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


Abstract

Altered microRNA (miRNA; miR) expression is associated with tumour 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 (ALDH$^{high}$) strongly affected the invasive cytokeratin, 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 $\alpha_v$- and $\alpha_6$-integrins. Taken together, our observations suggest that miR-25 is a key regulator of invasiveness in human prostate cancer through its direct interactions with $\alpha_v$- and $\alpha_6$-integrin expression.

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′-translated 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 EZH2−/−CD133− 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 αv- and α6-integrins linked to the cytoskeletal organization and invasive behavior in vitro. In line with these observations, we further demonstrate that miR-25 targeted αv- and α6-integrins in selected ALDHhigh subpopulation 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; ALDHhigh = highest 10% ALDH; ALDHlow = lowest 10% ALDH− cells). miRNA expression profiling was performed using RT2 miRNA PCR array (SA-biosciences) according to the manufacturer’s protocol. Data were normalized using SNORD48 and U6 RNA housekeeping genes. Inclusion criteria were CFI 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 cells were maintained in DMEM with 10% FCS, 1% penicillin–streptomycin (Life Technologies) and 0.8 mg/mL neomycin and T-medium DMEM (Sigma-Aldrich) with 20% F-12K nutrient mixture (Gibco-BRL), 10% insulin–transferrin–selenium, 0.125 mg/mL biotin, 12.5 mg/mL adenine, 6.825 ng/mL T3 and 1% penicillin–streptomycin, respectively. Cells were maintained at 37°C with 5% CO2.

For transient transfection, Lipofectamine 2000 (Invitrogen) was used according to the manufacturer’s protocol with pre-miR-25 (ID: PM10584; Life Technologies) and pre-miRNA negative control (scramble; ID, AM17110; Life Technologies). Total RNA was used according to the manufacturer’s protocol (Milteny Biotec; refs. 5, 12, 14). Cultured cells were harvested at passage 2 and total RNA was extracted using the miRVana Kit (Life Technology). Agilent V3 arrays were used to perform miRNA microarray analysis and the data were processed using Agilent Feature extraction software. The data were quantile normalized, and RMA summarized.

**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).

**RNA isolation and real-time qPCR**

Total RNA was isolated using TRIzol (Invitrogen), cDNA was synthesized by reverse transcription (Promega) according to the manufacturer’s protocol and qRT-PCR performed with Bio-Rad CFX36 system (Bio-Rad). Expression was normalized to GAPDH (Primer sequences in Supplementary Table S1).

**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 (Sigma-Aldrich) 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 × 106 cells were incubated for 45 minutes at 4°C in FACS wash buffer containing PBS + 1% FCS + 0.1% Natriumazide NaN3 and 10 μL antibody (rV-PE, a2-FITC, o6-APC; Milteny). Cells were washed with PBS, protein measured with FACS Calibur (BD Biosciences) and data analyzed with FCS express software (De Novo software).

**Phalloidin 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 phalloidin (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. x 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).

Figure 1.
Differential expression of miR-25 in prostate cancer, prostate cancer stem-like cells, and benign prostate epithelial stem cells. A, miR-25 expression in ALDHhigh versus ALDHlow subpopulation isolated from PC-3M-Pro4Luc2 cells measured with real time PCR-based miRNA expression profiling; error bars, ±SEM (n = 2). B, relative array expression of miR-25 in BPH, PCa (prostate cancer), and CRPC samples isolated from patients. C, relative array expression of miR-25 in BPH, PCa (prostate cancer), and CRPC samples isolated from patients. D, also referred to as stem-like cells, TA cells, and CB cells, respectively (22), isolated from BPH (n = 5 patients); PCa (prostate cancer; n = 5 patients; D), and CRPC (n = 3 patients; E); error bars, ±SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
compared with the ALDH\textsuperscript{low} subpopulation of cells (log\textsubscript{10} 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: α2β1\textsuperscript{hi}/CD133\textsuperscript{a}, α2β1\textsuperscript{hi}/CD133\textsuperscript{b}, and α2β1\textsuperscript{low}, 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 α2β1\textsuperscript{hi}/CD133\textsuperscript{a} population compared with the α2β1\textsuperscript{hi}/CD133\textsuperscript{b} and α2β1\textsuperscript{low} 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 α2β1\textsuperscript{hi}/CD133\textsuperscript{a} 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.

