**Доклади на Българската академия на науките**
**Comptes rendus de l'Académie bulgare des Sciences**
*Tome 76, No 8, 2023*

*MEDICINE*
*Immunology*

# COMBINED microRNA-141 RESCUE AND MAPK1 SILENCING AS PUTATIVE STRATEGY TO SUPPORT CHEMOTHERAPY IN TRANSLATIONAL STAGE TOWARDS METASTATIC CASTRATION-RESISTANT PROSTATE CANCER – AN IN VITRO MODEL STUDY

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*Received on May 4, 2023*
*Presented by B. Petrunov, Member of BAS, on May 30, 2023*

**Abstract**

Tumourigenesis is associated with disruption of cell differentiation, proliferation, migration, and abnormal DNA methylation. Deregulation of transcription factors MAPK1 and NF-κB and other post modification factors play an important role in these processes. Using AR-positive and AR-negative cell line models (LNCaP-p53$^{+/+}$ and PC3-p53$^{-/-}$), we found MAPK1 siRNA silencing as potentially productive approach to decrease castration-resistant cell line invasiveness, but not sufficient to abrogate anti-apoptotic, pro-inflammatory by NF-κB signalling, and so we combined this approach with miR-141 rescue (mimic) and inhibition to further modulate autophagy signalling and eventually block MAPK1/NF-κB/ROS pathways. We found miR-141 rescue to upregulate AR and NF-κB inductor ACT1 and to promote total DNA demethylation, in LNCaP, but not in PC3 cells, suggesting microRNA-141 rescue as putative enhancer to MAPK1 blockade in conjunction to pro-apoptotic chemotherapy, but only in transitional towards castration-resistance stages.

**Key words:** prostate cancer, inflammation, migration, DNA methylation, NF-κB, MAPK1, microRNA-141

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The research was implemented under research infrastructure funded by the National Roadmap for RI of the Ministry of education and science of Bulgaria, grant agreements DO1-308/20.12.2021, DO1-183/01.08.2022) and Bulgarian National Science Fund, grant KP-06-H33/4, 2019.

DOI:10.7546/CRABS.2023.08.15

**Introduction.** The most common cancer diagnosis in men is prostate cancer (PCa), which is also the second leading cause of death globally [1]. The development of castration-resistant phenotype in PCa is related with increased metastatic activity, constant stable androgen receptor (AR) transcriptional activity associated with loss of androgen receptivity and further acquisition of TMPRSS2:ERG and other gene fusion phenomena promoting metastasis, result of harsh genome perturbation. We have previously shown that the non-coding micro-RNA miR-204 is able to regulate TMPRSS2:ERG promoter depending on the castration-resistance phenotype [1]. Recently, it was shown that MAPK1 (ERK2/MEK/p42), a member of mitogen-activated protein kinase (MAPK) family, has been associated with tumour progression, invasion, and metastasis [2]. MAPK1 was frequently overexpressed in castration-resistant prostate cancer [3]. MAPK1 (ERK2) can also activate the transcription factor NF kappa B (NF-κB) by phosphorylating its inhibitor I kappa B alpha (IKBα), which results in its ubiquitination and degradation, allowing for NF-κB nuclear translocation and subsequent target genes activation [4]. NF-κB was found constitutively activated in prostate cancer, promoting the initiation and further progression towards castration-resistant prostate cancer [5], by enhancing cell survival, tumour invasion, metastasis, and chemotherapy resistance.

Autophagy is responsible for degrading and recycling damaged components, including mitochondria, thus participating in DNA damage control and cellular stress response, and cell fate regulation [6]. In tumours it is often context-dependent, ambiguously modulated, sustaining tumour progression by both being suppressed enhancing the genomic instability and inflammation, or being activated to promote tumour growth via nutrient provision and toxic substance and excessive ROS elimination, culminating in therapy resistance [6]. MAPK1 can promote tumour progression via autophagy inhibition, inactivating a key autophagy initiator like ULK1, but it could also enhance autophagy in other tumour contexts [7]. Reactive oxygen species (ROS) contribute to tumourigenesis through mechanisms such as DNA, protein, and mitochondrial damage, and promote inflammation either acting as second messengers or by the recruitment of inflammatory cells. In solid tumours, infiltrating myeloid cells frequently produce ROS, which activate pro-inflammatory transcription via the NF-κB pathway. Moreover, ROS derived from both tumour and host inflammatory cells can interact with autophagy, promoting cancer cell survival in response to various stressors, including chemotherapy and oncogenic pressure. Autophagy role in tumours is often context dependent, as its inhibition could potentiate DNA and cell damage, ROS formation and pro-inflammatory signalling promoting metastasis, but in other cases autophagy is being reactivated in tumours to prevent excessive ROS formation and cell death, extending tumour cell survival [8].

