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Original Article

miR-199a and miR-34c enhance the migration of prostate cancer stem cells but inhibit migration of PC3 prostate cancer cells

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Abstract: Cancer stem cells (CSCs) have been identified in several cancers, including prostate cancer (PCa). Although prostate cancer stem cells (PCSCs) may differentiate into the various cells that constitute the PCa tumor, PCSC biology is markedly distinct from that of the PCa tumor mass. Delineating these differences could hold the key to understanding PCa progression, including epithelial-to-mesenchymal transition (EMT) and metastasis. Because CSCs are modulated by microRNAs (miRNAs), which may influence resistance to treatment and metastasis, we investigated the differential expression of miRNAs in PCSCs and in PC3 cells and their effect on cell invasion and migration. We analyzed the miRNA expression profiles by microarray, and a subset was confirmed using qRT-PCR. Twenty-three miRNAs were differentially expressed of which seven were validated by qRT-PCR, including has-miR199a, has-miR155, let-7d, has-miR34b, has-miR-34c, has-miR-505, and has-miR-196a. We then selected two miRNAs, miR-199a and miR-34c, that were highly expressed in PCSCs, and investigated their effect on PCSC invasion and migration using Matrigel invasion assays and scratch wound assays, respectively. Neither miR-199a nor miR-34c inhibition altered PCSC or PC3 cell invasion; however, inhibition of miR-199aor miR-34c reduced PCSC migration and enhanced PC3 cell migration. The effects of miR-199a and miR-34c inhibition on PCSC and PC3 cell migration suggest they may represent potential biomarkers and therapeutic targets in the treatment of PCa, which requires validation in further studies.

Keywords: Invasion, migration, miRNA, prostate cancer, stem cells

Introduction

Prostate cancer (PCa) is the sixth leading cause of cancer-related death in men globally, and the second in the United States [1]. Current treatment options include surgery, radiation therapy, proton beam therapy, chemotherapy, androgen deprivation therapy (ADT), and cryosurgery [2]. Although ADT is highly effective, it often results in tumor recurrence, which may be caused by prostate cancer stem cells (PCSCs) [3].

Cancer stem cells (CSCs) have the capacity to replicate and to differentiate into the various cells that comprise the heterogeneous tumor-mass [4]. CSCs may arise from normal stem cells by mutation and also from mutated progenitor cells [5]. CSCs have been identified in several cancers, including blood, breast, brain, colon, melanoma, pancreatic, prostate, ovarian, and lung cancers [6, 7], and have been associated with the chemoresistance and radioresistance [8]. Relative to the tumor mass, CSCs comprise a very small pool of cells. In PCa, PCSCs comprise only 0.01-1.1% of the cells in core biopsies from primary human prostatectomies [9]. This small reservoir of drug-resistant cells is, however, responsible for relapse after chemotherapy-induced remission and has been implicated in distant metastases [4]. The expression of the stem cell markers, CD133, CD44, and ABCG2, varies between the various Gleason grades and metastatic PCa samples, with the highest levels occurring at the medium grade just prior to metastasis [10], coinciding with epithelial-to-mesenchymal transition (EMT).

In EMT, epithelial cells lose their cell polarity, adherens junctions, and tight junctions, and

gain migratory and invasive properties to become mesenchymal cells [11]. Kong et al. [12] showed that aPCa cell line with an EMT phenotype displayed stem cell-like features, characterized by increased expression of Sox2, Nanog, Oct4, Lin28B and/or Notch 1. miRNA modulation can reverse EMT in PCa cells [13]. miRNAs have also been shown to promote and inhibit metastasis of PCSCs [14, 15]; therefore, they may serve as potential biomarkers and therapeutic targets in PCa.

Several miRNAs are significantly up- or down-regulated in PCa with important implications in tumor progression and pathogenesis [16]. For example, miR-21 is over-expressed in PCa and effects tumorigenesis, invasion, and metastasis by inhibiting the synthesis of proteins in those pathways. It also induces cancer cell motility and inhibits apoptosis [17]. Also, over-expression of miR-30 in PCa cells suppresses EMT, inhibiting cell migration and invasion [18]. Additionally, miR-154 regulates EMT, and up-regulation of miRNA-143 in PCSCs promotes PCa metastasis [14, 19]. Also, miR-320 suppresses the stem cell-like characteristics of PCa cells and decreases tumorigenesis and chemoresistance [20]. Finally, miRNAs are involved in the acquisition of the EMT phenotype by epithelial tumor cells [21]. Studying the differential expression of miRNAs in PCSCs could, therefore, provide important information on metastasis, recurrence, and resistance to treatment in PCa.

