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MiR-33a and statins collaboratively reduce the proliferative capacity of prostate cancer cells

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ABSTRACT

Objective: Prostate cancer (PCa) is one of the leading causes of cancer deaths among men in the developed countries. Accumulating data suggests a high-cholesterol Western diet as an important risk factor for PCa. Besides,significant evidencesassociate increased serum cholesterol levels with PCa development and progression.In this study, we aimed at investigating the collaborative roles of cholesterol analogs, cholesterol-lowering drugs, and miR-33a, which is an important microRNA involved in regulation of cholesterol metabolism,on the cellular phenotypes associated with PCa progression.

Methods: We evaluated the effects of low-density lipoprotein (LDL) cholesterol, 25-hydroxycholesterol (25-HC), mevastatin and simvastatin on their ownand together with miR-33a on the proliferation, invasion and anchorage independent growthcapacity of PCa cells using Cell Counting Kit-8, Matrigel invasion, and soft agar assays, respectively.

Results: We show that cholesterol analogs significantly promoted proliferative, invasive, and clonogenic potential of PCa cells, while cholesterol loweringstatins demonstrated opposite effects. Moreover, LDL and 25-HC reversed the tumor suppressive potential of miR-33a and statin treatment promoted the proliferation inhibitory effect of miR-33a on PCa cells.

Conclusions: We demonstrated that statins inhibited the cellular phenotypes associated with PCa progression and miR-33a treatment strengthens the impacts of statins on cellular proliferation. These findings suggest that statins alone and together with miR-33a might be a useful tool for effective and successful eradication of PCa cells.

Keywords: Prostate cancer, microRNA, miR-33a, cholesterol, statins

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Prostate cancer (PCa) is one of the most frequently diagnosed malignanciesin men in the developed countries [1]. Hormone ablation therapy, radical prostatectomy, and radiotherapy are considered as the first line treatment options for clinically localized PCa,however, they do not necessarily provide improved survival rates and almost one third of the patients develop clinical recurrence [2, 3]. For advanced tumors, chemotherapy, the only therapeutic alternative, is not curative [4]. Therefore, a comprehensive understanding of the molecular contributors to the prostate carcinogenesis is necessary to develop novel therapeutic modalities.

Accumulating data suggest a high-cholesterol Western diet as a critical risk factor for many solid tumors including PCa [5]. Although conflicting

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findings are present in terms of association of serum cholesterol levels with PCa risk, significant evidences support an important role for high-density lipoprotein (HDL), low-density lipoprotein (LDL), and total cholesterol in PCa development and progression [6-9]. Prostate tumor cells might acquire castration-resistance through activating intrinsic androgen biosynthesis pathway, which might be via acquisition of capability to synthesize androgens from its precursors including cholesterol [10]. Statins, as cholesterol-lowering drugs, might be considered as effective adjuvant therapeutic agents against PCa [5]. Interestingly, several epidemiological studies reported a significant association between statin use and overall reduced cancer risk and mortality, including PCa [11-13].

Furthermore, an intronic microRNA, miR-33a, together with its host sterol-response-element-binding protein gene *(SREBF2)*, play important roles in the control of lipid and cholesterol metabolism [14]. It has been reported to be predominantly coexpressed with its host gene both in human and mouse tissues [15] and has been recently demonstratedto act as a tumor suppressor in PCa along with several other tumor types [16-23]. MiR-33a is involved in regulation of cholesterol metabolism through targeting 3' untranslated regions of genes like *ABCA1,CPT1A* and *HADHB* [24].

In this study, we aimed at investigating the roles of LDL, 25-hydroxycholesterol (25-HC), mevastatin, and simvastatin on the cellular phenotypes associated with PCa progression, and evaluating their collaborative actions with increased miR-33a expression on the proliferative potential of PCa cells. We found that cholesterol analogs significantly promoted proliferative, invasive, and clonogenic potential of PCa cells, while statins inhibited those features. Besides, LDL and 25-HC reversed the tumor inhibitory potential of miR-33a and statin treatment increased the inhibitory effects of miR-33a on PCa cells. These findings suggest that miR-33a and statins might be useful tools for effective and successful eradication of PCa cells.

