miR-25 Modulates Invasiveness and Dissemination of Human Prostate Cancer Cells via Regulation of αvβ6-Integrin Expression

E. Zoni1, G. van der Horst1, A.F. van de Merbel1, L. Chen2, J.K. Rane3, R.C.M. Pelger1, A.T. Collins3, T. Visakorpi4, B.E. Snaar-Jagalska2, N.J. Maitland3, and G. van der Pluijm1

Abstract

Altered microRNA (miRNA; miR) expression is associated with tumor formation and progression of various solid cancers. A major challenge in miRNA expression profiling of bulk tumors is represented by the heterogeneity of the subpopulations of cells that constitute the organ, as well as the tumor tissue. Here, we analyzed the expression of miRNAs in a subpopulation of epithelial stem/progenitor-like cells in human prostate cancer [prostate cancer stem cell (PCSC)] and compared their expression profile to more differentiated cancer cells. In both cell lines and clinical prostate cancer specimens, we identified that miR-25 expression in PCSCs was low/absent and steadily increased during their differentiation into cells with a luminal epithelial phenotype. Functional studies revealed that overexpression of miR-25 in prostate cancer cell lines and selected subpopulation of highly metastatic and tumorigenic cells (ALDHhigh) strongly affected the invasive cytoskeleton, causing reduced migration in vitro and metastasis via attenuation of extravasation in vivo. Here, we show, for the first time, that miR-25 can act as a tumor suppressor in highly metastatic PCSCs by direct functional interaction with the 3′-untranslated regions of proinvasive αvβ6 and αv-integrins. Taken together, our observations suggest that miR-25 is a key regulator of invasiveness in human prostate cancer through its direct interactions with αvβ6 and αv-integrin expression. Cancer Res; 75(11) June 1, 2015; 2326–36. ©2015 AACR.

Introduction

Prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of death from cancer in males worldwide (1). Despite the progress in the pathogenesis, detection, and treatment of primary tumor, the main problem for prostate cancer patients remains the risk of metastasis formation and tumor recurrence after surgical removal and/or treatment of the primary tumor.

From a hierarchical point of view, normal and transformed epithelial tissues are indeed characterized by a cellular heterogeneity, in which different cell types contribute to the maintenance of the complexity of tissues (2). One of the major challenges in the field of new therapy development for advanced cancer is to specifically target “driver” cancer cell subpopulations, that seem to be involved in tumor maintenance, metastasis and therapy resistance (3). Accumulating evidence shows that prostate cancer stem/progenitor-like cells play key roles in tumor initiation, local, and distant relapse, metastasis, and castration and chemotherapy resistance (4, 5). One of the driving forces of oncologic transformation of normal epithelial stem cells into cancer stem cells (CSC), is the deregulated gene expression of tumor suppressors and oncogenes (6). Furthermore, oncologic research has highlighted an emerging role for microRNAs (miRNA; miR) as crucial regulators of such oncogenes and tumor suppressors in cancer (7). miRNAs are a class of small non-coding RNA molecules (18–25 nucleotides long), which modulate gene expression by binding to the 3′-untranslated regions (3′-UTR) of target mRNAs and promoting mRNA degradation or translational repression (8). Several studies have delineated and compared the expression of miRNAs in bulk tissues from human prostate cancer and normal prostate and have shown significant correlations between miRNAs levels, prostate cancer progression, and response to chemotherapy (9, 10). In addition, these studies have highlighted the diagnostic and prognostic value of miRNAs detection in blood and urine, suggesting the possible relevance of the use of miRNAs as prostate cancer biomarkers.

Most attempts to decipher the miRNAs signatures have been performed in clinical samples of bulk tumor tissues or heterogeneous prostate cancer cell lines. In these heterotypic and heterogeneous cell populations, this strategy cannot clearly discriminate between the “driver” subpopulation and other, nontumorigenic and more differentiated cancer cell subpopulations. In bulk tumor tissues, it is even more difficult to discriminate between tumor-derived and stroma-derived miRNA expression profiles.

Here, we examined the expression of miRNAs in the “driver” subpopulation of human stem/progenitor-like prostate cancer cells (cell lines and patient samples) that was previously shown to...
drive tumorigenesis and metastasis in preclinical prostate cancer models of bone metastasis in vivo (11). On the basis of the list of differentially expressed miRNAs, miR-25 was selected because a number of its putative target genes are predicted to be involved in the stimulation of cancer invasiveness. In both clinical prostate cancer specimens and prostate cancer cell lines, we found that miR-25 is low/absent in the alDH<sup>hi</sup>/CD133<sup>+</sup> compartment, also referred to as stem-like cells in a recent publication (12), and steadily increases during differentiation into luminal epithelial cells in clinical samples. Here, we validate, for the first time, the direct functional interaction between miR-25 and α<sub>v</sub>- and α<sub>β</sub>-integrins linked to the cytoskeletal organization and invasive behavior in vivo. In line with these observations, we further demonstrate that miR-25 targeted α<sub>v</sub>- and α<sub>β</sub>-integrins in selected alDH<sup>hi</sup>/subset of cancer stem/progenitor cells and reduced invasion by blocking the extravasation of human prostate cancer cells in the intact organism.