DNA methylation landscape and total methylation level ($m^5C$ contents) is often changed in cancer, being related to silencing or re-expression of cancer-related

genes [9]. Due to the lack of specific demethylating enzymes, demethylation is only carried out via Ten-Eleven Translocation (TET) enzymes mediated oxidation or via spontaneous oxidation by ROS action [10]. ROS-induced damage to DNA and cellular components, promotion of epigenetic alterations, interaction with oncogenes and tumour suppressor genes, and modulation of immunological responses contribute to global hypomethylation of cellular DNA, making total DNA methylation a sensitive marker for carcinogenesis and a reflection of oxidative stress levels in the cell. DNA methyltransferase inhibitors, such as decitabine, show potential in mitigating inflammation by targeting the NF-κB signalling pathway [10,11], downregulating inflammatory cytokine levels, and thus inhibiting cancer progression.

A member of the miR-200 family, miR-141, is implicated in the epithelial-to-mesenchymal transition and has been recently observed to be downregulated in osteosarcoma [9], correlating with suppressed autophagy, and enhanced proliferation and invasiveness. miR-141 was found to alter mitochondrial function, increasing ATP and ROS production, and down-regulating total antioxidant cell activity, inducing oxidative stress by directly silencing PTEN, in an obesity model [12]. Notably, miR-141 downregulation has also been detected in castration-resistant AR-negative prostate cancer cells [9] and cancer stem cells, while exhibiting upregulation in AR-positive cells and in situ tumour epithelia [13]. The application of decitabine unveiled differential methylation patterns in miR-141 promoters between AR-positive and AR-negative metastatic cell lines, implying a microRNA with potential prognostic and therapeutic implications, contingent upon cancer stage, DNA methylation, autophagy, and metastatic capacity.

Since both MAPK1 and NF-κB pathways were shown tightly intertwined between each other and with oxidative cell stress and autophagy signalling, combining inhibitors of these pathways was recently explored as chemotherapy augmenting strategy in castration-resistant stages of PCa. In this paper we aim to investigate if a combined strategy of rescuing miR-141 and suppressing MAPK1, could have potential for further therapeutic exploration to support chemotherapy.

**Materials and methods.** Both cell lines were obtained from American Type Culture Collection (ATCC). Lymph node metastasis derived cell line LNCaP-p53$^{+/+}$ was cultured in RPMI-1640 medium (Sigma-Aldrich®) with L-glutamine and sodium bicarbonate, supplemented with D-(+)-glucose 4.5 g/L (Sigma-Aldrich®), 10% FBS (Sigma-Aldrich®) and 1% Ab/Am (Sigma-Aldrich®). Bone metastasis PC3-p53$^{-/-}$ cell line was cultured in DMEM medium with high glucose (4.5 g/L), L-glutamine, sodium bicarbonate (1.5 g/L) and 1 mM sodium pyruvate, supplemented with 10% FBS (Sigma-Aldrich®) and 1% Ab/Am (Sigma-Aldrich®). LNCaP and PC3 cell lines were cultured in 6/12 well plates in incubator with 5% $CO_2$ at 37 °C and routinely passaged. Cell lines were transfected for 24/48 h with hsa-miR-141-3p mimic (5 nmol) with seq. UAACACUGUCUGGUAAAGAUGG, (Qiagen) and siMAPK1 (5 nmol), with target seq. AAT-

GCTGACTCCAAAGCTCTG, (Qiagen) by PureFection™ Transfection Reagent (System Bioscience, Palo Alto, CA). Two hours before transfection, the culture medium was replaced. Cells were cultured for at least 24 h before transfection.

***Total DNA methylation assay.*** Total genomic DNA was extracted from PC3 and LNCaP cell lines by Quick-DNA MiniPrep plus kit (ZymoResearch) and DNA concentration was measured by Qubit 2.0 Fluorometer. DNA ELISA kit (ZymoResearch) was used for DNA methylation detection with optimal concentration 100 µg. The absorbance of the samples was measured at 30 min with an ELISA Reader (FLUOstar OPTIMA), at 405 nm in a 96-well plate (strip type).

