miRNA-128 Suppresses Prostate Cancer by Inhibiting BMI-1 to Inhibit Tumor-Initiating Cells

Min Jin1,3, Tao Zhang3, Can Liu1, Mark A. Badeaux1, Bigang Liu1, Ruifang Liu1,4, Collene Jeter1, Xin Chen1, Alexander V. Vlassov2, and Dean G. Tang1,4

Abstract

MicroRNA-128 (miR128) is reduced in prostate cancer relative to normal/benign prostate tissues, but causal roles are obscure. Here we show that exogenously introduced miR128 suppresses tumor regeneration in multiple prostate cancer xenograft models. Cancer stem-like cell (CSC)-associated properties were blocked, including holoclone and sphere formation as well as clonogenic survival. Using a miR128 sensor to distinguish cells on the basis of miR128 expression, we found that miR128-lo cells possessed higher clonal, clonogenic, and tumorigenic activities than miR128-hi cells. miR128 targets the stem cell regulatory factors BMI-1, NANOG, and TGFBR1, the expression of which we found to vary inversely with miR128 expression in prostate cancer stem/progenitor cell populations. In particular, we defined BMI-1 as a direct and functionally relevant target of miR128 in prostate cancer cells, where these genes were reciprocally expressed and exhibited opposing biological functions. Our results define a tumor suppressor function for miR128 in prostate cancer by limiting CSC properties mediated by BMI-1 and other central stem cell regulators, with potential implications for prostate cancer gene therapy. Cancer Res 74(15): 4183–95. ©2014 AACR.

Introduction

Prostate cancer is the most common malignancy afflicting American men with estimated 238,590 new cases and ~29,720 deaths in 2013, accounting for 10% of all male cancer-related deaths (1). Development of castration-resistant disease and distant metastasis are the most common causes of prostate cancer patient death (2). At the cellular level, most patient tumors are heterogeneous containing phenotypically differentiated cancer cells as well as a population of self-renewing tumorigenic cells termed cancer stem cells (CSC), which are implicated in tumor initiation, metastasis, recurrence, and resistance to conventional therapies (3–5). Deciphering novel molecular mechanisms associated with prostate cancer progression, especially those regulating the "stemness" of CSC, is of great importance and may provide insight into developing novel therapeutics.

MicroRNAs (miRNA) are small noncoding RNAs, roughly 19 to 22 nucleotides in their mature form that mediate the instability and degradation of target mRNAs in a sequence complementarity-dependent fashion (6, 7). As endogenous regulators of gene expression, miRNAs play vital roles in diverse physiologic processes, including proliferation (8, 9), apoptosis (10, 11), senescence (12), cell identity (13), and stem cell maintenance (14). Dysregulation of miRNA expression or function is involved in various malignancies (15). Systematic miRNA profiling and characterization studies have reported differentially regulated miRNAs in human cancers. For example, >50 miRNAs are aberrantly expressed in prostate cancer with tumor-suppressive miRNAs (e.g., miR34a and Let-7) repressed or deleted (16, 17) and oncogenic miRNAs (e.g., miR21, miR125b, and miR221) overexpressed or amplified (18–20). Our group has reported miR34a and let-7 as prostate cancer-suppressive miRNAs that can inhibit tumor regeneration and metastasis (21, 22). Recently, a proteomics-based study found that miR128 expression was progressively lost from normal/benign prostate tissues to adenocarcinoma to metastatic prostate cancer (23). Another microarray study revealed that miR128 mRNA was reduced by 40% in prostate cancer samples compared with the normal prostate tissues (24). These observations would suggest tumor-suppressive functions of miR128 in prostate cancer but up to now this suggestion has not been rigorously tested.

The goal for our current study is to investigate the biological functions of miR128 in prostate cancer cells and to explore the underlying mechanisms of action. By performing a spectrum of gain-of-function, miR128 reporting sensor, and loss-of-function studies in both bulk and fractionated stem/progenitor cell-enriched prostate cancer cells, we provide direct evidence...
for prostate cancer–suppressive functions of miR128 in vitro and in vivo. We further show that the tumor-suppressing effects of miR128 are associated with its inhibition of CSC via targeting critical molecules such as BMI-1.