**Materials and Methods**

**ALDEFLUOR assay and real-time PCR-based miRNA expression profiling**

Aldehyde dehydrogenase (ALDH) activity of the cells was measured using the ALDEFLUOR Assay Kit (StemCell Technologies) according to the manufacturer's protocol (11). ALDH substrate was added to the cells and converted by intracellular ALDH into a fluorescent product. For FACS sorting, cells were labeled with the ALDEFLUOR Kit and sorted using FACS ARIA cell sorter (BD Biosciences; ALDH<sup>hi</sup> = highest 10% ALDH<sup>+</sup>; ALDH<sup>low</sup> = lowest 10% ALDH<sup>−</sup> cells). miRNA expression profiling was performed using RT<sup>2</sup> miRNA PCR array (SA-biosciences) according to the manufacturer's protocol. Data were normalized using SNORD48 and U6 RNA housekeeping genes. Inclusion criteria were C<sub>q</sub> value <35, fold induction >2 and < −2 and similar data in two independent experiments.

**Prostate cancer cell lines and transfection with miR-25 precursor molecule**

Human osteotrophic prostate cancer cell lines PC-3M-Pro4Luc2 and C4-2B were maintained in DMEM with 10% FCS, 1% penicillin–streptomycin (Life Technologies) and 0.8 mg/mL norepinephrine and T-medium DMEM (Sigma-Aldrich) with 20% F-12K (Life Technologies) and 10% sodium azide. PC-3M-Pro4Luc2 transfected with pre-miR-25 (PM10584; Life Technologies) and pre-miRNA–negative control (scramble; ID, AM17110; Life Technologies). Total RNA was collected after 72 hours.

**Collection of samples from patient, isolation of subpopulation of cancer stem/progenitor cells and reduced invasion by blocking the extravasation of human prostate cancer cells in the intact organism**

**RNA isolation and real-time qPCR**

Total RNA was isolated using TRIzol (Invitrogen), CD133<sup>+</sup> subpopulation of cancer stem/progenitor cells and reduced invasion by blocking the extravasation of human prostate cancer cells in the intact organism.

**miRNA target prediction and bioinformatic analysis of cluster of genes**

Targetscan v6.2, miRDB (15) and microT-CDS (16) were used to identify novel miR-25–predicted targets. Functional annotation was performed using DAVID Bioinformatics Resources 6.7 (17, 18) and KEGG database (19).

**Migration assay**

Cells were starved overnight in medium containing 0.3% serum and then seeded in medium containing 0.3% serum in a Transwell chamber (Corning 8-µm pore size). The lower chamber was filled with medium containing 10% serum. After 18 hours of incubation, cells on the upper side of the filters were removed and cells migrated to the lower side were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet and counted.

**Proliferation assay**

Cells were seeded at density of 2,000 cells per well 24 hours and allowed to grow for 24, 48, 72 hours. After incubation, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium was added and mitochondrial activity was measured after 2 hours incubation at 37°C (CellTiter96 Aqueous Non-radioactive Cell proliferation assay).

**FACS analysis**

Protein expression was measured with flow cytometry. A total of 1 × 10<sup>5</sup> cells were incubated for 45 minutes at 4°C in FACS wash buffer containing PBS + 1% FCS + 0.1% Natriumazide NaN<sub>3</sub> and 10 µL antibody (eB-PE, eB-APC, Milteny). Cells were washed with PBS, protein measured with FACS Calibur (BD Biosciences) and data analyzed with FCS express software.

**Phallloidin staining**

PC-3M-Pro4Luc2 transfected with pre-miR-25 and scramble-negative control were seeded onto glass slides, fixed with 4% paraformaldehyde and stained with 0.25 µmol/L phallloidin (Life Technologies). TO-PRO-3 (Life Technologies) was used for nuclei visualization. Images were acquired with confocal microscope Leica SP5 (Leica) and analyzed with ImageJ (NIH).

