthetic mimics and miR-204 suppression using specific inhibitor and validated their functional efficiency using Luciferase reporter bearing miR-204 specific seed region (Figure 1).

We found that E-cadherin was upregulated in LNCaP cells and downregulated in all other cell lines, while N-cadherin was upregulated in all four cell lines after miR-204 mimic transfection. SLUG was upregulated only in VCaP cells (Figure 1). VCaP and NCI-H660 cell lines harbor the TMPRSS2-ERG fusion and have an advanced genome perturbation status. The downregulation in NCI-H660 was stronger than in other cell lines (Figure 1), and it could be explained with additional E-cadherin alternative splicing exon-11 exclusion, associated with prostate and other malignant tumors [33]. E-Cadherin is downregulated, while N-cadherin is upregulated during EMT [5].

3.2. E-Cadherin Differential Regulation by miR-204 in LNCaP Cells Probably Depends on Its Different Splicing Variant Switch. We found additionally that E-cadherin is alternatively spliced in LNCaP and all other cell lines: PC3, VCaP, and NCI-H660. This was manifested by a different melting curve peak when E-cadherin was amplified in either LNCaP and PC3 or other two cell lines (Figure 2). This alternative splicing might account for the E-cadherin downregulation during cancer progression and metastasis and could further support the differential miR-204 suppressive effect exerted in prostate cancer cell lines discussed above.

This could be explained by the different events of alternative isoform expression like alterations in tandem 3′ untranslated regions that correlate to the different cancer phenotypes [34].

3.3. miR-204 Upregulates SOX9 and Regulates VEGFA in an AR Sensitivity Dependent Manner in Prostate Cancer Cell Line Model. SOX-9 was upregulated by miR-204 in all cell lines, while SOX9 downregulation by miR-204 inhibition was lost in the fusion harboring cell lines (Figure 1). The importance of miR-204 implication in Sox9 control is related to the finding of the role of Sox9 as imperative for both early prostate development and prostate cancer (TRAMP and Hi-Myc) initiation as demonstrated by studies utilizing its deletion. This is due to its role in multiple cytokeratins and cell adherence/polarity regulation [2]. Moreover, SOX9 is found as critical downstream effector of ERG in TMPRSS2:ERG
**Figure 1:** Luciferase reporter assay validation of syn-hsa-miR204 and anti-hsa-miR204 efficiency. Luminescence readings (arbitrary units) of miR-204 seed specific LightSwitch 3′ UTR GoClone Luciferase reporter cotransfected with MiScript Inhibitor Negative Control (targeting only AllStars Negative siRNA) and miR-204 mimic, or miR-204 inhibitor, normalized to AllStars Negative siRNA cotransfection. miR-204 mimic binding to the reporter 3′ UTR results in decreased Luciferase signal.

Effect of miR-204 on EMT. AllStars Negative siRNA, MiScript Inhibitor Negative Control, miR-204 mimic, or miR-204 inhibitor transfected LNCaP, PC3, VCaP, and NCI-H660 cell lines were harvested for total RNA. qPCR was performed for SLUG, ECDH, NCDH, VEGFA, SOX9, and the relative gene expression (Pfaffl, $2^{-\Delta\Delta CT}$), normalized to PGK1, was assessed for LNCaP, PC3, VCaP, and NCI-H660 cell lines. **$P < 0.01$, *$P < 0.05$.** Error bars represent s.d., $n = 3$ independent biological repeats.
Figure 2: E-Cadherin has differential splicing variant expression in LNCaP cells compared to all other cell lines: PC3, VCaP, and NCI-H660. Dissociation and amplification curves (Stratagene MX3005P) of ECDH specific primers amplified product in total RNA from LNCaP, PC3, VCaP, and NCI-H660 cells (AllStars Negative siRNA, MiScript Inhibitor Negative Control, miR-204 mimic, or miR-204 inhibitor). Different Tm in dissociation curves are highlighted using vertical black bars.

fusion-positive PCa like VCaP. Since ERG is overexpressed in these fusion cells, and ERG redirects AR to a set of genes including SOX9 that are not normally androgen stimulated. This further supports miR-204 deregulation role in cancer progression [35].

We followed the role of miR-204 in VEGFA transcript expression since many metastases secrete VEGFA to produce neovascularization [36]. miR-204 modulated VEGFA in AR-sensitivity dependent manner. It downregulated VEGFA in LNCaP and VCaP cells and upregulated VEGFA in castration resistant cell lines as PC3 and NCI-H660. The inhibition of miR-204 had a reciprocal effect on VEGFA transcripts (Figure 1). This miR is known to regulate the vascularization in cornea [37] by inhibiting Angiopoetin-1 and also to play substantial role in pulmonary arterial hypertonia [38], where it is downregulated.

3.5. RUNX2, ETS1, and cMYB Are Required for SLUG Expression in TMPRSS2-ERG Free Cell Lines LNCaP and PC3. SLUG is in vivo recruited by the promoters of Runx2 and Sox9 genes, thus acting as both positive and negative transcription regulator of these genes, respectively [39]. We followed how the transcription factors RUNX2, ETS1, and cMYB were involved in prostate cancer related EMT.