***MAPK1 gene silencing.*** MAPK1 siRNA (siMAPK1, Qiagen) was transfected for 48 h using PureFection™ Transfection Reagent (System Bioscience, Palo Alto, CA), following manufacturer's protocol.

***NF-κB activation assessment.*** PC3 cell line with a stable reporter plasmid pNIFTY-SEAP (InvivoGene) were cultured in a 6-well plate and transfected for 48 h with siMAPK1, according to the kit protocol. NF-κB activity was assessed using a colourimetric assay detecting cell media released SEAP by activated reporter plasmid. The reaction was read at 405 nm, using Fluorescence plate reader (FLUOstar OPTIMA).

***In vitro cell migration assay (Scratch test).*** Cell lines (LNCaP and PC3) were seeded in a 12-well plate. After reaching 80% confluency for LNCaP cell line and 60% for PC3 cell line the cells were transfected with siMAPK1 for 48 h. Wells were scratched down the midline with a 100 µL pipette tip and migration of LNCaP and PC3 cells was assessed with an inverted light microscope (Leica). Photographs were taken at different time intervals (for LNCaP at 0, 4, 6, 24 h and for PC3 at 0, 4, 8, 10, 24 h). Cell migration was assessed using wound distance reduction ratio, where initial wound distance was assumed as 100% and at each time point, the current wound distance was calculated as current wound distance between migrating cell contours and initial wound distance, represented as percentage.

***Flow cytometry.*** Intracellular flow cytometry protocol was used as described elsewhere [14], with anti-human/mouse androgen receptor monoclonal IgG, Alexa Fluor 405-conjugated and anti-human ACT1 IgG, Alexa Fluor 647-conjugated.

**Results and discussion.** We first silenced MAPK1 (ERK2) for 48 h in an in vitro lymph node AR-positive (LNCaP, $AR^{+/+}$, $p53^{+/+}$) and bone AR-negative (PC3, $AR^{-/-}$, $p53^{-/-}$) metastatic model to follow the cell invasiveness using cell migration assay carried out for 24 h. We found that MAPK1 silencing reduced cell migration, represented as wound distance ratio (in percentage), most notably in AR-negative PC3 cell line by the impressive 46.5 % (19% vs. 65.5%), compared to only 6.8% (79.6% vs. 86.4%) reduction in AR-positive LNCaP cell line (Fig. 1). MAPK1 was frequently overexpressed in castration-resistant prostate cancer [3], since its activation was found crucial for androgen receptor transcriptional ac-