**Reporter constructs and luciferase assay**

497-bp and 485-bp nucleotide sequences corresponding to portion of the 3′-UTR of ITGA6 and ITGAV, respectively, including the conserved predicted binding site (seed sequence) for miR-25, were cloned downstream of the firefly luciferase2 sequence in a
PGL4.10 vector (Promega) using XbaI (Promega) and FseI (New England Biolabs) restriction enzymes. 1184-bp sequence of human elongation factor 1α (hEF1α) promoter was inserted into the multiple cloning site (MCS) of the PGL4.10 upstream the luciferase2 sequence using KpnI and HindIII (Promega). Mutagenesis was performed using QuikChange (Stratagene) site-directed mutagenesis approach (Primer sequences in Supplementary Table S2).

Zebrafish maintenance
Tg(mpo:GFP)i114 zebrafish line (20, 21) was handled compliant to local animal welfare regulations and maintained according to standard protocols (www.ZFIN.org).

Zebrafish embryo preparation and tumor cell implantation
Two days post-fertilization (dpf), dechorionized zebrafish embryos were anaesthetized with 0.003% tricaine (Sigma) and placed on a 10-cm Petridish coated with 3% agarose. PC-3M-Pro4mCherry cells were transfected 48 hours before implantation. Single-cell suspensions were resuspended in PBS, kept at room temperature before implantation and implanted within 3 hours. The cell suspension was loaded into borosilicate glass capillary needles (1 mm O.D. × 0.78 mm I.D.; Harvard Apparatus) and the injections were performed using a Pneumatic Picopump and a manipulator (WPI). Approximately 400 cells were injected at around 60 μm above the ventral end of the duct of Cuvier (DoC), where the DoC opens into the heart. After implantation with mammalian cells, zebrafish embryos (including non-implanted controls) were maintained at 33°C, to compromise between the optimal temperature requirements for fish and mammalian cells (22). Data are representative of at least two independent experiments with at least 50 embryos per group. Experiments were discarded when the survival rate of the control group was less than 80%.

Statistical analysis
Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad software) using the *t* test for comparison between two groups. Data are presented as mean ± SEM. *P* values ≤ 0.05 were considered to be statistically significant (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

Results
miR-25 expression is downregulated in normal and transformed prostate stem cells and steadily increases upon luminal differentiation
To investigate the expression of miRNAs in prostate cancer stem cells (PCSC), we used the ALDEFLUOR assay, which involves viable cell sorting based on ALDH enzyme activity (23, 24). After viable sorting, ALDHhigh and ALDHlow subpopulations of PC-3M-Pro4Luc2 were used to identify the differential miRNA expression profiles of cancer stem cells (ALDHhigh) and committed nontumorigenic and nonmetastatic (ALDHlow) cells (11). Real-time PCR-based miRNA expression profiling revealed that miR-25 was the most downregulated miRNA in cancer stem cells (ALDHhigh)
compared with the ALDHlow subpopulation of cells (log10 fold change, -2.76; P value = 0.05; Fig. 1A).

We next investigated miRNA expression in subpopulations isolated from prostate tissue and primary epithelial cultures derived from patients with benign prostatic hyperplasia (BPH), hormone-naive prostate cancer (> Gleason 7), and castration-resistant prostate cancer (CRPC; ref. 12). First, we quantified the expression of miR-25 in unfractonated soft tissue collected from BPH, prostate cancer, and CRPC and found increased miR-25 expression upon tumor progression (Fig. 1B). Then, after expansion of primary prostate cells in culture, we compared miR-25 expression in three cell populations: α2β1hi/CD133+, α2β1low/CD133−, and α2β1low, also referred to as stem-like cells, transit-amplifying (TA) cells and committed basal (CB) cells, respectively (5, 12, 14, 25). Interestingly, miR-25 is significantly and strongly downregulated in the α2β1hi/CD133+ population compared with the α2β1low/CD133− compartments, irrespective of their pathologic status (i.e., BPH, prostate cancer, and CRPC; Fig. 1C–E).

Taken together, our data show consistent relative low expression levels of miR-25 in the cancer stem/progenitor subpopulation of cells in both human prostate cancer cell lines and the compartment defined as α2β1hi/CD133+ cells isolated from prostate cancer patients (12, 25). The expression of miR-25 steadily and consistently increases during epithelial differentiation in patient-derived benign prostates (BPH) and malignant prostate samples.

Figure 2.