METHODS

Chemicals and Reagents

Fetal bovine serum (FBS, Gibco, Gaithersburg, MD, USA), penicillin/streptomycin (Invitrogen, Waltham, MA, USA), RPMI-1640 medium (GenDepot, Barker, TX, USA), Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Waltham, MA, USA), and lipoprotein deficient serum (LPDS, Kalen Biomedical, Germantown, MD, USA) was used for cell culture experiments. LDL, 25-HC, and simvastatin were purchased from Sigma and Mevastatin was purchased from MedChemExpress.

Cell Culture

LNCaP and VCaP cells with miR-33a expression reduced by more than 50% compared toimmortalized benign prostate epithelial cell line PNT1a were utilized in this study [16]. Cell lines were acquired from American Type Culture Collection (ATCC, Manassas, VA, USA) and their authentication was performed with STR analysis at MD Anderson Cancer Center Characterized Cell Line Core Facility. LNCaP cells were grown within RPMI-1640 medium containing 10% FBS and 1% penicillin/streptomycin. VCaP cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin. Cells treated with cholesterol analogs and statins were grown in medium supplemented with 10% LPDS. Cells were maintained at 37 °C in a humidified 5% CO2 incubator.

MicroRNA Transfection

Transfection of miR-33a and non-targeting control microRNA (Invitrogen) was performed with Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions.Briefly, appropriate number ofLNCaP or VCAP cells were seeded in 96 well plates to be 60-80% confluent at the time of transfection. Lipofectamine® RNAiMAX Reagent (Invitrogen, Waltham, MA, USA)and 60 pmol miR-33a mimic were diluted in Opti-MEM® Medium(Gibco, Gaithersburg, MD, USA). Diluted miR-33a mimic and Lipofectamine® RNAiMAX Reagent were mixed in 1:1 ratio and incubated for 5 minutes at room temperature. Then, microRNA-lipid complex was added to the medium of cells.

RNA Isolation, cDNA Synthesis and Quantitative Real-Time PCR

Total RNA from reagent treated and/or

microRNA-transfected samples were isolated using TRIzol (Invitrogen, Waltham, MA, USA) reagent. The concentrations and purities of RNA samples were evaluated spectrophotometrically with NanoDrop ND-2000c (Thermo Fisher Scientific, Wilmington, DE, USA).

MicroRNA first strand DNA (cDNA) synthesis from equal amounts of total RNA was carried out with microRNA specific primers (Applied Biosystems, Foster City, CA, USA) and "TaqMan MicroRNA reverse transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA) and microRNA probes (Applied Biosystems, Foster City, CA, USA) were used for microRNA expression analysis. Each experiment was performed in triplicates and microRNA expression data were normalized to RNU43.

For gene expression analysis, equal amounts of total RNA samples were reverse transcribed using "amfiRivert cDNA Synthesis Platinum Master Mix" (GenDepot, Barker, TX, USA) following the manufacturers' protocol. SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used for quantitative real time PCR (qRT-PCR). Each experiment was performed in triplicates andnormalization of gene expression was done comparingß-actin expression.

Primer sequences used to evaluate gene expressions are listed in Table 1. qRT-PCR was performed in a StepOnePlus™ real-time thermal cycler (Applied Biosystems, Foster City, CA, USA) with standard parameters. 2- $^{\Delta\Delta CT}$ method was utilized to calculate the alterations in expression levels.

Cell Proliferation Test

The proliferation capacities of LNCaP and VCaP cells in the presence of the reagents alone (LDL in 50 µg/ml [25], 25-HC in 1 µg/ml [25], Mevastatin in 10 µM [26], and Simvastatin in 10 µM [26] final concentration) or in combination with miR-33a mimic (60 pmol final concentration) were evaluated as follows. Cells were seeded in 96 well plates and after 24 hours, cells were transfected with miR-33a mimic or non-targeting control. Then 3 hours later, cells were treated with reagents to assess their effects on cell proliferation alone or in combination with miR-33a.