![miR-25 Regulates Invasion of Human Prostate Cancer Cells](image-url)

**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).
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 target genes, 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 that the regulation of F-actin cytoskeleton was one of the predicted pathways linked to invasion and pathways related to prostate cancer and cell shape). PIP4K2C and PIP5K1C (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). Pikfyve (which plays a role in endosome-related membrane trafficking), PPP1R12A (regulates myosin phosphatase activity), SLC9A1 (sodium/hydrogen exchanger involved in focal adhesion), ITGAV (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 ITGA6 (a.k.a. vitronectin receptor), ITGA6 ( laminin-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, ITGA6 and ITGAV were also identified as miR-25–predicted targets using miRDB (15) and microTCS (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
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 ITGAV 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 ALDH<sup>high</sup> subpopulation

To investigate the functional interaction between miR-25 and the predicted target genes, we isolated ALDH<sup>high</sup> and ALDH<sup>low</sup> from the PC-3M-Pro4Luc2 prostate cancer cell line by flow cytometry. As expected, the clonogenic and migratory potential of ALDH<sup>hi</sup> versus ALDH<sup>lo</sup> cells was higher (Supplementary Fig. S1B and S1C; ref. 11). Furthermore, ITGAV and ITGA6 expression was also higher in ALDH<sup>hi</sup> versus ALDH<sup>lo</sup> cells as expected, thus confirming the inverse correlation with miR-25 expression (Supplementary Fig. S1D; ref. 11). We used ITGAV2, an established PCSC marker, as positive control and confirmed its increased expression in ALDH<sup>hi</sup> 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 (ALDH<sup>high</sup> stem/progenitors and ALDH<sup>low</sup> 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<sup>P = 0.05</sup> and 0.001, respectively; ITGA6<sup>P = 0.01</sup> 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 ($P = 0.01$ and 0.001, respectively) and reduced levels of ITGA3, ITGB1, and ITGB4 in C4-2B ($P = 0.01$, 0.001 and 0.05, respectively; 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 ALDHlow and highly aggressive ALDHhigh subpopulation of stem/progenitor cells transfection 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 ALDHhigh subpopulation of stem/progenitor-like cells transfected after viable cell sorting ($P < 0.001$; 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 ($P = 0.01$; Fig. 4E and F). In addition, migration was monitored in ALDHhigh and ALDHlow subpopulation 4 days after sorting (i.e., 72 hours after transfection of selected subpopulation) and confirmed significantly higher conserved migratory potential in ALDHhigh cells compared with ALDHlow (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 ALDHhigh 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 αv-integrins (Supplementary Fig. S2D and S2E; ref. 31).

miR-25 directly targets proinvasive αv- and α6-integrins

Next, we investigated the putative direct functional interaction between miR-25 and its predicted ITGA6 and ITGAV 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 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 1α (hEF1α) promoter was inserted into the MCS of the pGL4.10 upstream to the luciferase sequence. The reporter
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

Figure 6. miR-25–overexpressing cells injected in zebrafish circulation show reduced extravasation. Of note, 100 embryos per group were injected and the percentage of embryos with invasion at CHT (invasion defined as >3 cells extravasating/embryo) was counted at day 1 post-injection (1 dpi; A) and day 2 postinjection (2 dpi; B); error bars, ±SEM (n = 2 experiments). C, representative confocal images of zebrafish embryos injected with PC-3M-Pro4mCherry cells overexpressing miR-25 or negative control. Cells overexpressing miR-25 were lodged into circulation, whereas cells overexpressing negative control started to show extravasation, full out of CHT and from CHT into the neighboring tail fin at 1 dpi. *, P < 0.05.
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.

**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). 100 embryos per group were injected; error bars indicate ± SEM (n = 2 experiments). 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.

**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 α2β1high/CD133− cell subpopulation, but its expression steadily increased during differentiation to α2β1high/CD133+ (TA) cells and α2β1low (CB) cells committed for terminal differentiation (12). Here, we identified, for the first time, the proinvasive αv- 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 αv- 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 strongly 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 cell cycle line 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 α2β1high/CD133+, α2β1high/CD133+ 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 α2β1high/CD133− cells as a cellular subpopulation that "drives" tumorogenesis 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 α2β1high/CD133− 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 α2β1high/CD133−, α2β1low/CD133+, and α2β1low cell compartment...
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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 α₅-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, α₅β₁ 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). Out 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 (ALDH₁, high) suggests 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 ALDH₁, high 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 vivo.

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 ALDH₁, high 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.

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