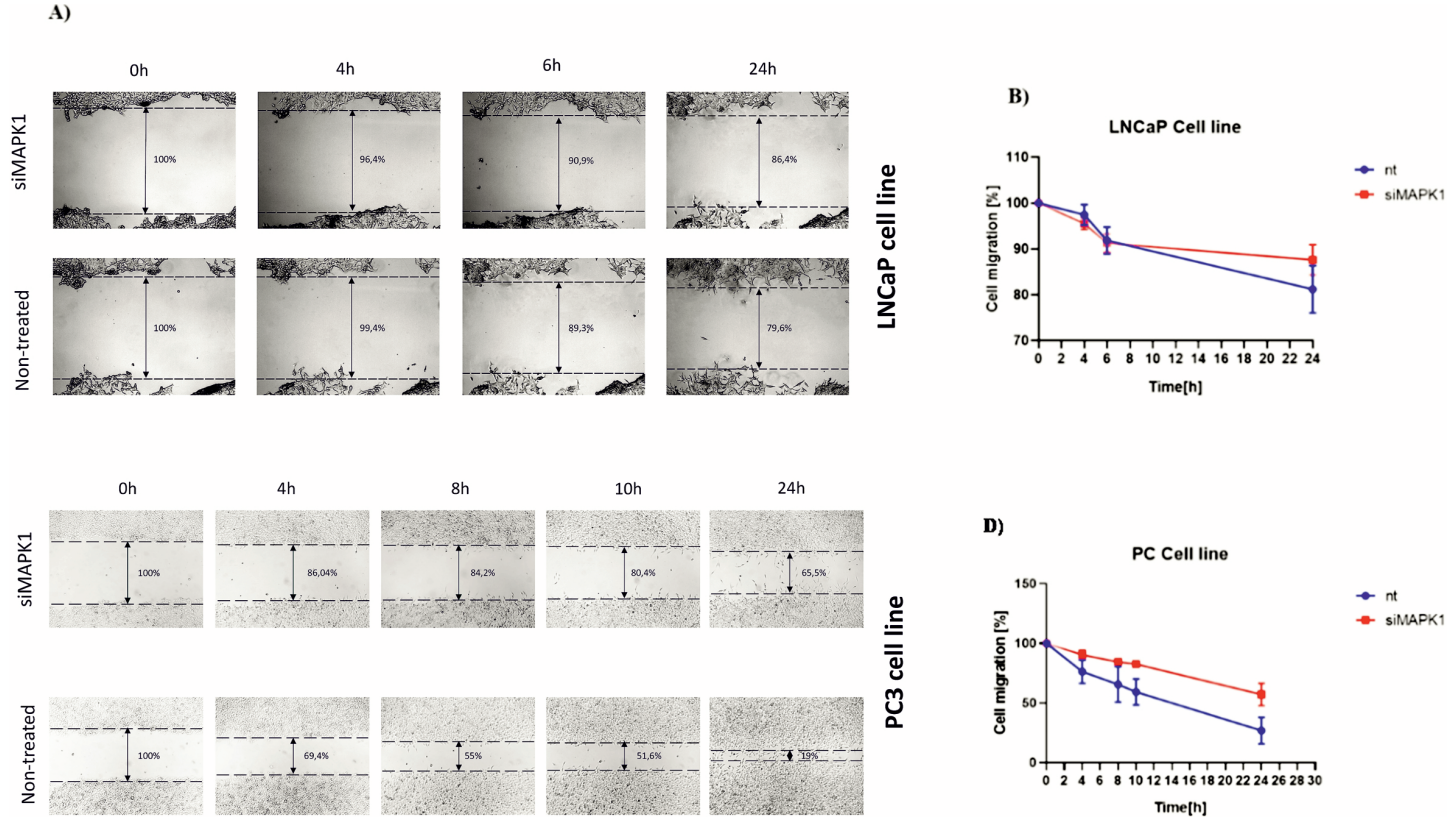



Fig. 1. A) Representative results of the scratch assay. Cell migration distance of LNCaP cell line after MAPK1 gene silencing for 0, 4, 6 and 24 h. B) The results of the scratch assay were presented as a percentage [%] of the ratio of the current wound distance to initial wound distance. The wound distance is presented as mean ± SD. C) Representative results of the scratch assay. Cell migration level of PC3 cell line after MAPK1 gene silencing for 0, 4, 8, 10 and 24 h. D) The results of the scratch assay were presented as a percentage [%] of the ratio of the current wound distance to initial wound distance. The wound distance is presented as mean ± SD

tivation in AR-negative prostate cancer cells as demonstrated by the beneficial MAPK1 inhibition resulting in a reduced expression of AR target genes, such as PSA and TMPRSS2 and sensitization to anti-androgen therapy [15], in AR negative, but not in AR positive cells [3]. We found in similar fashion that MAPK1 silencing affects mostly AR-negative castration-resistant bone metastasis derived PC3 cell line, but not the AR-positive lymph node cell line LNCaP. Since we aim to aid the castration-resistant phenotype chemotherapy, MAPK1 silencing seems to be a viable approach.

Unfortunately, silencing MAPK1 for 48 h in castration-resistant PC3 cell line ($AR^{-/-}$, $p53^{-/-}$) resulted in an increased NF-$\kappa$B activation, as observed using highly sensitive NF-κB reporter vector possessing three transcription factor response elements (Fig. 2). Previous studies have shown that NF-κB signalling can be activated by MAPK kinases [16], but our findings reveal that NF-κB signalling was not dependent only on MAPK1 signalling, or rather has become independent of MAPK1 induction in this castration-resistant model. Constitutive NF-κB activity found in AR-negative, but not in AR-positive cells of metastatic prostate cancer, was found accompanied by several pro-inflammatory signalling pathways constantly activated, thus promoting continuous NF-κB activation in PCa, like IL-6/ERK1/ERK2, TNF-$\alpha$/NIK, TNF/p38, IL-1/NIK or IL-1/p38 [17]. Along with pro-inflammatory signalling supporting NF-κB expression, autophagy was found cytoprotective in castration-resistant PC3 cells against both apoptosis inducing chemotherapeutics – celecoxib and docetaxel [8]. In the second case, the cell survival protection was related to increased mitophagy, that was later selectively linked to MAPK1/3 [7], suggesting that in castration-resistant PCa MAPK1 has