**Materials and Methods**

**Cells, animals, and reagents**

PC3, Du145, PPC-1, C4-2, and LNCaP cells were cultured in RPMI-1640 plus 7% heat-inactivated fetal bovine serum (FBS), 100 mg/mL streptomycin, and 200 U/mL penicillin (Life Technologies). VCaP cells were cultured in DMEM supplemented with 10% FBS and antibiotics. The nontumorigenic human prostate epithelial cell line 9 (NHP9) was maintained in serum-free PrEBM medium (Clonetics) supplemented with insulin, epidermal growth factor, hydrocortisone, bovine pituitary extract, and cholera toxin. LAPC9 and LAPC4 were xenograft tumors (21, 22). NOD/SCID mice were purchased from the Jackson Laboratory and breeding colonies maintained in our animal facility under standard conditions. All animal experiments were approved by Institutional Animal Care and Use Committee. Antibodies were active caspase-3 (polyclonal antibody (pAb), R&D), Ki67 (monoclonal antibody (mAb), DAKO), BMI-1 (mAb, Cell Signaling), TGFBR1 (pAb, Abcam), EGFR (mAb, Cell Signaling), GAPDH (mAb, Santa Cruz), bromodeoxyuridine (BrdUrd; mAb, Sigma), CD44 (mAb, BD Pharmingen), CD133 (mAb, Miltenyi), and α2β1 (mAb, Chemicon). Isotype control antibodies and FITC- or PE-conjugated secondary antibodies were from Chemicon. Other secondary antibodies were purchased from GE Healthcare.

**Oligonucleotides, plasmids, and transfection**

Lipofectamine RNAiMAX (Invitrogen) was used to transfect prostate cancer cells with 30 nmol/L (nmol/L) miR128 mirVana mimic or nontargeting negative control (mir-NC) oligonucleotides (Life Technologies) according to the manufacturer’s instructions. The mirVana mimics are double-stranded oligonucleotides mimicking mature microRNA, which also have novel chemical modifications that allow for higher potency and specificity over the early-generation pre-miR precursor products (Ambion), mirVana miR128 inhibitor (anti-miR128) or nontargeting negative control miRNA inhibitor (anti-NC) was used in some experiments to inhibit miR128 expression, under the same transfection conditions. Lipofectamine 2000 (Invitrogen) was used to transfect cells with vectors in medium without antibiotics. The miR128 sensor, which contains four copies of miR128 at the 3′-UTR was amplified from Du145 genomic DNA by PCR. PCR products were cloned downstream of the firefly luciferase gene in pMIR-REPORT (Ambion) to obtain wild-type pMIR-REPORT-BMI-1 3′-UTR or pMIR-REPORT-NANOG 3′-UTR. To construct mutant vectors, putative miR128 binding sites in BMI-1 and NANOG 3′-UTR were mutated using Quick-Change Site-Direct Mutagenesis Kit (Stratagene). All inserts were sequenced to verify the mutations. Primers used for PCR and sequencing are presented in Supplementary Table S1. For luciferase assays, Du145 cells were plated in 24-well plates and, 24 hours later, cotransfected with 30 nmol/L miR128 or NC mimic, 1 μg pMIR-REPORT or vectors containing wild-type or mutant BMI-1 or NANOG-3′UTR, together with 0.5 μg pMIR-Renilla expressing vector (transfection control). Forty-eight hours later, luciferase activities were measured using Dual Luciferase Reporter Assay Kit (Promega) on a Gen-Probe chemiluminometer.