In this study, we investigated the differential expression of miRNAs in PCSCs and PC3 cells, in search of miRNAs that effect invasion and migration. We analyzed the miRNA expression profiles by microarray, and a subset was confirmed using qRT-PCR. We then selected two miRNAs, miR-199a and miR-34c, that were highly expressed in PCSCs, and investigated their effect on PCSC invasion and migration. Both miR-199a and miR-34c inhibition suppressed the migration of PCSCs; however, their inhibition enhanced PC3 cell migration. Therefore, miR-199a and miR-34c may represent potential biomarkers and therapeutic targets in the treatment of PCa.

Materials and methods

Cell lines

PCSCs were purchased from Celprogen (San Pedro, CA, USA). They were isolated as primary cells from human adult PCa tissue based on the expression of the adult stem cell marker, CD133 [22]. They were also positive for the following markers: CD44, SSEA3/4, octamer-binding transcription factor 4 (Oct4), alkaline phosphatase, aldehyde dehydrogenase, and telomerase. Furthermore, the manufacturer observed tumorigenicity with <1000 cells. The PCSCs were maintained in the PCSC media as recommended by Celprogen. The PC3 prostate cancer cell line was used as a control and was purchased from ATCC (Manassas, VA, USA; Cat No. CRL-1435).

Profiling of miRNA by miRNA microarray

RNA was extracted as reported by Ambs et al. [23]. Total RNA was isolated using mirVana™-miRNA isolation kit according to the manufacturer's instructions (Ambion, Austin, TX, USA; #AM1561). In brief, the sample was first lysed in a denaturing lysis solution, and the lysate was then extracted once with acid-phenol: chloroform. The sample was purified over a glass-fiber filter to yield total RNA. RNA integrity was analyzed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

For the miRNA microarray, an Affymetrix Flash Tag Biotin HSR (Santa Clara, CA, USA) was used for miRNA labeling and hybridization onto the microarray. An AffymetrixGeneChip® miRNA 3.0 array (2 arrays) was performed according to the manufacturer's instructions. Primary data were analyzed using the Gene Chip-compatible™ software program and Affymetrix Expression Console software. The miRNA expression profiles of PC3 cells and PCSCs were compared using a normalization factor and clustering (expression in PC3 was set to 1 for normalization). A miRNA was considered to be differentially expressed when a *P*-value <0.05 was obtained.

Verification of identified miRNAs

The identified miRNAs were verified using quantitative real-time PCR (qRT-PCR). Seven miRNAs, including has-miR199a, has-miR155, let-7d, has-miR34b, has-miR-34c, has-miR-505, and has-miR-196a, were selected for verification based on their relatively higher expression levels. The selected miRNAs were measured using the stem-loop TaqMan MicroRNA Assay kit (Applied Biosystems, Foster City, CA, USA), according to a protocol published by Chen et al.

Table 1. Differentially expressed miRNAs with more than a 20-fold difference in expression level

miRNA	Fold-difference in expression level (PCSC/PC3)
Let-7d	25.9
miR-125b-2	60.4
miR-146b	187.7
miR-155	26.1
miR-192	22.6
miR-196a	134.5
miR-199a	145.3
miR-199b	181.6
miR-2467	99.2
miR-346	39.2
miR-34b	70.1
miR-34c-3p	250.7
miR-34c-5p	518.2
miR-3935	41.2
miR-4505	27.1
miR-4532	224.7
miR-4656	233.8
miR-4743	65.0
miR-486	61.2
miR-505*	24.4
miR-505	24.2
miR-885	20.5

*Passenger strand or anti-sense strand.