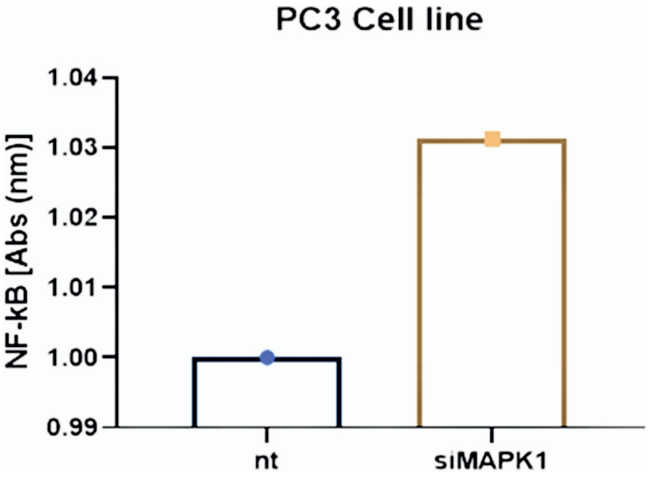



Fig. 2. Levels of active NF-κB in PC3 cell line after 48 h MAPK1 gene silencing, estimated by colourimetric SEAP reporter plasmid. Values were normalized to a control of 1, readings were taken on the fortieth minute at $\lambda_{em} = 405$ nm

been "reprogrammed" to support NF-κB anti-apoptotic activities. Other studies with docetaxel also supported that, showing cytopathic effect in LNCaP, but not in PC3 cells, with MAPK1 activation causing therapeutic resistance to antimitotic drug [8].

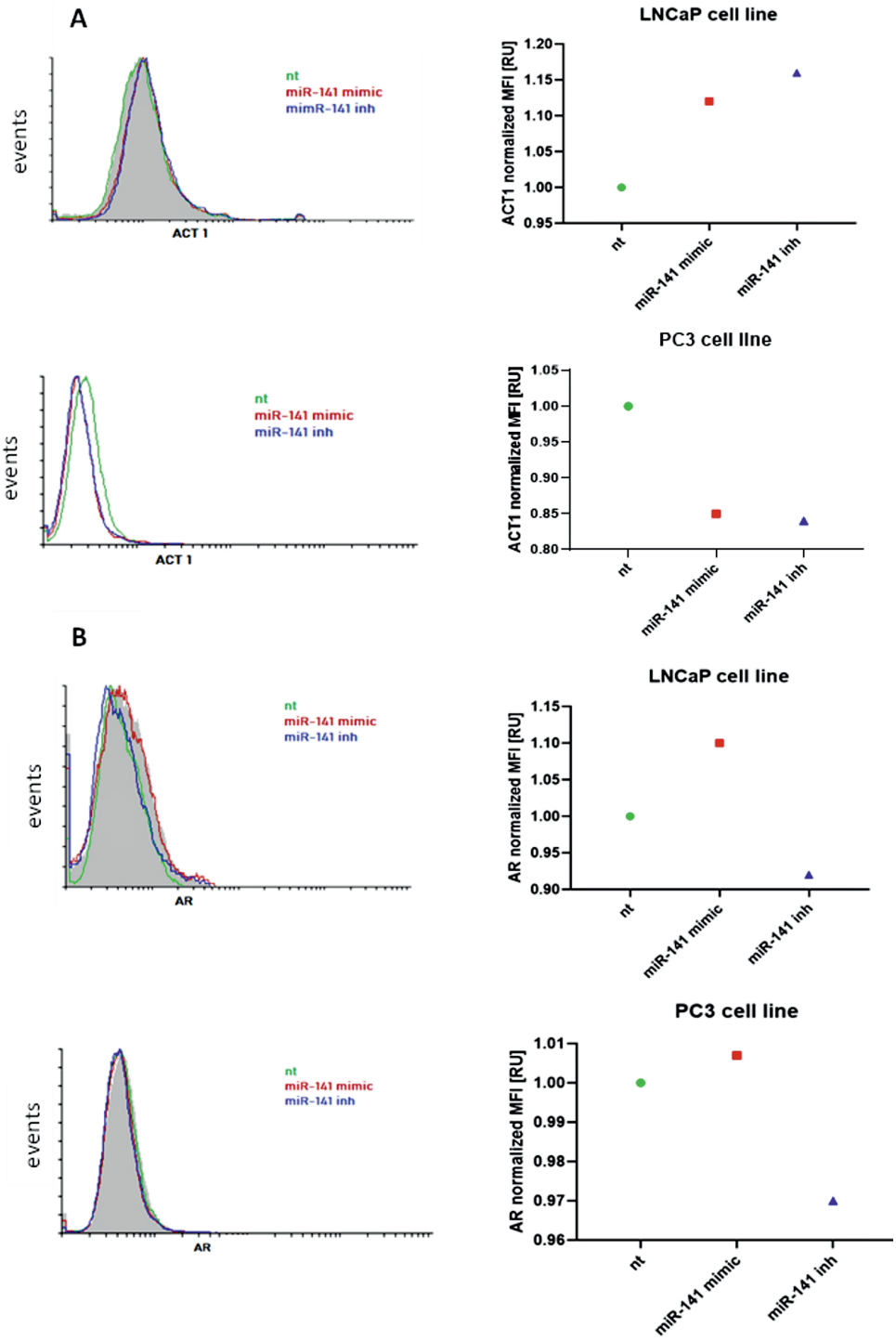
Since MAPK1 silencing in castration-resistant PC3 cell line fall short in reducing NF-κB activation, we further continued our efforts for counteracting NF-κB pro-oncogenic activities by rescuing miR-141, targeting both its mitochondrial activity and PTEN/ROS production activities.