Proliferation capacities were evaluatedat the end of each day for 4 days with the Cell Counting Kit-8 (Dojindo, Rockville, MD, USA) according the manufacturer's protocol and the absorbances were measured with a VERSAmax Tunable microplate reader (Conquer Scientific, San Diego, CA, USA).

Table 1. qRT-PCR primer sequences

Gene	Sequence
Beta-actin-F	5'-GCCTCGCCTTTGCCGATC-3'
Beta-actin-R	5'-CCCACGATGGAGGGGAAG-3'
CPT1A-F	5'-GGCAAGTTTTGCCTCACATACG-3'
CPT1A-R	5'-GAACAACTTCAGCCTCTGTTCC-3'
HADHB-F	5'-AAACCAAGGTTGGATTGCCTC-3'
HADHB-R	5'-CACTATCATAGCATGGCCCTG-3'

Matrigel Invasion Test

Invasion capacities of cells were evaluated with BD BioCoat Matrigel invasion chambers (Becton Dickinson, Franklin Lakes, NJ, USA). After treated with reagents alone (LDL in 50 µg/ml, 25-HC in 1 µg/ml, Mevastatin in 10 µM, and Simvastatin in 10 µM final concentration) or in combination with overexpression of miR-33a mimic or non-targeting control (60 pmol final concentration) for 48 hours, LNCaP cells were harvested and plated into invasion chambers in triplicates. After 24 hours, cells on the upper surface of the chamber were removed and the cells on the lower side of the filter were initially fixed inmethanol and stained with 0.3% crystal violet and 2% ethanol containing solution for 20 min. Then, relative invasion ratios were calculated through counting cells.

Soft Agar Colony Formation Test

LNCaP cells were suspended in 0.3% agar diluted in RPMI at a density of 3x103 cells/ml and seeded on a 0.6% base agar in 6-well culture plates in triplicates. Then cells were cultured in the presence or absence of reagents (LDL in 50 µg/ml, 25-HC in 1 µg/ml, Mevastatin in 10 µM, and Simvastatin in 10 µM final concentration) at 37°C incubator for 2 to 3 weeks.

Cells were fixed and stained with 0.01% crystal violet and 10% ethanolcontaining solution. Relative colony numbers were calculated through counting colonies with a dissecting microscope.

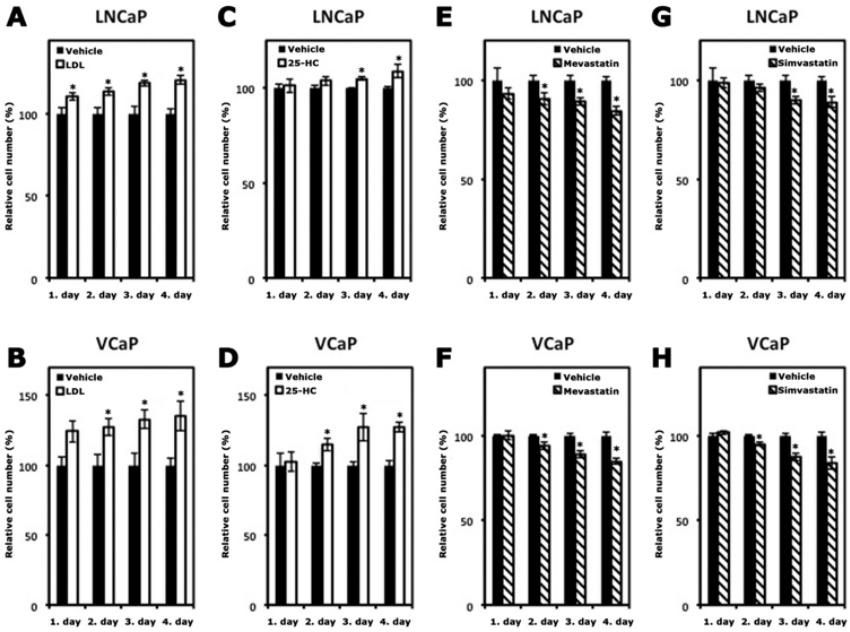

Figure 1. Impacts of cholesterol analogs and statins on the proliferative potential of PCa cells. Proliferation of LNCaP cells treated with LDL (a), 25-HC (c), Mevastatin (e), Simvastatin (G). Proliferation of VCaP cells treated with LDL (b), 25-HC (d), Mevastatin (f), Simvastatin (h). Mean +/- SEM is shown *p <0.05; t-test

Statistical Analysis

Data were plotted as ± mean standard error of mean (SEM) and the statistical significances were tested using Student's t test. A p value of 0.05 or below was accepted as statistically significant.