In silico analysis for predicted pathway identification and validation by RT-qPCR. A, interaction between miR-25 and predicted target genes overlaid on KEGG regulation of the actin cytoskeleton pathway. B, clustergram of mRNA expression assessed by RT-qPCR for selected target among those represented in A. Analysis performed in PC-3M-Pro4Luc2 and C4-2B cells (N = 5). Colors match with those represented in B, D, E, F, G, H (green, downregulation; red, upregulation). C, mRNA regulation of selected target genes on PC-3M-Pro4Luc2-overexpressing miR-25, regulation is highlighted in green (down) and red (up). Colors are matched with scatter plot (D) with threshold selected (threshold value = 2) and significant values are represented in volcano plot (E). F, mRNA regulation of selected target genes on C4-2B-overexpressing miR-25, regulation is highlighted in green (down) and red (up). Colors are matched with scatter plot (G) with threshold selected (threshold value = 2) and significant values are represented in volcano plot (H).
Identification and transcriptional analysis of miR-25–predicted target genes

Next, Targetscan (release 6.2) was used to identify novel miR-25–predicted target genes (26). Using this approach, we identified 893 conserved putative target genes, with a total of 992 conserved sites and 211 poorly conserved sites. Among the list of predicted targets, 63 genes were mapped, using the database for annotation, visualization and integrated discovery DAVID (17, 18), in processes linked to invasion and pathways related to prostate cancer and bone metastasis (regulation of F-actin cytoskeleton, ECM-receptor interaction, TGFβ signaling pathway, MAPK signaling pathway, and cell cycle). Interestingly, our in silico analysis showed that the regulation of F-actin cytoskeleton was one of the predicted pathways that is potentially affected by miR-25 (P = 2.1E−2). Mapping of the predicted miRNA targets to the regulation of F-actin cytoskeleton, the KEGG pathway identified multiple genes involved in important processes for cell motility, migration, invasion, and cytoskeleton dynamic (Fig. 2A). Strikingly, miR-25 was predicted to target IQGAP2 (a GTP-dependent protein involved in the cytoskeletal reorganization), WASL and CFL2 (involved in the actin polymerization and depolymerization), CDC42 (required for rounded/ameoboid movements of single tumor cells), MYH9 (cellular myosin with a role in cytokinesis and cell shape), PIP4K2C and PIKS51C (kinase that mediates RAC1-dependent reorganization of actin filaments; ref. 27). RAC1 is involved in focal adhesion and is required for mesenchymal movements of single tumor cells (28). PKFYV (which plays a role in endosome-related membrane trafficking), PPP1R12A (regulates myosin phosphatase activity), SLC9A1 (sodium/hydrogen exchanger involved in focal adhesion), ITGA5 (a.k.a. fibronectin receptor, significantly downregulated by miR-25 in both cell lines as shown by qRT-PCR analysis; Fig. 2B-E and Fig. 2F-H) and ITGAV (a.k.a. vitronectin receptor). ITGA6 (faminolin-10/11 receptor), and vinculin (VCL), however, not affected by miR-25 as shown by Western blot analysis (Supplementary Fig. S1A) that are all involved in cell–matrix interactions and adhesion. Among the target genes involved in the regulation of the F-actin cytoskeleton, ITGAV and ITGA6 are members of the integrin family of transmembrane receptors that regulate cell adhesion, migration, and remodeling of the ECM (29–31). In addition, TIMP and TACE were also identified as miR-25–predicted targets using miRDB (15) and microTCDS (16). Moreover integrin–transmembrane receptors regulate the activation of Rho-GTPases, RAC1 and CDC42 (32). Interestingly, our mRNA analysis (Fig. 2B) revealed that miR-25 significantly downregulated CDC42 and its effector proteins CDC42BPA and CDC42EP2 and decreased mRNA of

![Image](https://example.com/image.png)

**Figure 3.** miR-25 overexpression decreases ITGAV and ITGA6 expression at mRNA and protein level in prostate cancer cells and selected ALDHlow subpopulation of cells. A, PC-3M-Pro4Luc2 and C4-2B-overexpressing miR-25; relative expression is compared with scramble-negative control and all values were normalized to GAPDH; error bars, ±SEM (n = 3). B, mean intensity of ITGAV and ITGA6 fluorescence in PC-3M-Pro4Luc2- and C4-2B-overexpressing miR-25 compared with scramble-negative control determined by FACS analysis; error bars, ±SEM (n = 3). C, histogram of flow cytometric analysis of ITGAV and ITGA6 protein expression. Expression levels in cells overexpressing miR-25 (continued line) are compared with scramble-negative control (dashed line). D and E, mean intensity of ITGAV and ITGA6 fluorescence in ALDHlow, versus ALDHlow-overexpressing miR-25 compared with scramble-negative control determined by FACS analysis; error bars, ±SEM (n = 2). F, histogram of flow cytometric analysis of ITGAV and ITGA6 protein expression. Expression levels in ALDHlow versus ALDHlow-subpopulation overexpressing miR-25 (continued line) are compared with scramble-negative control (dashed line). * and $, P < 0.05; ** and $$$, P < 0.01; $$$, P < 0.001.
RAC1 (Fig. 2C–E and Fig. 2F–H). In addition, our RT-qPCR analysis revealed that miR-25 could significantly decreased PIP5K1C, kinase involved in RAC1 signaling and predicted target of miR-25 in both cell lines (Fig. 2C–E and Fig. 2F–H).