[24]. Briefly, the miRNAs were reverse-transcribed into cDNA from 10 ng of total RNA with mature microRNA-specific looped RT primers from the TaqMan MicroRNA Assay kit and with reagents from the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) following the manufacturer's instructions. Real-time PCR was performed on the 5'-extended cDNA with Applied Biosystems 2× Taqman Universal PCR Master Mix and the appropriate 5× Taqman MicroRNA Assay Mix for each miRNA. The threshold cycle (Ct) was calculated for each sample using ABI 7500 Sequence Detection System software. Standard curves were used to determine the miRNA concentration in the samples, which were then normalized to U6 RNA.

miRNA knockdown

For the miRNA knockdown, has-miR199a and has-miR-34c were synthesized using the miScriptmiRNA inhibitor (Qiagen), and each synthesized miRNA and inhibitor were co-transfected into PCSCs. A GFP reporter was also transfected to monitor transfection efficiency. PCSCs were seeded at 0.4-1.6×10^5 cells per well in a 24-well plate and cultured in the appropriate culture medium containing serum and antibiotics in a volume of 500 μL. The cells were incubated under normal growth conditions until transfection with 50 nM miRNA inhibitor diluted in 50 μL of culture medium without serum and 1.5 Ml of HiPerFect Transfection Reagent. The cells were incubated with the transfection complexes under their normal growth conditions, and gene expression was monitored 6 to 72 h after transfection. Within 24 h, transfection efficiency was tested by flow cytometry. RT-PCR was performed to check for knockdown efficiency.

Invasion assay

Invasion assays were performed in 96-well plates. For the invasion assays, cells (5×10^4 cells/200 μL) were resuspended in a serum-free RPMI1640 medium and seeded onto the upper chambers of Matrigel-coated filter inserts (8-μm pore size; BD Biosciences, San Jose, CA, USA). Serum-containing medium was added to the lower chambers. After 24 h of incubation, the number of cells that invaded the lower surface of the filter was counted as previously described [25].

Migration assay

Cells (5×10^4 cells/200 μL) were seeded in multi-well plates and cultured until confluent. A micropipette tip was used to scratch the confluent cells to simulate a wound. Images were taken immediately after the scratches and again at 24 h after the scratches. The widths of the scratches were also measured immediately after the scratches were made and at subsequent experimental points. The number of particles in the scratched areas was also determined using the ImageJ software.

Statistical analysis

Data were performed utilizing Microsoft Excel 2010. The relative expression of miR-34c and miR-199a was shown. Data were represented as a bargraph for the observed relative expression value.

Table 2. Candidate miRNA validation by real-time PCR

Sample	Assay target	Ct1	Ct2	Mean Ct	ΔCt	ΔΔCt	-ΔΔCt	$2^{-\Delta\Delta CT}$
PC3	U6	16.08	16.18	16.13				
PC3	Let 7d	26.25	26.13	26.19	10.06	0.00	0.00	1.00
PC3	miR-155	27.16	27.98	27.57	11.44	0.00	0.00	1.00
PC3	miR-199a	34.65	35.70	35.17	19.04	0.00	0.00	1.00
PC3	miR-34b	N/A	N/A					-
PC3	miR-34c	N/A	N/A					-
PC3	miR-505	N/A	N/A					-
PC3	miR-196a	24.75	25.60	25.18	9.04	0.00	0.00	1.00
PCSC	U6	19.02	18.07	18.55				
PCSC	Let 7d	24.77	26.22	25.49	6.94	-3.11	3.11	8.64
PCSC	miR-155	29.71	30.65	30.18	11.63	0.20	-0.20	0.87
PCSC	miR-199a	30.22	29.56	29.89	11.34	-7.69	7.69	207.15
PCSC	miR-34b	26.27	26.32	26.30	7.75	7.75	-7.75	-
PCSC	miR-34c	23.37	23.67	23.52	4.97	4.97	-4.97	-
PCSC	miR-505	N/A	N/A					-
PCSC	miR-196a	25.42	24.00	24.71	6.16	-2.88	2.88	7.37

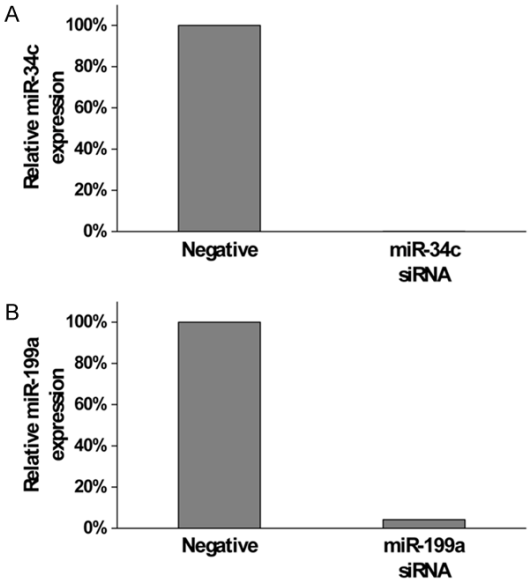


Figure 1. Candidate miRNAs were specifically knocked down in PCSCs. The relative expression of (A) miR-34c and (B) miR-199a after knocking down with their specific inhibitors. The data is presented as expression in the knockdown groups relative to expression in untransfected PCSCs (negative control).