**Quantitative RT-PCR and Western blot analysis**

Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion). Levels of mature miR128 were measured using TaqMan MicroRNA Assay (Applied Biosystems) by normalizing to the levels of RNU48. SYBR Green PCR Kit (TAKARA) was used to quantify the mRNA levels of several miR128 targets by normalizing to GAPDH. The PCR reactions were performed and analyzed using ABI 7900 system. Western blots were performed as described previously (21). Briefly, total protein was separated on a precast 4% to 15% polyacrylamide gel and blotted with antibodies for BMI-1, EGFR, TGFBR1, and GAPDH. Densitometric analysis of protein bands was performed via Image J software.

**Clonal, clonogenic, and sphere-formation assays**

Basic procedures have been described (21). For clonal experiments, cells were seeded at low density (100 cells/well) in a 6-well plate and allowed to grow until visible colonies appeared. Clones were counted within 2 weeks. For clonogenic assays, 100 μL of cells (300 cells/well) was mixed with 100 μL of cold Matrigel and then plated around the rim of a 24-well dish. After solidification at 37°C for 15 minutes, 200 μL warm PrEBM was added in the center of the dish. Colonies were enumerated in 1 to 2 weeks. For sphere formation assay, 500 to 800 single cells/well are seeded in serum-free PrEBM supplemented with 1 × R27 (Life Technologies), 20 ng/mL epidermal growth factor, and 20 ng/mL basic fibroblast growth factor in ultralow attachment (ULA) plate. Medium was replenished every 4 days and spheres counted within 2 weeks. For secondary (2nd) sphere formation assay, the 1st spheres were trypsinized into single cells and reseeded (500 cells/well) in the ULA plate. The 2nd spheres were counted in ~10 days.

**Dual-luciferase assays**

For BMI-1 and NANOG, fragments containing the predicted binding sites for miR128 at the 3′-untranslated regions (UTR) were amplified from Du145 genomic DNA by PCR. PCR products were cloned downstream of the firefly luciferase gene in pMIR-REPORT (Ambion) to obtain wild-type pMIR-REPORT-BMI-1 3′-UTR or pMIR-REPORT-NANOG 3′-UTR. To construct mutant vectors, putative miR128 binding sites in BMI-1 and NANOG 3′-UTR were mutated using Quick-Change Site-Direct Mutagenesis Kit (Stratagene). All inserts were sequenced to verify the mutations. Primers used for PCR and sequencing are presented in Supplementary Table S1. For luciferase assays, Du145 cells were plated in 24-well plates and, 24 hours later, cotransfected with 30 nmol/L miR128 or NC mimic, 1 μg pMIR-REPORT or vectors containing wild-type or mutant BMI-1 or NANOG-3′UTR, together with 0.5 μg pMIR-Renilla expressing vector (transfection control). Forty-eight hours later, luciferase activities were measured using Dual Luciferase Reporter Assay Kit (Promega) on a Gen-Probe chemiluminometer.
The purity of the HPCa cells from xenografts was generally achieved using a Lineage-Depletion Kit (Miltenyi; refs. 21, 27, and 28). For invasion assays, prostate cancer cells were transplanted with miR128 or NC mimic for 48 hours, after which 50,000 cells in serum-free medium were seeded in the top chamber of 24-well transwell units (BD Pharmingen) with RPMI-1640 containing 15% FBS added to the bottom chambers. Cells were allowed to migrate for 20 hours at 37°C, and then cells in the top chambers were removed and cells that invaded into the bottom chamber were fixed, stained, and quantified.