Previously, we have shown that ITGAV is required for the acquisition of a stem/progenitor phenotype (31). In addition, ITGAV and ITGA6 are highly expressed in prostate progenitor cells and ITGA6 has been established as maker for progenitor cells in prostate and breast cancer. These results suggested a functional link between miR-25 and ITGAV and ITGA6 expression regulation in prostate cancer, for which no information is currently available.

miR-25 overexpression downregulates αv- and α6-integrins in human prostate cancer cell lines and selected ALDHhigh subpopulation

To investigate the functional interaction between miR-25 and the predicted target genes, we isolated ALDHhigh and ALDHlow from the PC-3M-Pro4Luc2 prostate cancer cell line by flow cytometry. As expected, the clonogenic and migratory potential of ALDHhigh versus ALDHlow cells was higher (Supplementary Fig. S1B and S1C; ref. 11). Furthermore, ITGAV and ITGA6 expression was also higher in ALDHhigh versus ALDHlow cells as expected, thus confirming the inverse correlation with miR-25 expression (Supplementary Fig. S1D; ref. 11). We used ITGA2, an established PCSC marker, as positive control and confirmed its increased expression in ALDHhigh cells (Supplementary Fig. S1D; refs. 5, 11).

The functional interaction between miR-25 and ITGAV and ITGA6 expression in human prostate cancer cell lines and selected subpopulation of cells (ALDHhigh stem/progenitors and ALDHlow committed cells) was evaluated by transfection with 60 nmol/L of pre–miR-25 or prenegative control sequence. Overexpression of miR-25 significantly attenuated ITGAV and ITGA6 mRNA expression in PC-3M-Pro4Luc2 and C4-2B (ITGAV P = 0.05 and 0.001, respectively; ITGA6 P = 0.01 and 0.001, respectively; Fig. 3A). Interestingly, forced overexpression of miR-25 also led to a...
significant reduction in ITGA5 expression in both cell lines \((P = 0.01\text{ and }0.001, \text{ respectively})\) and reduced levels of ITGA3, ITGB1, and ITGB4 in C4-2B \((P = 0.01, 0.001\text{ and }0.05, \text{ respectively})\; \text{Supplementary Fig. S1E and S1F}\) and Fig. 2B–H). As expected, no consistent inhibitory effect was observed on ITGAV expression.

Strikingly, upon transfection of PC-3M-Pro4Luc2 and C4-2B cells with pre–miR-25 (or prenegative control) for 72 hours, ITGAV and ITGA6 protein expressions were also significantly downregulated not only in the bulk cell lines (Fig. 3B and C), but also in selected ALDH\textsuperscript{low} and highly aggressive ALDH\textsuperscript{high} subpopulation of stem/progenitor cells transfected with pre–miR-25 (or prenegative control) after viable cell sorting (Fig. 3D–F).

miR-25 overexpression decreases migration of metastasis-initiating human prostate cancer cells and affects cytoskeleton dynamics

Prostate cancer cell migration in both PC-3M-Pro4Luc2 and C4-2B cells was significantly attenuated upon miR-25 overexpression (PC-3M-Pro4Luc2, 88% decrease, \(P = 0.001\); C4-2B, 49% decrease, \(P = 0.01\) after 72 hours; Fig. 4A and B).

Strikingly, miR-25 was able to strongly and significantly reduce migration also in selected highly migratory ALDH\textsuperscript{high} subpopulation after viable cell sorting \((P < 0.001; \text{Fig. 4C and D})\).

In contrast with migration, cell proliferation was not affected by forced miR-25 overexpression compared with the scrambled-negative control sequence (Supplementary Fig. S2A and S2B). miR-25 also induced a switch to a less invasive phenotype characterized by a dramatic change in cell morphology (Supplementary Fig. S2C). Phalloidin staining revealed an almost complete loss of actin filopodia and cytoskeletal reorganization associated with a strong decrease in the average F-actin fluorescence \((P = 0.01; \text{Fig. 4E and F})\). In addition, migration was monitored in ALDH\textsuperscript{high} and ALDH\textsuperscript{low} subpopulation 4 days after sorting (i.e., 72 hours after transfection of selected subpopulation) and confirmed significantly higher conserved migratory potential in ALDH\textsuperscript{high} cells compared with ALDH\textsuperscript{low} (Fig. 4G and H).