RESULTS

LDL and 25-HC promoted and Statins inhibited the proliferation, invasion, and clonogenic capacity of PCa cells

To explore the biological relevance of cholesterol analogs and statins in PCa, we initially evaluated their effects on the proliferative capacity of PCa cells. Treatment of LNCaP and VCaP cells with LDL significantly promoted proliferation by up to 20% (Figure 1a) and 35% (Figure 1b), respectively. 25-HC treatment also resulted in increased proliferation in both cell lines (Figures 1c and 1d). On the contrary, cholesterol-lowering drugs, Mevastatin and Simvastatin, reduced proliferation by up to 16% (Figure 1e) and 12% (Figure 1g) in LNCaP cells, respectively. Similarly, statin treatment caused significant decrease in proliferation of VCaP cells (Figures 1f and 1h).

Moreover, LDL significantly increased the invasive and clonogenic potential of LNCaP cells, while statins demonstrated opposite effects (Figures 2a and 2b). 25-HC treatment also resulted in enhanced invasive potential (Figure 2b), however, it did not result in a significant change in colony formation ability of LNCaP cells (Figure 2b). This might be due to accumulation of 25-HC within the cells during soft agar assays, which could make the concentration of 25-HC reach the toxic levels for LNCaP cells. These overall findings point the potential of statins to inhibit cellular phenotypes associated with PCa progression.

MiR-33a reverses the effects of cholesterol analogs and strengthens the impacts of statins on cellular proliferation

To evaluate the collaborative action of miR-33a with cholesterol analogs and statins on the proliferative potential of PCa cells, we treated cells that are overexpressing miR-33a with LDL, 25-HC, mevastatin, and simvastatin and evaluated their dual impact on the proliferation of both LNCaP and VCaP cells. We initially investigated the efficiency of miR-33a transfection in cells, which showed that miR-33a was overexpressed up to ~40 and ~130 fold in LNCaP and VCaP cells, respectively, relative tocells

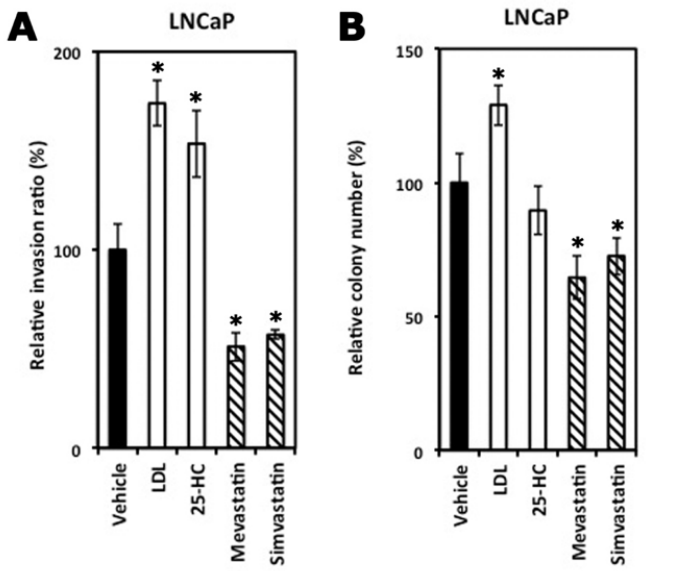

Figure 2. Impacts of cholesterol analogs and statins on the invasive and clonogenic potential of PCa cells. Invasion (a) and anchorage independent growth (b) of LNCaP cells treated with LDL, 25-HC, Mevastatin, Simvastatin. Mean +/- SEM is shown *p <0.05; t-test