Cell-cycle analysis, BrdUrd incorporation assay, immunofluorescence, and immunohistochemistry

These procedures were previously detailed (21). For cell-cycle analysis, cells were transfected with miR128 or NC mimics for 72 hours and fixed with cold 70% ethanol overnight followed by staining in PPR buffer (0.02 mg/mL RNase A, 0.5% BSA, 0.5% Tween-20, and 0.05 mg/mL propidium iodide) at 37°C in the dark for 40 minutes. DNA contents were determined on a BD Aria FACS machine and data were analyzed by Flowjo software. For BrdUrd incorporation assays, cells were transfected with mimics or vectors for 72 hours and incubated in 10 μmol/L of BrdUrd for 4 hours. At the end, cells were fixed with 4% paraformaldehyde (PFA), denatured with 6 M HCI, and then incubated with anti-BrdUrd antibody followed by Alexa Fluor 594 labeled secondary antibody. A total of 500 to 1,000 cells per coverslip were counted to determine the percentage of BrdUrd positive cells with two coverslips counted for each condition. Immunofluorescence (IF) staining of BMI-1 required permeabilization and denaturation (0.5% Triton) pretreatment. For immunohistochemistry, tumor tissue sections were cut and stained for hematoxylin and eosin, Ki67, and active caspase-3. The IF images were captured using an Olympus IX 50 inverted epi fluorescence microscope.

Tumor transplantation experiments

The lab routinely maintains LNCaP, Du145, PC3, PPC-1, LAPC9, and other human prostate cancer xenografts in NOD/SCID or NOD/SCIDγ mice (21, 27, 28). Human prostate cancer cells in these xenografts were purifed out using a Histopaque-1077 gradient followed by removing murine cells with a Lineage-Depletion Kit (Miltenyi; refs. 21, 27, and 28). The purity of the HPCa cells from xenografts was generally ~100%. The 1st tumor transplantation experiments were performed by subcutaneously injecting purified human prostate cancer cells in 60-μl medium-Matrigel mixture (1:1) into NOD/SCID mice. For 2nd PC3 tumor transplantation, 1st tumors were dissociated into single cells, human tumor cells were purified out and then retransplanted with the NC or miR128 mimics. The 2nd tumor transplantation was performed using strategies similar to the primary implantation.

Statistical analysis

In general, unpaired 2-tailed Student t test was used to compare significance between groups for cell numbers, tumor weights, the percentages of Ki67+, caspase-3+, BrdUrd+, and S-phase cells, cloning, and sphere-forming efficiencies, and RNA levels of miR128 and its targets. The Fisher exact test and χ2 test were used to compare tumor incidence. All results were presented as mean ± SD or mean ± SEM with a P value < 0.05 considered statistically significant.

Results

Overexpression of miR128 by oligonucleotide transfection inhibits xenograft tumor growth in vivo

Mature miR128 can derive from either pre-miR128a (i.e., miR128-1) or pre-miR128b (miR128-2) locus located at two different chromosomal regions (Supplementary Fig. S1). miR128 levels were recently reported to be downregulated in prostate cancer and their metastases (23, 24). Consistent with these reports, when we quantified miR128 mRNA levels in eight prostate cancer cell lines, including PPC-1, LAPC4, LNCaP, VCaP, PC3, DU145, LAPC9, and LNCaP-C4-2 (a “castration-resistant” LNCaP subline), we found that the miR128 levels were lower in these prostate cancer cells compared with an immortalized nontumorigenic prostate epithelial cell line, NHP9 (Supplementary Fig. S2), which was established in our lab (21). These observations (23, 24; Supplementary Fig. S2) prompted us to investigate the functions of miR128 in prostate cancer cells by first performing gain-of-function studies. To this end, we transfected synthetic miR128 mimic or the negative control (NC) oligonucleotides (30 nmol/L, 48 hours) into PC3, PPC-1, Du145, or LNCaP cells. As expected, cells transfected with miR128 mimic showed miR128 mRNA levels several orders of magnitude higher than cells transfected with miR-NC (Supplementary Fig. S3A). Strikingly, when miR128 transfected PC3, PPC-1, and Du145 cells were implanted subcutaneously into NOD/SCID mice, tumor regeneration was significantly inhibited in every case (Fig. 1A; Supplementary Fig. S3B). Specifically, miR128 mimics significantly inhibited tumor growth as well as tumor incidence in PPC-1 cells (Fig. 1A). miR128 overexpression inhibited both primary (1st) and secondary (2nd) PC3 tumor growth with a trend of reduced tumor incidence (Fig. 1A). In three independent experiments, miR128 overexpression inhibited Du145 tumor growth with a trend of reduced tumor incidence (Fig. 1A; Supplementary Fig. S3B). Taken together, these tumor regeneration experiments provide the first in vivo evidence that miR128 possesses prostate cancer-suppressive effects.