Runx2, a bone-specific transcription regulator, is abnormally expressed in highly metastatic prostate cancer cells, promoting metastasis, driving the metastatic homing to the bone marrow hematopoietic stem cell niche, and facilitating local osteolysis [40]. This is achieved through large tumors
miR-204 decreases LNCaP and PC3 cell viability but has no effect on VCaP and NCI-H660 viability. Changes in cell viability of LNCaP, PC3, VCaP, and NCI-H660 cells transfected for 24 h with either AllStars Negative siRNA, MiScript Inhibitor Negative Control, miR-204 mimic, or miR-204 inhibitor in increasing dosages (2, 10, 20 nM) were assessed using cell viability kit CCK-8. Colorimetric assay was read on 450 nm. Scale bar corresponds to 50 μm. **P < 0.01, *P < 0.05. Error bars represent s.d., n = 3 independent biological repeats.
development, increased expression of matrix metalloproteinases (MMP9, MMP13), osteominalization inhibition (Osteopontin), bone-resorbing factors (PTHrP, IL8), and VEGF, a key angiogenic factor [36, 40]. Key event to this process is the induction of EMT-related molecules (SOX9, Snail2/SLUG/SNAI2, and SMAD3) by RUNX2 that potentially enhance invasion of cancer cells [36, 41].

As SLUG is reported to upregulate RUNX2 [42], we decided to follow the reverse pathway of interaction, namely, if RUNX2 affects SLUG levels. Our results showed that SLUG...

Figure 4: miR-204 suppresses cell migration in TMPRSS2:ERG fusion-negative cell lines. Cell migration assay was done for cells transfected with syn-hsa-miR204, AllStars Negative siRNA, anti-hsa-miR204, or MiScript Inhibitor negative control. Morphometric measurement of the scratch width at 0 h and 18 h time points, represented as relative change in arbitrary units for each treatment. Error bars represent s.d., n = 3 independent biological repeats.
**Figure 5:** Transcription factors cMYB, RUNX2, and ETS1 siRNA knockdown validation. qPCR validation. Cell lines LNCaP, PC3, VCaP, and NCI H660 were analyzed for cMYB, RUNX2, and ETS1 mRNA expression by RT-qPCR after specific siRNA or AllStars Negative siRNA transfection. qPCR data are represented as a relative fold change normalized to nontreated cells (100%). FCS validation. Cell lines LNCaP, PC3, VCaP, and NCI H660 were analyzed for cMYB, RUNX2, and ETS1 protein expression after specific siRNA or AllStars Negative siRNA transfection using FCS. Expression is presented as FCS overlay histograms of isotype control, AllStars Negative siRNA, and specific siRNA transfected cells. MFI bar chart represents the mean fluorescent intensity index of each FCS overlay histogram, normalized to MFI of AllStars Negative siRNA transfected cells (100%).
Figure 6: Transcription factors cMYB, RUNX2, and ETS1 are EMT regulators. LNCaP, PC3, VCaP, and NCI-H660 cells transfected for 72 h using either AllStars Negative siRNA or siRNA specific for RUNX2, ETS1, or cMYB, were all harvested for total RNA. qPCR was performed for SLUG, E-CADH, N-CADH, VEGFA, SOX9, and the relative gene expression represented as log₁₀ change (Pfaffl, 2ΔΔCT), normalized to PGK1, was assessed for LNCaP, PC3, VCaP, and NCI-H660 cell lines. **P < 0.01, *P < 0.05. Error bars represent s.d., n = 3 independent biological repeats.

was downregulated after RUNX2 silencing in all four cell lines (Figures 5 and 6), suggesting that there is a positive feedback loop between RUNX2 and SLUG. Indeed, recent data show that RUNX2 is able to bind AR and detach it from its target genes like some tumor suppressors, but at the same time, the two together, RUNX2 and AR, are able to bind the SLUG enhancer region and result in an increased invasiveness [43].

ETS1 silencing resulted in SLUG downregulation in both LNCaP and PC3 cells, while there was no significant effect in NCI H660 cells. Surprisingly, in VCaP cells ETS1 silencing resulted in SLUG upregulation (Figures 5 and 6). cMYB silencing resulted in SLUG downregulation in all lines, but VCaP, where it had no significant effect (Figures 5 and 6).

3.6. RUNX2, ETS1, and cMYB Are Involved in the Regulation of E-Cadherin, N-Cadherin, and VEGFA Expression. Silencing ETS1, we found it to be a positive E-cadherin and a negative N-cadherin regulator, accordingly, thus preventing EMT. Silencing of RUNX2 resulted in downregulation of N-cadherin in all cell lines. Interestingly, it resulted in concomitant upregulation of E-cadherin in PC3 and NCI H660 AR responsive cell lines, but not in AR sensitive LNCaP and VCaP cell lines (Figures 5 and 6).