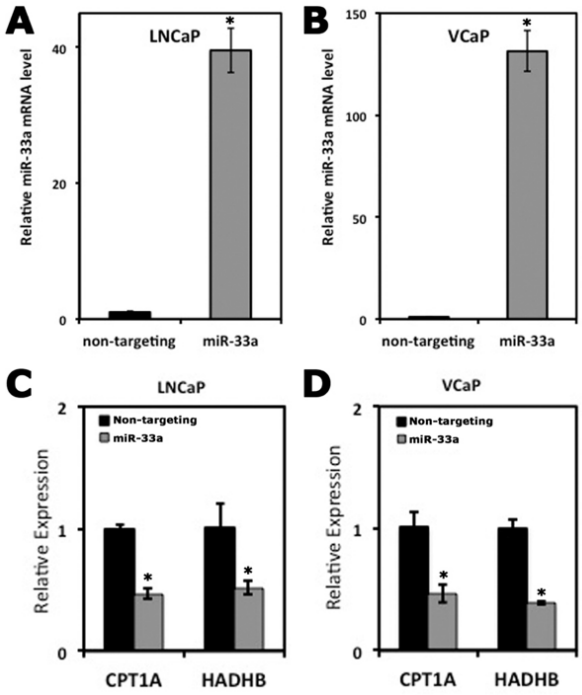

Figure 3. Overexpression of miR-33a. Relative miR-33a level in miR-33a transfected LNCaP (a) and VCaP (b) cells. qRT-PCR validation of CPT1A and HADHB as direct targets of miR-33a involved in cholesterol metabolism in miR-33a transfected LNCaP (c) and VCaP (d) cells. MiRNA levels are demonstrated as fold change and were normalized to RNU43, mRNA levels are demonstrated as fold change and were normalized to ß-actin. Mean +/- SEM is shown *p < 0.05; t-test

overexpressing non-targeting controls (Figures 3a and 3b).

We then validated the downregulation of CPT1A and HADHB, which are direct targets of miR-33a as implicated in cholesterol metabolism, at the mRNA level upon ectopic miR-33a overexpression (Figures 3c and 3d), and confirmed the inhibitory role of miR-33a on the proliferative capacity of both LNCaP and VCaP cells (Figure 4).

Furthermore, treatment of LNCaP and VCaP cells ectopically overexpressing miR-33a with LDL and 25-HC reversed the inhibitory potential of miR-33a and resulted in increased proliferation (Figures 4a, 4b, 4c and 4d). To explore the collaborative action of miR-33a and statins on PCa cell inhibition, we also treated PCa cells ectopically overexpressing miR-33a with statins and demonstrated that treatment of cells with either Mevastatin or Simvastatin along with miR-33a overexpression significantly enhanced the proliferation inhibitory potential of miR-33a.This points the power of utilization of miR-33a and statins together as a tool for effective and successful eradication of PCa cells.

DISCUSSION

High cholesterol, also known as hypercholesterolemia, is a worldwide problem with a global prevalence of about 39% among adults [27]. Adults with high cholesterol are considered as at higher risk for cardiovascular diseases as well as several cancers, and recent data suggest that hypercholesterolemia might be implicated with the risk of PCa development [7, 8, 28, 29].

3-Hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors, commonly referred as statins, are effective lipid-lowering drugs, which are generally administered in Western countries [27]. Accumulating evidences like (i) significant reduction of PSA levels, which is in parallel with the amount of LDL decline, in PCa-free men after starting a statin [30], (ii) profound inhibition of cellular growth upon combinatorial therapy with atorvastatin and celecoxib, but not upon their individual use, in PCa xenograft models [31, 32], and (iii) associations between statin use and a decline in the advanced PCa incidence and recurrence risk after treatment compared to controls