We performed immunohistochemical staining of Ki67 and active caspase-3 in the endpoint tumors. The results revealed reduced Ki67+ cells in all three miR128-transfected tumor systems and increased caspase-3+ cells in miR128-overexpressing PC3 and PPC-1 tumors (Fig. 1B and C). Du145 tumors had a low percentage of caspase-3+ cells, which did not differ between miR128 and miR-NC–transfected tumors (Fig. 1B and C). These results suggest that the tumor-inhibitory effects of miR128 may be associated with...
suppression of proliferation and promotion of apoptosis. Preliminary results revealed that miR128 might also regulate prostate cancer cell differentiation as miR128 mimic increased both the mRNA and protein levels of differentiation markers androgen receptor (AR) and prostate-specific antigen (PSA; data not shown).

**miR128 overexpression suppresses cell proliferation and invasion in vitro**

To further help elucidate the mechanism of action of miR128, we assessed the impact of miR128 on several biological properties of prostate cancer cells in vitro. miR128 overexpression inhibited prostate cancer cell growth (Fig. 2A) and proliferation as determined by both BrdUrd incorporation assays (Fig. 2B) and cell-cycle analysis (Fig. 2C). miR128 mimic decreased the percentage of S-phase cells from 30.3% to 18.6% in PC3, and from 23.1% to 9.5% in Du145 cells, compared with the corresponding NC mimic-transfected cells (Fig. 2C). miR128 also significantly inhibited the Matrigel invasion in three of the four prostate cancer cell lines tested except PPC-1 (Fig. 2D).

**miR128 overexpression inhibits prostate cancer cell clonal, clonogenic, and sphere-forming activities in vitro**

Our previous studies indicate that a fraction of prostate cancer cells in culture, xenografts, and patient tumors possesses certain stem cell properties such as high capacity to found hologones in 2D cultures, 3D colonies in matrices such as Matrigel or methylcellulose, or anchorage-independent floating spheres in ULA plates (21, 22, 27–29). Here we analyzed the effects of miR128 on these properties of prostate cancer cells. As shown in Fig. 3A–F, miR128 overexpression, compared with miR-NC transfection, greatly inhibited the formation of hologones, which are enriched in self-renewing CSCs.

![Figure 1. miR128 overexpression attenuates prostate tumor growth in vivo. A, miR128 overexpression inhibits prostate cancer xenograft growth in three tumor models. Shown on the left are images of prostate tumors from the indicated cell types that were transfected with miR128 mimics or miR-NC oligonucleotides, with cell numbers injected and time in days (d) when tumors were harvested shown for each panel. Tumor incidences (#tumors/#injections), endpoint tumor weights (mean ± SD), and the corresponding P values were indicated on the right. For 20 tumor transplantsations, one NC and miR128 tumor each (indicated by a red box) was dissociated into single cells and purified human prostate cancer cells were retransfected with the miR128 or NC mimics, respectively, and transplanted into the NOD/SCID mice. B, representative immunohistochemical micrographs of Ki67 and active caspase-3 in endpoint tumor tissues. Original magnification, ×200. C, qualification of Ki67⁺ (top) and active caspase-3⁺ (bottom) cells in endpoint tumors. Bars, mean ± SD. *P < 0.05; **P < 0.01.](https://cancerres.aacrjournals.org/content/74/15/4186)
A differential enumeration in Du145 cells indicated that miR128 shifted holoclone to meroclone formation (Fig. 3F). Similarly, miR128 mimic inhibited clonogenic (in Matrigel) and sphere-forming capacities of both PC3 and Du145 cells (Fig. 3G–J). Serial sphere propagating assays revealed that miR128 also impaired the stem cell trait of self-renewal as assessed by secondary prostasphere establishment in Du145 cells (Fig. 3J).