Taken together, these results suggest a critical role of miR-25 in the regulation of an invasive phenotype by modulating cytoskeletal integrity, organization, and motility in human prostate cancer cell lines and selected aggressive tumor- and metastasis-initiating ALDH\textsuperscript{high} subpopulation \((11)\). The miR-25–induced change to a less invasive phenotype does not coincide with major changes in the expression of epithelial markers, suggesting that the observed morphologic changes are most likely due to altered integrin expression as we demonstrated previously for \(\alpha\)-integrins (Supplementary Fig. S2D and S2E; ref. 31).

miR-25 directly targets proinvasive \(\alpha_6\) and \(\alpha_6\)-integrins

Next, we investigated the putative direct functional interaction between miR-25 and its predicted ITGAV and ITGA6 target genes. For this, we cloned 497-bp and 485-bp nucleotide sequences corresponding to a portion of the 3′-UTR of ITGA6 and ITGAV, respectively, including the conserved predicted binding site (seed sequence) for miR-25, downstream of the firefly luciferase\textsuperscript{2} sequence in a pGL4.10 vector background (see Materials and Methods; Fig. 5A). To achieve high expression of the reporter system, a 1184-bp sequence corresponding to human elongation factor 1\(\alpha\) (hEF1\(\alpha\)) promoter was inserted into the MCS of the pGL4.10 upstream to the luciferase\textsuperscript{2} sequence. The reporter

**Figure 5.**

miR-25 directly regulates ITGAV and ITGA6. **A**, portion of 3′-UTR of ITGAV or ITGA6 containing the miR-25 predicted–binding site was cloned in pGL4-basic vector modified with hEF1\(\alpha\) promoter. Scramble-negative control or pre–miR-25 was cotransfected with WT or MUT construct for ITGA6 (B) or ITGAV (C) together with CAGGS-renilla plasmid. RLU is calculated as ratio luciferase/renilla and normalized for scramble-negative control; error bars, ±SEM (\(n = 3\); *, \(P < 0.05\).
constructs, containing mutant miR-25–binding site in the 3'-UTR of the described genes, were also generated and used as a control. Transfection of pre-miR-25 resulted in a significant reduction of luciferase activity in the wild-type but not in the mutant 3'-UTR of the ITGA6 and ITGAV genes (P = 0.05 for both genes; Fig. 5B and C). These results, combined with the transcriptional and translational analysis described above, show for the first time that miR-25 directly targets ITGA6 and ITGAV expression.

miR-25 inhibits distant metastasis of human prostate cancer cells in zebrafish

To investigate the ability of miR-25 to interfere with migration and invasion in the intact organism PC-3M-Pro4 prostate cancer cells, that stably express the NIRF protein mCherry, were injected into the circulatory system of zebrafish embryos and their tumor extravasation and distant metastasis formation was examined (33). The embryonic vascular system of zebrafish is fully functional and allows efficient detection of extravasating tumor cells (34). In addition, in the Tg(mpo:GFP)i114, the embryos are transparent and the immune system is not fully developed permitting successful xenotransplantation of human tumor cells (22). This makes the zebrafish model system highly appropriate for observing interaction between tumor cells and vasculature at the single cell level (35). We transfected PC-3M-Pro4mCherry cells to overexpress pre-miR-25 (or prenegative control) and inoculated the cancer cells into the DoC of 2-day-old zebrafish embryos (100 embryos injected/group; ref. 33). Disseminated cells were arrested in the host vasculature in the first hours, and extravasation was detected from 12 hpi (hours post-implantation). Perivascular tumor cells were observed in multiple foci, including the optic veins, the intersegmental vessels, the dorsal aorta and the caudal vein. However, exclusively at the posterior ventral end of the caudal hematopoietic tissue (CHT, as indicated in Fig. 6) in the tail, perivascular tumor cells were able to invade into the neighboring tail fin. At day 1 post-implantation (1 dpi) miR-25 overexpression caused a robust and significant reduction in the distal colonization and invasion from CHT into the tail fin compared with the scramble control cells (Fig. 6A). miR-25 was able to completely abolish invasion that was detected in 20% of the embryos injected with cells transfected with prenegative
control. At day 2 post-implantation (2 dpi), 40% of embryos injected with cells overexpressing the negative control showed invasion from CHT, compared with 20% of embryos injected with cells overexpressing miR-25 (Fig. 6B and C). In addition, miR-25 was able to significantly reduce the number of tumorigenic foci/embryo at 1 dpi, whereas no significant difference was measured at 2 dpi (Fig. 7A–C).

Taken together, our experimental metastasis data support the findings in vitro and indicate that miR-25 negatively regulates the acquisition of an invasive, metastatic phenotype in human prostate cancer cells.