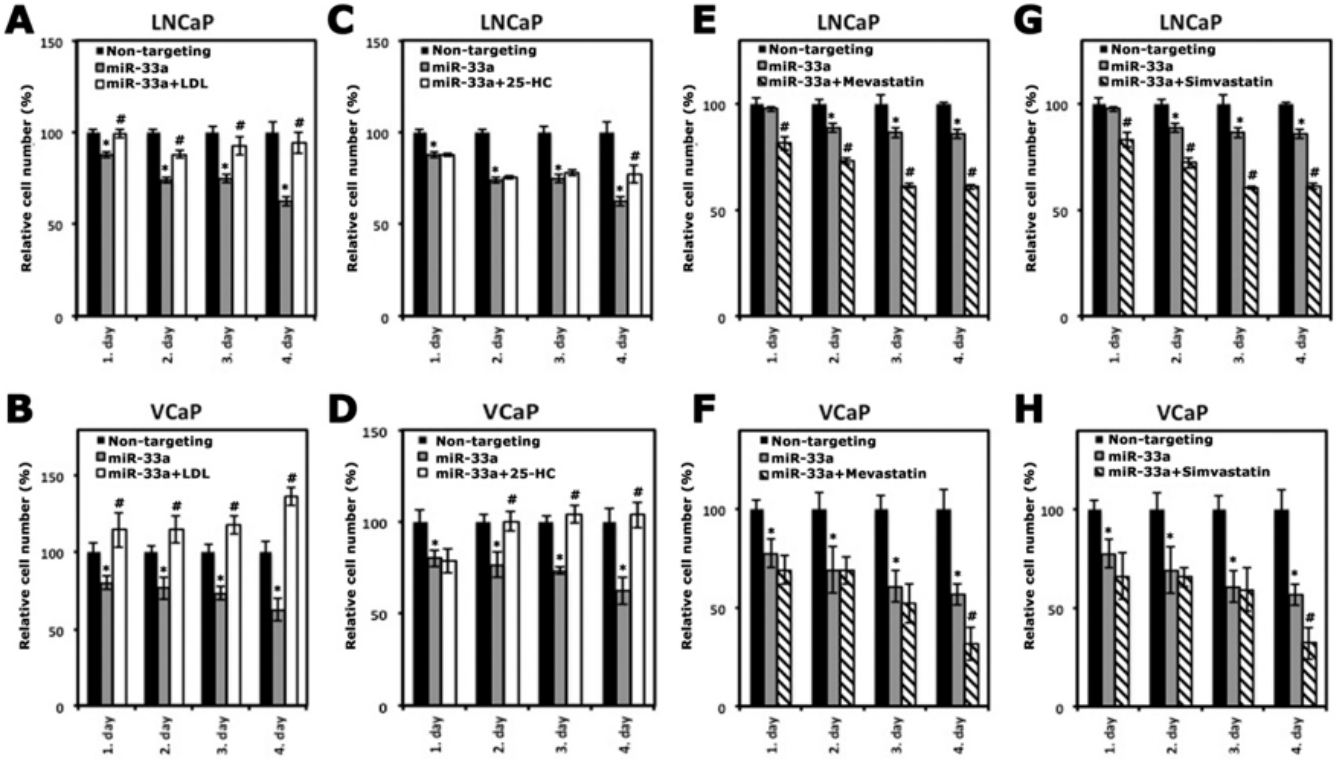

Figure 4. Collaborative effects of miR-33a with cholesterol analogs and statins on cellular proliferation. Proliferation of LNCaP cells overexpressing miR-33a treated with LDL (a), 25-HC (b), Mevastatin (c), Simvastatin (d). Proliferation of VCaP cells overexpressing miR-33a treated with LDL (e), 25-HC (f), Mevastatin (g), Simvastatin (h). Mean +/- SEM is shown *$p < 0.05$; t-test

[27], suggest that development of cholesterol-dependent therapeutic approaches may be key to enhance the fight against PCa.

Our results demonstrated that introduction of cholesterol analogs significantly promoted proliferation, invasion, and clonogenic capacity of PCa cells, whereas, statins inhibited their proliferation, invasion, and anchorage independent growth, which is in line with previous findings [25, 33]. Interestingly, in those studies, both Mevastatin and Simvastatin have been shown to display toxic effects to RWPE-1 to some extent, however, they were found to have selective inhibition against PCa cells and reduced potency against benign epithelial RWPE-1 cells compared to PCa cells [26, 34].