Results

miRNA expression profiles in PCSC and PC3

There were 129 miRNAs with higher expression level than the background in PC3 cells and 111 miRNAs with higher expression level than background in PCSCs. The miRNA expression profiles of PC3 cells and PCSCs were next compared. In PCSCs, 23 differentially expressed miRNAs with a fold-change ≥20 relative to PC3 cells were identified, including let-7d, miR-125b-2, miR-146b, miR-155, miR-192, miR-196a, miR-199a, miR-199b, miR-2467, miR-346, miR-34b, miR-34c-3p, miR-34c-5p, miR-3935, miR-4505, miR-4532, miR-4656, miR-4743, miR-486, miR-505*, miR-505, and miR-885 (**Table 1**).

Candidate miRNA validation

Seven candidate miRNAs, miR199a, miR155, let-7d, miR34b, miR-34c, miR-505, and miR-196a, were selected from the miRNA microarray because their expression levels were relatively higher, and their inhibitors were commercially available (**Table 1**). After real-time PCR validation, miR-199a and miR-34c were selected for subsequent analysis in functional assays based on (1) their expression level, (2) their role in tumor metastasis [26, 27], and (3) the availability of commercial inhibitors. As shown in **Table 2**, miR-199a expression levels was >200-fold higher in PCSCs compared to its expression PC3 cells. miR-34c was also highly expressed in PCSCs, but undetectable in PC3 cells (**Table 2**).

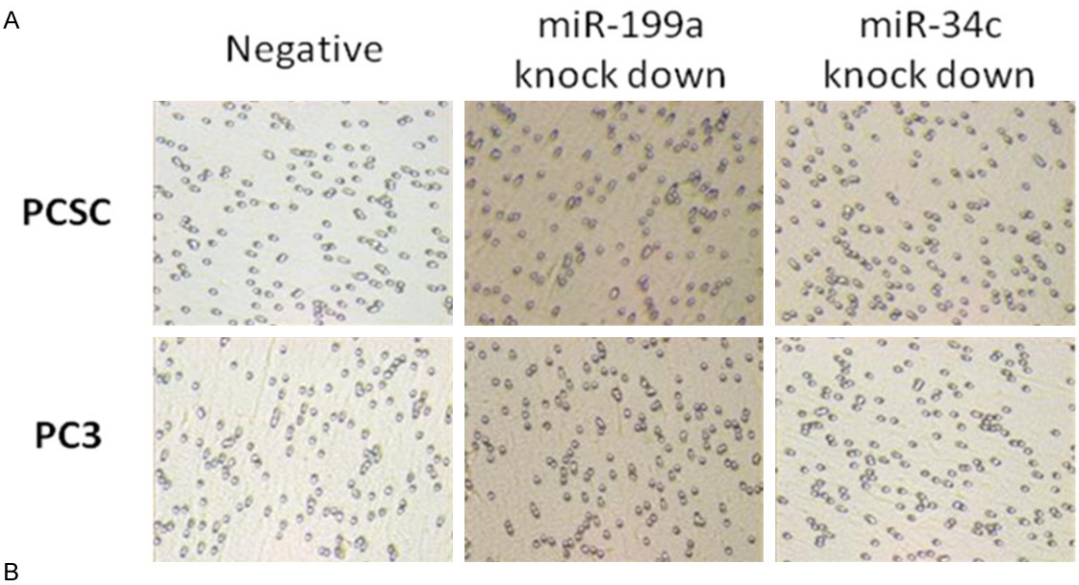
Candidate miRNA knockdown

In PCSCs, miR-199a and miR-34c were specifically suppressed using miRNA inhibitors. The expression of miR-34c was completely knocked down with the miR-34c inhibitor, and the expression of miR-199a was almost completely knocked down (96%), as compared to negative control cells (**Figure 1**).

miR-199a and miR-34c do not alter PCSC proliferation

To determine if miR-199a and miR-34c impact the invasion of PCSCs, we transfected specific

A



B

Cells	miRNA inhibitor	Invaded cell number/field	ImageJIntDen value
PCSC	control	175	25668.955
	miR-199a	153	25026.116
	miR-34c	188	26169.546
PC3	control	172	25668.955
	miR-199a	193	30587.512
	miR-34c	192	31912.240

Figure 2. miR-199a and miR-34c do not effect invasion of PCSCs and PC3 cells. A. Representative images of PCSCs and PC3 cells that invaded the matrix in Matrigel invasion assays. B. Quantification of the invaded cells.