In noncancer mouse mammary cells, Ets1 was found to facilitate TGF-β induced EMT and E-cadherin down-regulation specifically, by upregulation of direct E-cadherin transcription repressors [44]. Our data show an inverted ETS1 action in human prostate cancer, most likely driven by the androgen and TMPRSS2:ERG fusion signaling.

cMYB silencing revealed that it is also a positive E-cadherin regulator, except in VCaP cells. We found that cMYB is required for EMT in LNCaP and PC3 cells, as cMYB silencing downregulated N-cadherin. This dependence was lost in TMPRSS2-ERG fusion harboring cells like VCaP and NCI-H660 (Figures 5 and 6).
Like data reported in breast cancer models, ETS1 silencing demonstrated that it is a VEGFA positive regulator in prostate cancer cell line model. Runx2 was also reported as a positive VEGF inducer [40], a finding we also confirm in this study. cMYB silencing suggested it has differential role on VEGF induction, being positive regulator only in TMPRSS2-ERG fusion-free prostate cancer cell line models (LNCaP, PC3). In fusion positive cell lines, cMYB had a restrictive role (Figures 5 and 6).

3.7. RUNX2 Knockdown Results in SOX9 Downregulation, While ETS1 and cMYB Silencing Result in SOX9 Upregulation in VCaP Cells. Following SOX9 dependency on these transcription factors, we silenced RUNX2 and found it to be a positive SOX9 inducer only in VCaP cell line. In other cell lines it had no significant effect (Figures 5 and 6). RUNX2 and SOX9 were found by Cheng and Genever 2010 [45] to physically interact during mesenchymal stem cell differentiation. SOX9 inhibited the transactivation of RUNX2 and vice versa. The divergence in SOX9 dependence on RUNX2 in other cell lines investigated is most likely related with the different state of global gene network and genome deregulation.

On the contrary, SOX9 dependency on ETS1 was significant in PC3 and VCaP cell lines. Silencing ETS1 abrogated significantly SOX9 expression in PC3 cell line, while in VCaP cell line, ETS1 silencing resulted in significant SOX9 upregulation (Figures 5 and 6). Gao et al., 2010 [46], found Sox9 upregulated in Ets1-deficient hearts in knockout mouse model, confirming the dependency found by us in VCaP cell line.

cMYB silencing resulted in SOX9 downregulation in PC3 cells, while in VCaP cells cMYB silencing resulted in significant SOX9 upregulation. There was no significant change in SOX9 level upon cMYB silencing in LNCaP and NCI-H660 cells. c-Myb transcription factor is associated with...
proliferation of undifferentiated cells in number of tissues, but recent data suggests its role also in differentiation. c-Myc is important in formation of the cartilage, bone, and apparently also hard tissue mineralization [47]. Transient transfection of embryonic micromasses using constructs carrying siRNA c-Myc treated cultures expresses significantly lower level of Sox9. The c-Myc and Sox9 have probably negative regulatory feedback loop [48].

3.8. Global Demethylation Reveals SLUG and N-Cadherin Expression to Be Methylation Dependent. Using 5-aza-cytidine (5-AzaC), an inhibitor of DNA methyltransferase, we followed the expression of the EMT related genes. We found similar pattern of expression of SLUG, E-cadherin, N-cadherin, and VEGFA in AR preserved signaling cell lines LNCaP and VCaP. SLUG and VEGFA were upregulated in LNCaP, PC3, and VCaP cells. E-Cadherin was downregulated, while N-cadherin was upregulated in LNCaP, VCaP, and NCI H660 cells. Sox9 was downregulated in TMPRSS2-ERG fusion free cell lines (LNCaP, PC3) after 5-AzaC treatment (Figure 7).

4. Conclusions

miR-204 gradually loses its tumor-suppressor activities and becomes deregulated in more advanced fusion positive cell lines, promoting EMT in AR dependent and fusion dependent manner. The different models of lymph node and bone marrow metastasis have distinct patterns of expression for various EMT markers and TFs and provide initial means for adapted therapeutic approaches of selective silencing of noncoding RNAs and TFs.

Abbreviations

PCa: Prostate cancer (carcinoma)
AR: Androgen receptor
TF: Transcription factor
EMT: Epithelial to mesenchymal transition
miR: MicroRNA
siRNA: Small interfering RNA
RT-qPCR: Reverse transcriptase quantitative real-time polymerase chain reaction
s.d.: Standard deviation
5-AzaC: 5-Aza-cytidine
cMYB: v-myb avian myeloblastosis viral oncogene homolog
RUNX2: runt-related transcription factor 2
ETSI: v-ets avian erythroblastosis virus E26 oncogene homolog
ERG: v-ets avian erythroblastosis virus E26 oncogene homolog
PGKI: Phosphoglycerate kinase 1.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution

Krassimira Todorova and Soren Hayrabedyan designed the study, acquired and analyzed the data, and wrote the paper. Krassimira Todorova did the transfection experiments, qPCR, and FCS. Krassimira Todorova, Diana Zasheva, and Kristiyan Kanev did the cell culturing, cell migration, and CKK-8 assay.

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