Differential clonogenic and tumorigenic capacities of prostate cancer cells expressing low and high levels of endogenous miR128

All the preceding experiments studied the impact of exogenously overexpressed miR128 on the biological and tumorigenic properties of prostate cancer cells. Next, we investigated whether prostate cancer cells that express different levels of endogenous miR128 may possess intrinsically different biological and tumorigenic potential. To this end, we utilized a miR128 sensor construct (25), which contains four copies of miR128 perfect complementary binding sequences in the 3'-UTR of GFP cDNA (Supplementary Fig. S4A). The rationale was that the cells that express high endogenous levels of miR128 (i.e., miR128-hi) will be identified as GFP⁻ as miR128 will target the binding sites, leading to extinguishment of the GFP signal. By contrast, prostate cancer cells that express low levels of endogenous miR128 (i.e., miR128-lo) will be identified as GFP⁺. When the control plasmid pEGFP-N1 was transfected into PC3 and Du145 cells, an average of ~80% of the cells were
and this percentage did not change in the presence of increasing amount of exogenous miR128a mimic (Fig. 4A and B; Supplementary Fig. S4A). These results suggest that our transfection efficiency was 80%. In contrast, when PC3 and Du145 cells were transfected with the miR128 sensor construct, we observed that 40% cells were GFP+ in the absence of exogenous mimic (Fig. 4A and B; Supplementary Fig. S4B), suggesting that both cell types contain a fraction of miR128-hi cells, leading to increased GFP+ cells. Two pieces of evidence support that the miR128 sensor faithfully reports endogenous levels of miR128. First, when increasing amounts of exogenous miR128 mimics were cotransfected, together with the sensor construct, into PC3 and Du145 cells, we observed decreasing numbers of GFP+ cells (Fig. 4B; Supplementary Fig. S4C). Second, qPCR analysis in four sensor-transfected prostate cancer cell types demonstrated that GFP+ cells expressed lower levels of miR128 compared with the corresponding GFP− prostate cancer cells (Fig. 4C).

Subsequently, GFP+ (miR128-lo) and GFP− (miR128-hi) cells were sorted out by fluorescence-activated cell sorting (FACS) and used in clonal, clonogenic, and tumor regeneration assays. The results demonstrated that miR128-lo prostate cancer cells possessed higher clonal (Fig. 4D and E), clonogenic (Fig. 4F), and tumorigenic (Fig. 4G) potentials. Because the miR128 sensor construct contains four copies of the binding sequences that perfectly match the miR128 seed sequence, we utilized the miR128-sensor construct as a “decoy” for loss-of-function studies (i.e., to sequester endogenous miR128). As shown in Supplementary Fig. S5, compared with the bulk PC3 and Du145 cells transfected with pEGFP-N1, cells transfected with the miR128 sensor constructs displayed increased clonal and sphere-forming capacities.
miR128 targets a cohort of stem cell regulatory factors

To investigate the molecular mechanisms through which miR128 exerts its prostate cancer-inhibitory effects, we screened its potential targets using several bioinformatics tools, including TargetScan, Microcosm V5, and miRanda. Based on the sequence complementarity to miR128 seed sequence, we chose five genes as our interest of research, including BMI-1, NANOG, TGFBR1, EGFR, and E2F3 (Fig. 5A), all of which are known oncogenic and stem cell regulators implicated in prostate cancer initiation and progression. We found that miR128 overexpression reduced the mRNA levels of different molecules in a cell type–dependent manner (Fig. 5B). In PC3 cells, miR128 significantly decreased NANOG and TGFBR1 levels; whereas in PPC-1 cells, miR128 additionally reduced E2F3 mRNA levels (Fig. 5B). In Du145 cells, miR128 attenuated mRNA levels of all molecules except EGFR (Fig. 5B). In sharp contrast, in LNCaP cells, miR128 only reduced the mRNA levels of BMI-1 and EGFR (Fig. 5B).