MicroRNA-141 levels are increased in LNCaP cell line compared to bone marrow metastatic PC3 cell line. This is inversely related to the methylation of the microRNA-141 promoter, which is unmethylated in LNCaP cell line, but hypermethylated in PC3 cell line [13]. The tumour suppressive role of miRNAs in prostate oncogenesis, including microRNA-141, is crucial in preventing epithelial-to-mesenchymal transition (EMT) [13]. Using both miR-141 mimic and inhibitor we did functional studies to follow the expression of both Act1 inductor of NF-κB and AR. Act1 is one of the essentials for NFκB activity, as it was shown to be an NFκB activator via TRAF6, acting through binding to the NFκB depressors like IKK-gamma [18]. We found miR-141 mimic rescue to upregulate both Act1 and AR in LNCaP AR-positive cell line, while same miR inhibition had no significant effect on expression. In castration-resistant PC3 cells miR-141 rescue suppressed Act1 expression, with no effect on AR, which is lacking per se (Fig. 3), suggesting potential for adding miR-141 mimic in castration-resistant therapeutic approaches. This option is only available in AR-negative PCa, like metastatic and cancer stem cell driven tumours, where miR-141 downregulation promotes bone metastasis by upregulating TRAF5 and TRAF6, resulting in sustained NF-κB signalling activity and inflammation [19]. Early stage, AR-negative PCa has increased miR-141 targeting PTEN and increasing ROS [12], and the approach is not viable. Our data showed that miR-141 is most likely implicated in NF-κB upregulation mediated via AR suppression and miR-141 inhibition resulted in declined AR expression (Fig. 3).

AR signalling has complex participation in PCa transition towards castration-resistance, initially increased AR signalling is replaced with detached and reprogrammed AR transcription control, to promote further EMT [1]. miR-141 is therefore more important for this transitional, rather than the early PCa stages.

DNA methyltransferase inhibitors, such as decitabine, showed potential in mitigating inflammation by targeting the NF-κB signalling pathway [11], down-

Fig. 3. Levels of active ACT1 (A) and AR (B) assessed using intracellular flow cytometry in LNCaP and PC3 cell lines after 48 h transfection with either miR-141 mimic or miR-141 inhibitor. Expression is assessed using MFI parameter of FCS histogram. ACT1 and AR → expression are assessed as normalized MFI to non-treated control and presented in arbitrary units


A
events
nt
miR-141 mimic
mimR-141 inh
ACT 1
LNCaP cell line
ACT1 normalized MFI [RU]
1.20
1.15
1.10
1.05
1.00
0.95
nt
miR-141 mimic
miR-141 inh
PC3 cell line
ACT1 normalized MFI [RU]
1.05
1.00
0.95
0.90
0.85
0.80
B
AR
AR normalized MFI [RU]
1.15
1.10
1.05
1.00
0.95
0.90
1.01
0.99
0.98
0.97
0.96