**Discussion**

In this study, miR-25 was identified as an important regulator of the invasive program in nontransformed and malignant human prostate epithelial tissues. In human prostate cancer cell lines and patient-derived primary prostate tumors, miR-25 expression was low/absent in the α2β1hi/CD133low cell subpopulation, but its expression steadily increased during differentiation to α2β1hi/CD133hi (TA) cells and α2β1low (CB) cells committed for terminal differentiation (12). Here, we identified, for the first time, the proinvasive α5- and α6-integrins as functional target genes of miR-25. Forced overexpression of miR-25 in human prostate cancer cells and in highly metastatic and aggressive subpopulation of cells (ALDHhigh) leads to a strong and significant decline in α5- and α6-integrin–driven invasive behavior in vitro and blockage of metastatic colonization in the intact organism.

Consistent with these observations, overexpression of miR-25 decreased migration and slightly affected cell morphology of prostate cancer cells through its direct effect on the cytoskeletal arrangement and dynamics. miR-25 may, therefore, represent one of the key regulators of the invasive program in the human prostate epithelium, in particular in the maintenance of an aggressive phenotype in human prostate cancer "driver" subpopulation of stem/progenitor-like cells.

The results from this study support the notion that the stem/progenitor subpopulation in human prostate cancer displays increased clonogenic, migratory properties in vitro and stronger tumor- and metastasis-initiating properties in preclinical in vivo models (11).

miR-25 is part of the miR-106b-25 cluster that was previously reported to be upregulated in primary tumors and distant metastasis in prostate cancer (36–40). A likely explanation for these apparent contradictory observations is that cancer cell lines and bulk tumor tissues are not homogeneous and consist of a mixture of heterogeneous subpopulations of cells (2). The findings reported here suggest that cellular heterogeneity may limit the appropriate interpretation of RNA expression-based analysis data obtained from bulk tissues. The cellular composition and proportion of α2β1hi/CD133low, α2β1hi/CD133hi and α2β1low, also referred to as stem-like cells, TA cells, and CB cells (12) in the normal prostate epithelium versus prostate cancer epithelium is indeed generally very different (5). For instance, the "driver" stem/progenitor subpopulation in the human prostate often represents only 0.02% of all prostate epithelial cells (5, 41). The increase in absolute expression levels of miR-25 in bulk tissues during prostate cancer progression may, therefore, be indicative of an increase in the proportion of more differentiated, less invasive, miR-25high luminal epithelial cells.

Here, we focused primarily on the differential miRNA expression in α2β1hi/CD133low cells as a cellular subpopulation that "drives" tumorigenesis and metastasis (14). Our findings, indeed, confirmed that miR-25 is overexpressed in hormone-naïve and castration-resistant prostate cancer as previously reported by others (38, 42). Intriguingly, we found that despite the previously observed upregulation of miR-25 in bulk prostate cancer tissues (38), the expression of miR-25 in the α2β1hi/CD133low cells isolated from prostate cancer patients matched its expression in the tumor- and metastasis-initiating ALDHhigh prostate cancer stem/progenitor subpopulation (11). Our analysis on the α2β1hi/CD133low, α2β1hi/CD133hi, and α2β1low cell compartment

---

Figure 7. miR-25-overexpressing cells injected in zebrafish circulation show reduced tumor foci (colonization). A, number of tumor foci is reduced in embryos injected with cells overexpressing miR-25 compared with negative control at 1 day post-injection (1 dpi). B, no difference in the number of foci from embryos injected with cells overexpressing miR-25 and scr was measured at day 2 post-injection (2 dpi). C, representative confocal images of zebrafish embryos injected with PC-3M-Pro4mCherry cells overexpressing miR-25 or negative control at 1 dpi and 2 dpi. ***, P < 0.001.
enriched from primary prostate cancer samples supports the notion that miR-25 is downregulated in the stem/progenitor cell compartment and that its expression steadily increases during differentiation. Consistent with our findings, the expression of the miR-106b-25 cluster appears to mediate neuronal differentiation of adult neural stem/progenitor cells and, interestingly, induction of miR-106b-25 in hypoxic conditions was recently linked to increased expression of neuronal markers in prostate cancer cell lines (43, 44).

Thus, our work and these results suggest that lower miR-25 expression is needed to maintain stem/progenitor phenotype and its increase is associated with cellular differentiation.