Western blotting analysis of BMI-1, TGFBR1, and EGFR (Fig. 5C) overall corroborated the qPCR results. Specifically, miR128 overexpression reduced BMI-1 protein levels, to 30% to 50% of the NC-transfected cells, in all four cell types (Fig. 5C). TGFBR1 protein levels were also reduced by miR128 (Fig. 5C), even in LNCaP cells in which its mRNA levels were not altered by miR128 (Fig. 5B), suggesting that miR128 might regulate TGFBR1 in LNCaP cells through translational mechanisms. miR128 reduced EGFR protein levels only in PPC-1 cells (Fig. 5C).

Figure 4. Endogenous miR128 levels in prostate cancer cells inversely correlate with their clonogenic and tumorigenic potential. A–C, miR128 sensor reports endogenous miR128 mRNA levels in prostate cancer cells. Shown in A are images of PC3 and Du145 cells transfected with either parent pEGFP-N1 or miR128 sensor constructs in the absence of exogenous miR128 mimic (72 hours; original magnification, ×400). The corresponding images in the presence of 1 to 30 nmol/L of exogenous miR128 mimic are presented in Supplementary Fig. S4C. Shown in B is quantification of GFP* percentages in PC3 and Du145 cells transfected with the control (pEGFP-N1) or sensor construct in the presence of 0 to 30 nmol/L of exogenous miR128 mimic. Data represent the results from two independent experiments. The GFP* cells/total number of cells counted are presented in bar graphs. In C, levels of endogenous miR128 were determined by qRT-PCR in purified GFP* and GFP− prostate cancer cells 48 hours after transfection. Data were normalized to RNU48 and presented as values relative to corresponding GFP− cells. *, P < 0.05 and **, P < 0.01. D–F, differential clonal (C and D) and clonogenic (E) potential of miR128-hi (GFP+) vs. miR128-lo (GFP−) PC3 (C) and Du145 (D and E) cells. Log-phase PC3 and Du145 cells were transfected with the miR128 sensor plasmids and GFP+/GFP− cells purified out and used in clonal and clonogenic assays. *, P < 0.05. G, miR128-lo PC3 cells are more tumorigenic than the corresponding miR128-hi cells. Data on the left represent the mean tumor weight (±SD). *, P < 0.05.
BMI-1 is a direct and functional target of miR128 in prostate cancer cells

In all 4 prostate cancer lines, miR128 reduced both mRNA and protein levels of BMI-1 (Fig. 5B and C), and the reduction of BMI-1 protein by miR128 was also confirmed by IF staining of nuclear BMI-1 (Fig. 6A). These results, together with recent reports on BMI-1 being an essential regulator of mouse prostate epithelial and other stem cells (30), prompted us to focus on BMI-1 in subsequent studies. In luciferase reporter assays in which we cloned the BMI-1 3’-UTR harboring the miR128 binding site downstream of the firefly luciferase gene (Fig. 6B, left), cotransfection of Du145 cells with the luciferase construct and miR128 mimic led to reduced luciferase activity (Fig. 6B, right). Mutation of the miR128 binding site in the BMI-1 3’-UTR abrogated the miR128 effects (Fig. 6B), testifying BMI-1 as a direct target of miR128. Similar luciferase assays also confirmed NANOG as a direct miR128 target (Fig. 6C).

To determine whether BMI-1 is a functional target of miR128, we performed “rescue” experiments by cotransfecting Du145 cells with miR128 mimic and BMI-1 overexpressing construct containing the BMI-1 cDNA lacking its 3’-UTR (26), which could not be inhibited by miR128. BMI-1 overexpression was able to promote both proliferation (Fig. 6D) and sphere formation (Fig. 6E) over miR128 and control vector cotransfected cells. Altogether, these data support the notion that BMI-1 is a direct and functional target mediating the anti-proliferative and antitumorigenic effects of miR128 in prostate cancer cells.