Statins are thought to eradicate PCa cells through (i) regulation of cholesterol levels required for signal transduction, and (ii) modulation of signaling pathways associated with several cellular phenotypes independent of cholesterol lowering, like cell proliferation, invasion, and migration [30, 35-37]. Moreover, statins inhibit proliferation via regulation of androgen receptor expression and activity [26,38]. Androgens are implicated in PCa progression and androgen deprivation therapies have been an effective means of treatment for disseminated PCa [39]. However, cancer cells are able to endure the androgen-depleted environment and develop castration resistant tumors through several androgen receptor related adaptive mechanisms like reactivation of androgen receptor and *de novo* intratumoral production of androgens [34, 40]. Considering the synthesis of androgens via multi-enzymatic conversion of cholesterol, use of statins might be useful for treating castration resistant PCa patients [41]. Besides, Simvastatin, as an FDA approved cholesterol-lowering drug, display well tolerated side-effect profiles, which proposes statins as an ideal combinatorial therapy option with chemotherapeutic agents with minimal additional risk to the patients [34, 42]. However, further studies are needed to determine the class and optimal *in vivo* doses of statins with minimum toxic effects to normal cells.

In this study, we also investigated the potential

collaborative inhibitory function of miR-33a and statins on the proliferative potential of PCa cells. In normal tissues, miR-33a, along with its host gene *SREBF2*, modulate regulation of cholesterol levels [43]. Downregulation of miR-33a allows upregulation of oncogenic genes such as PIM1 and causes increase of β-oxidation of fatty acids through upregulation of genes like HADHB and CPT1A [16, 24]. Such elevated β-oxidation might contribute to providing of energy to PCa cells. Another potential association of miR-33a to cholesterol metabolism is its targets that are involved in cholesterol transport such as *ABCA1, ABCG1,* and *NPC1* [24].

Several studies demonstrated that overexpression of miR-33a *in vitro* profoundly suppressed cholesterol export in various cell culture models [15, 44-46]. Further in vivo studies also demonstrated significant elevation in serum HDL cholesterol in miR-33a -/- mice [45].

Until now, there are numerous microRNAs that are proposed as putative therapeutic agents against PCa. Unraveling the mechanism of actions, finding their true targets, and determining accurate expression data are required to develop effective and practical therapies. For example, recently overexpression of the lethal-7 (let-7) was suggested as a therapeutic tool for PCa through utilization of either lenti- or adenoviruses to inhibit the survival and proliferation of tumor cells [47, 48]. In another study, a natural product, 3,3'-diindolylmethane (DIM), was given to patients prior to radical prostatectomy to evaluate its therapeutic role. The phase II clinical trial demonstrated that DIM intervention lead to increased levels of let-7 and reduced expression of its target, EZH2, in PCa cells, which resulted low self-renewal and clonogenic capacity [49]. This findings point the potential of treatment options targeting deregulated microRNAs in PCa.

The Limitations of the Study

Certain limitations should be considered when interpreting the results of our study. Analysis of the impacts of cholesterol analogs and statins along with miR-33a overexpression were performed only with two PCa cell lines, which does not effectively reflect the heterogeneity of the PCa. Moreover, utilization of only cancer cells but not a healthy prostate epithelial cell line restricts to foresee potential side effects of miR-33a overexpression and statin treatment. Lack of in vivo experiments prevents drawing a clear conclusion about the collaborative function of miR-33a and statins against PCa. Lastly, not utilization of control cell lines that are not associated with cholesterol would effect the specificity of the proposed mechanism. Therefore, further detailed in vitro research should be performed to reveal the possible mechanisms of differential expression of miR-33a and the role of deregulated cholesterol homeostasis in PCa as well as to show the importance of targeting these deregulations to eradicate PCa cells. Also, further in vivo and clinical researches are needed to clarify the potential of miR-33a and statins against PCa.

CONCLUSION

In conclusion, we demonstrated that LDL and 25-HC promoted and statins decreased the proliferation, invasion, and clonogenic features of PCa cells and miR-33a reverses the effects of cholesterol analogs and strengthens the affects of statins on the phenotypes related to PCa progression, which suggests that miR-33a and statins might be useful tools for effective and successful eradication of PCa cells. However, more evidences on the curative or preventive impacts of statins against PCa both *in vitro* and *in vivo* still needs to be provided.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

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