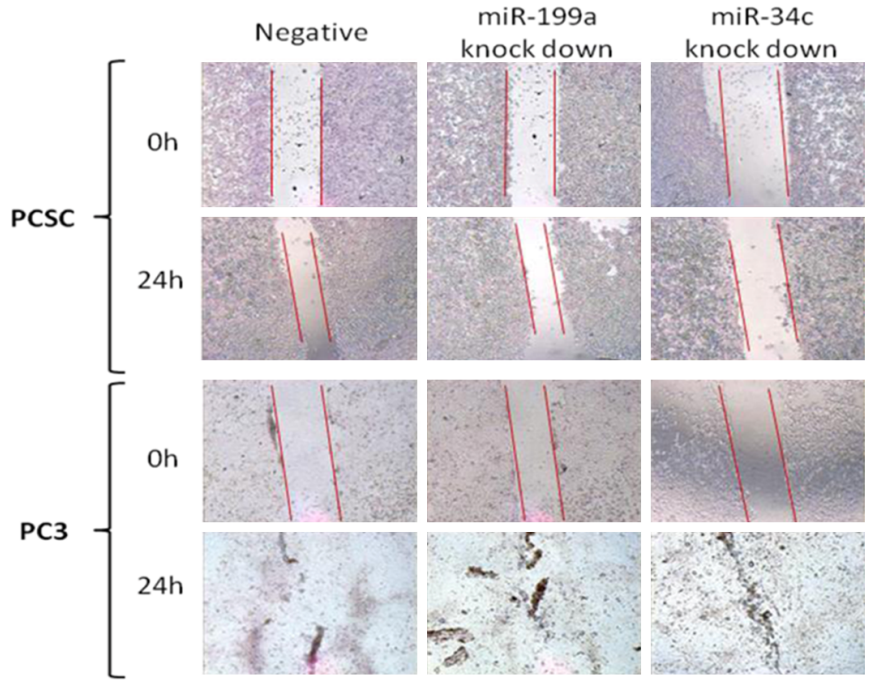
inhibitors of the miRNAs into PCSCs and PC3 cells and used the cells in invasion assays 24 h after transfection. Neither the knockdown of miR-199a nor miR-34c altered PCSC and PC3 cell invasion 48 h after seeding the cells on Matrigel (**Figure 2A**). Quantification of the cellular invasion showed that miR-199a and miR-34c did not effect PCSC proliferation, and a minimal increase in PC3 cell proliferation was detected (**Figure 2**).

Inhibition of miR-199a and miR-34c impaired PCSC migration and enhanced PC3 cell migration

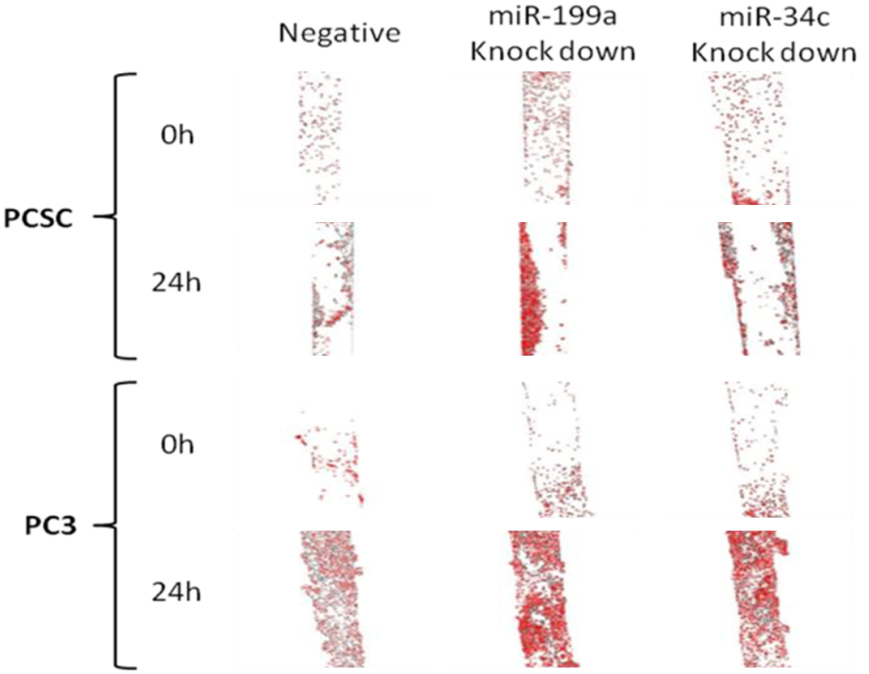
To determine if miR199a and miR-34c altered the migration of PCSCs, we transfected PCSCs and PC3 cells with specific inhibitors of these miRNAs and assessed their cell migration 24 h after transfection. In PC3 cells, the scratches in both knockdown and control groups were covered by cells after 24 h (**Figure 3A**). Inhibition of neither miR-199a nor miR-34c appeared to affect the scratch width (**Figure 3C**). In addition, PCSCs with miR-199a and miR-34c knockdown had a 0.37 and 0.25 unit decrease in scratch widths, respectively (**Figure 3C**).