In line with the miR-25 data presented in this study, we previously found that αv-integrins play a pivotal role in the acquisition of a migratory stem/progenitor phenotype, tumorigenicity and the formation of distant bone metastasis in vitro (31, 45). Moreover, αv-integrin expression has already been associated with prostate cancer invasion, metastasis, and disease progression (46–48). In addition, integrins provide a structural link between F-actin and the extracellular matrix and contribute to formation of focal adhesion points (49). Our confocal analysis showed that overexpression of miR-25 dramatically affected cell morphology and impaired F-actin polymerization, reducing focal adhesion sites. It seems, therefore, that miR-25 is a key player in the organization of the F-actin and exerts a crucial role in the regulation of an aggressive and migratory phenotype with its direct effect on integrin expression. In addition, organization of F-actin is linked to activation of integrin–transmembrane receptor that regulates the activation of Rho-GTPases, RAC1, and CDC42 (32). Aberrant migration and invasion of cancer cells are key components of their invasive-metastatic phenotype. Individual tumor cells with an elongated, morphology like PC-3M-Pro4-Luc2, often migrate in a “mesenchymal manner,” which requires activation of RAC1, decreased by miR-25 (28). In contrast, single-tumor cells with a less mesenchymal phenotype, like C4-2B, migrate with an “amoeboid mode” that requires signaling of CDC42, significantly downregulated after miR-25 overexpression (28). Our in silico analysis, revealed that PIP5K1C and PIP4K2C, kinases involved in RAC1 signaling, are also predicted target of miR-25. In addition, our transcriptional analysis indicated that miR-25 downregulates CDC42 mRNA together with CDC42BPA and CDC42EP2 mRNA (CDC42 effector proteins). This information combined with the evidence provided by our mRNA and protein analysis on integrin expression in bulk cell lines and selected subpopulation of highly metastatic cells (ALDHhigh), suggest that miR-25 could be a central player in F-actin organization and cytoskeletal dynamics. However, the observed miR-25–induced loss of a migratory phenotype, confirmed in selected ALDHhigh subpopulation of stem/progenitor-like cells, could not be fully explained by the acquisition of more epithelial characteristics (or blockage of EMT-like processes), despite the fact that E-cadherin is a validated target gene of miR-25 (50).

Consistent with our in vitro observations, complete blocking of metastasis by prostate cancer cells overexpressing miR-25 at 1 dpi and a strong reduction at 2 dpi was found in the embryonic zebrafish model (33). These observations indicate that the morphologic alterations produced by miR-25 disrupt extravasation and colonization at distant sites in vitro.

In conclusion, we identified—for the first time—a direct functional interaction between miR-25 and integrins as key regulators of prostate cancer invasiveness and metastasis. Our in vitro and in vivo data indicate that miR-25 can have a suppressor role in aggressive human prostate cancer cells (cell lines and selected subpopulation of ALDHhigh cells) by blocking invasion and metastasis by promoting prostate epithelial differentiation. From a therapeutic perspective, miR-25 seems an interesting small molecule for specific targeting of stem/progenitor-like cells in aggressive human prostate cancer.

Disclosure of Potential Conflicts of Interest

R.C.M. Pelger is a consultant/advisory board member for Amgen. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: E. Zoni, G. van der Horst, L. Chen, G. van der Pluijm


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Zoni, G. van der Horst, A.F. van de Meerb, L. Chen, J.K. Rane, A.T. Collins, T. Visakorpi

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Zoni, G. van der Horst, A.F. van de Meerb, J.K. Rane, G. van der Pluijm

Writing, review, and/or revision of the manuscript: E. Zoni, G. van der Horst, R.C.M. Pelger, T. Visakorpi, B.E. Snaar-Jagalska, N.J. Maitland, G. van der Pluijm

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Zoni, G. van der Horst, L. Chen, N.J. Maitland, G. van der Pluijm

Study supervision: B.E. Snaar-Jagalska, G. van der Pluijm

Acknowledgments

The authors thank Guido de Roo from the flow cytometry facility (Department of Hematology, LUMC, the Netherlands) and Dr. Twan de Vries (Department of Cardiology, LUMC, the Netherlands) for kindly providing construct with hEFlα promoter. The authors thank Chris van der Bent and Hettie Sips (Department of Endocrinology, LUMC, the Netherlands) for help and technical support.

Grant Support

The research leading to these results has received funding from the FP7 Marie Curie ITN under grant agreement no 264817—BONE-NET (E. Zoni) and no 238278—PRONEST (J.K. Rane). This project receives also additional support from Prostate Action UK (E. Zoni and J.K. Rane) and from the Dutch Cancer Society (UL-2011-4930; CdH1).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 23, 2014; revised February 18, 2015; accepted March 17, 2015; published OnlineFirst April 9, 2015.

References


Cancer Res; 75(11) June 1, 2015

miR-25 Modulates Invasiveness and Dissemination of Human Prostate Cancer Cells via Regulation of $\alpha_v$- and $\alpha_6$-Integrin Expression


_Cancer Res_ 2015;75:2326-2336. Published OnlineFirst April 9, 2015.

**Updated version**
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-2155

**Supplementary Material**
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2015/04/10/0008-5472.CAN-14-2155.DC1

**Cited articles**
This article cites 50 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/75/11/2326.full#ref-list-1

**Citing articles**
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/75/11/2326.full#related-urls

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/75/11/2326.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.