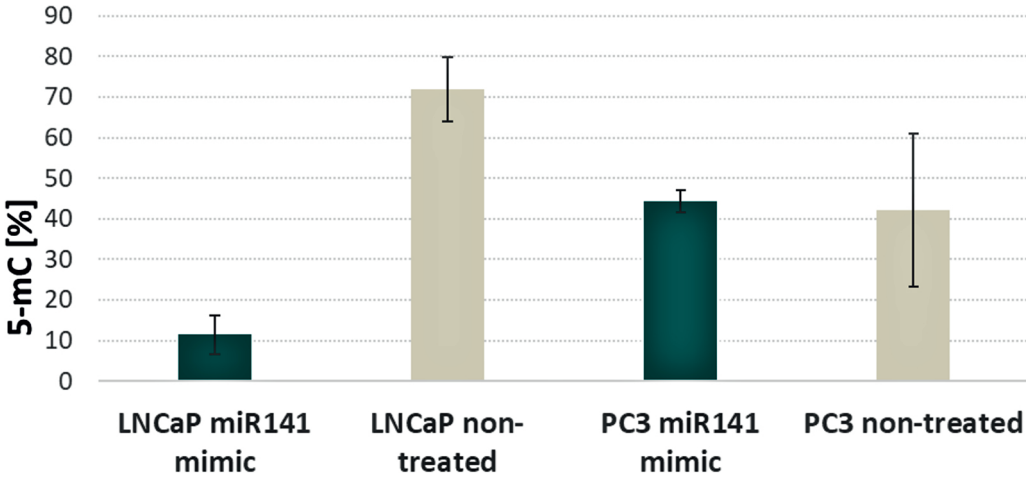



Fig. 4. Quantification of DNA methylation (i.e., 5-methylcytosine) by ELISA reader in LNCaP and PC3 metastatic prostate cancer cell lines after 24 h of miR-141 mimic treatment compared to untreated cells. The percentage 5-mC in a DNA sample is accurately quantified from a standard curve generated with specially designed controls. A logarithmic relationship was observed with a correlation of R2 = 0.993 and readings were taken on the thirtieth minute at Abs. = 405 nm. The results are presented as mean ± SD

regulating inflammatory cytokine levels, and thus inhibiting cancer progression. We rescued miR-141 using 24 h mimic transfection to follow its role also on total DNA methylation in LNCaP and PC3 cells. We found total DNA methylation levels significantly reduced in AR-positive LNCaP cell line, while the rescue did not have a significant effect in AR-negative PC3 cell line (Fig. 4).

It was shown before that microRNA-141 levels are androgen regulated and in vitro DHT (dihydrotestosterone) stimulation of LNCaP cell line, also increased microRNA-141 expression in dose-dependent fashion [20]. We found that miR-141 mimic increase (rescue) further increased AR expression, promoting positive vicious feedback (Fig 3). We hypothesize that androgen receptor overexpression in LNCaP cell line has direct effect on DNA demethylation via microRNA-141 in contrast to AR-negative PC3 cells, thus further promoting ROS pro-inflammatory signalling, caused by ROS demethylation. In castration- resistant PCa, like in PC3 cells [14], excessively increased ROS would severely damage the cell, and this signalling is abolished via MAPK1-mediated autophagy activation.

Lynch et al. [9] demonstrated that miR-141 promotor differential methylation is responsible for its downregulation in castration-resistant vs. AR-positive phenotypes, further supporting our observations in LNCaP cells for early stage PCa miR-141 promotion of global DNA demethylation that was found to silence the DNA-methyltransferase 1 (DNMT1), and hence enhance its promotor, activating another positive feedback loop. Considering the complex plasticity and multidimensional dysregulation that occur in prostate carcinogenesis, further studies

are required to elucidate the full mechanisms of microRNA-141 role in castration-resistant PCa.

**Conclusion.** We show that selective MAPK1 inhibition and/or microRNA-141 rescue could be employed potentially as supportive addition to chemotherapy as personalised approach in transitional stages towards castration-resistant prostate cancer. Further high-throughput and mechanistic studies elucidating the dysregulation in autophagy and pro-inflammatory signalling are required to fine tune this supportive approach.

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