Using ImageJ software to evaluate the particle count in the scratch areas, we observed that the number of migrated PCSCs was decreased in the knockdowns compared to the untransfected cells (**Figure 3B**). Specifically, there was a 63% and 50% decrease in migration in the

A



B



C

Cells	miRNA inhibitor	Scratch width		ImageJ particle count in scratch area (24h-0h)
		0h	24h	
PCSC	control	1.0	0.63	840
	miR-199a	1.0	0.63	309
	miR-34c	1.38	1.13	424
PC3	control	1.0	0.0	658
	miR-199a	1.13	0.0	2914
	miR-34c	0.88	0.0	1755

Figure 3. Knockdown of miR-199a and miR-34c reduce PCSC migration and enhance PC3 cell migration. A. Representative images showing PCSC and PC3 cell migration at 0 h and 24 h. B. Representative ImageJ-processed images showing PCSC and PC3 cell migration at 0 h and 24 h. Inhibition of miR-199a and miR-34c reduced the migration of PCSCs and enhanced the migration of PC3 cells. C. Quantification of cell numbers in the scratch width areas at 0 h and 24 h.

PCSCs with miR-199a and miR-34c knockdown, respectively (**Figure 3C**). In contrast, the number of migrating PC3 cells was increased with miR-199a and miR-34c knockdown as compared to the untransfected control cells (**Figure 3B**). As shown in **Figure 3C**, the number of migrating PC3 cells increased by 4.4- and 2.7-fold with miR-199a and miR-34c knockdown, respectively (**Figure 3C**). Thus, the particle count data indicated that miR-199a and miR-34c enhanced migration in PCSCs and inhibited it in PC3 cells.

Discussion

CSCs have been identified in several cancers and have a role in cancer initiation and progression [28]; they also provide a residual reservoir of cancer cells that cause resistance to treatment and relapse after an initial successful treatment [29, 30]. miRNAs have been implicated in multiple aspects of CSC biology, including drug resistance and metastasis [31]. In this study, we investigated the differential expression of miRNAs in PCSCs and PC3 cells and their effect on cell invasion and migration. Suppression of both miR-199a and miR-34c inhibited the migration of PCSCs and enhanced the migration of PC3 cells. Our study provides novel information about the differential migration capabilities between PCSCs and PC3 and the role of miR-199a and miR-34c. Thus, miR-199a and miR-34c could represent attractive therapeutic targets for PCSC-specific PCa treatment.

The existence of CSCs was first shown in acute myeloid leukemia and has since been shown in several other cancers [6, 7]. CSCs are capable of replication and differentiation into all of the cells that constitute the heterogeneous tissue mass. These stem cells have been shown to be resistant to traditional cancer treatments that effectively target the heterogeneous tumor mass [30, 31]. The resistance results in relapse, after an initial successful treatment, and highlights the need for new treatments that specifically target the CSCs. CSCs are also believed to be responsible for metastasis. It is, therefore, important to understand the differential biology of CSCs in order to develop treatments that target the stem cells. miRNAs appear to be promising therapeutic targets for CSC-specific treatments because they modulate key aspects of CSC biology [32]. According to cancer stem cell hypotheses, cancer stem cells are ideal seeds cells in metastasis. Liu et al. [28] suggested that not all CSCs are capable of migration. In this review, the low migration cancer stem cells were found in pancreatic cancer, breast cancer and liver cancer [28]. Our study provides novel information about the differential migration capacity between PCSC and PC3 cells the role of miR-199a and miR-34c.

miR-199a-5p downregulation in colorectal cancer may be associated with EMT via increased dicoidin domain receptors1 (DDR1) expression [33]. Furthermore, ectopic expression of miR-199a-5p reduced LOVO and LOVE1 colorectal cell invasion and migration [33]. Similarly, miR-34c suppressed EMT in MCT renal tubular cells [34], and miR-34a inhibited PCa metastasis via targeting CD44 [15]. In contrast, in the present study, the expression of both miR-199a and miR-34c was increased in PCSCs relative to PC3 cells, and their suppression inhibited PCSC migration, but not invasion. The differences in miR-199a expression may be due to differences in the cell types analyzed. Further studies will assess their role in PCSC-mediated EMT.

Analysis of PCa samples revealed that the expression of miR-34c was reduced as compared to adjacent normal samples [35, 36]. In the present study, inhibition of miR-199a and miR-34c increased PC3 cell migration, suggesting that these miRNAs could inhibit PC3 cell migration. This is consistent with studies in which miR-34c and miR-34a suppressed androgen-induced PCa cell proliferation by targeting the androgen receptor [37], and miR-34a inhibited the metastasis of CD44-positive PCa cells [15]. It is also consistent with studies in which tumor suppressor roles for miR-34c were observed in PCa cells by targeting E2F3, BCL-2 [38], and MET [39]. Although increased PC3 cell migration was observed with miR-34c

inhibition, the mechanism by which this occurs is not clear as miR-34c levels were already undetectable by real-time PCR analysis. It is possible that even extremely small levels of miR-34c that are below the limits of detection by the assay have biological significance.

We identified 23 miRNAs that are differentially expressed in PCSCs. Of these, several have been implicated in inducing or regulating EMT in other cancers, including let-7d, miR-192, miR-196a, miR-199a, miR-155, miR-34b, miR-34c-3p, miR-34c-5p, and miR-505 [40-46]. There is increasing evidence of the vital role of miRNAs in PCa. For example, miR-143 promotes PCa metastasis by repressing the expression of fibronectin type III domain containing 3B (FNDC3B) [14]. Also, miRNA-320 suppresses the stem cell-like characteristics of PCa cells by downregulating the Wnt/beta-catenin signaling pathway [20], and miR-34a inhibits PCSCs and metastasis by directly repressing CD44 [15]. Some of the 23 differentially-expressed miRNAs from our microarray could also be important in the regulation of PCa biology, and, therefore, will be analyzed in further studies.

This preliminary study has limitations that warrant further discussion. Although our findings suggest that miR-199a and miR-34c could regulate migration and perhaps EMT in PCSCs, we did not analyze the effects of their suppression on EMT markers, including vimentin, fibronectin and alpha-smooth muscle actin. In addition, the mechanism by which miR-199a and miR-34c alter migration in PCSCs and PC3 cells, including identifying their potential targets, was not determined. In colorectal cancer cells, miR-199a-3p targeted NLK, and its downregulation was associated with lymph node metastasis, venous invasion, liver metastasis, and TNM stage [26]. Moreover, the tumor suppressive effects of miR-34c in colorectal cancer were mediated by downregulation of KITLG, a protein involved in cell migration and invasion [27]. Therefore, further studies will assess the potential regulation of NLK and KITLG by miR-199a and miR-34c in PCSCs. Furthermore, given the roles of the homeobox factors, ZEB1 and ZEB2, in EMT and their regulation by other miRNAs [21], further studies will determine whether miR199-a and miR-34c regulate ZEB expression. Moreover, the effects of miR-199a and miR-34c on cell proliferation in the migration assays along with the migration rates were not determined in the present study. Finally, the expression of miR-199a and miR-34c in PCa tissues needs to be evaluated in further studies.

Conclusions

miR-199a and miR-34c differentially regulate the migration of PCSCs and PC3 cells. Further studies will assess whether they regulate migration through inducing and/or regulating EMT in PCSCs and metastasis in PCa as well as their possible role in suppressing metastasis from the PCa tumor mass.

Disclosure of conflict of interest

None.

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