Inverse correlation between miR128 and its targets in prostate CSC populations

We and others have characterized and reported on several tumor-initiating prostate cancer cell populations from both cultured cell lines and xenograft tumors (21, 22, 27, 28, 31, 32), which include CD44+ and CD133+ populations. We first determined the mRNA expression patterns of miR128 and its five targets (above) in some of these prostate cancer stem/progenitor cell populations. miR128 mRNA levels were lower in CD44+ and CD133+ Du145 cells (Fig. 7A, left) and in CD133+ LAPC9 cells (Fig. 7A, middle) compared with the corresponding marker-negative populations. Interestingly, in androgen-independent (AI) LAPC9...
xenograft tumors, which harbor significantly increased CD44^+ and PSA^+/lo prostate CSCs (PCSC) compared with the androgen-dependent (AD) xenografts (data not shown; ref. 28), the miR128 mRNA levels were also much lower (Fig. 7A, right). Overall, these data suggest that several PCSC populations in different prostate cancer models show reduced miR128 expression relative to non-CSC populations.

Consistent with miR128 expression patterns, the mRNA levels of its target molecules, that is, BMI-1, NANOG, TGFBR1, E2F3, and EGFR, showed upregulation in some of the PCSC populations (Fig. 7B). For example, in CD44^+ Du145 cells, all five molecules were overexpressed (Fig. 7B, left). In CD133^+ Du145 (Fig. 7B, middle) and LAPC9 (Fig. 7B, right) cells, three or four of these target molecules were overrepresented. These results (Fig. 7A and B), together, reveal an inverse correlation between miR128 and its targets in some PCSC populations.

Figure 6. BMI-1 is a direct target of miR128 and its reexpression partly rescued the decreased proliferation phenotype of miR128 overexpression. A, immunofluorescence staining of BMI-1 in three prostate cancer cell types. Cells transfected with miR-NC or miR128 mimic (30 nmoi/L, 48 hours) were cultured on coverslips overnight followed by immunostaining. Original magnifications, ×100. B and C, dual luciferase assays in DU145 cells. Schematic diagram of the BMI-1 and NANOG 3'UTR pMIR-REPORT constructs (left). Sequences were compared between mature miR128 and the wild-type (WT) or mutant (Mut) putative target sites in the 3'-UTR of BMI-1 (top) or NANOG (below). Cells were cotransfected with pMIR-REPORT containing the empty (Ctrl), wild-type, or mutant target site of the BMI-1 or NANOG 3'-UTR plus miR128 or NC mimic for 48 hours. The luciferase activity was normalized to the Renilla activity and presented as relative activity to the corresponding NC (assigned as value "1"). Values denote the mean ± SEM of three independent assays. *, P < 0.05.

D and E, effects of restoring BMI-1 expression on proliferation or sphere-formation ability in DU145 cells as determined by BrdU incorporation (D) and sphere formation (E) assays 72 hours after cotransfection with BMI-1 cDNA lacking the 3'-UTR and miR128 mimic. Error bars, mean ± SD obtained from two independent experiments. *, P < 0.05.

Negative regulation of PCSCs by miR128
Because miR128 was expressed at lower levels in some prostate cancer stem/progenitor cell populations, we asked whether miR128 might negatively regulate the tumor-regenerating capacity of these cells. To address this question, we manipulated miR128 expression in CD44^+ and CD44^-Du145 cells followed by tumor regeneration assays. As shown in Fig. 7C, overexpression of miR128 in purified CD44^-Du145 cells significantly inhibited tumor development in that fewer Du145 tumors were regenerated and the developed tumors were much smaller. In contrast, overexpression of anti-miR128 (i.e., anti-128) in CD44^-Du145 cells promoted tumor development (Fig. 7C).

Next, we manipulated the BMI-1 expression levels in purified CD44^- and CD44^+Du145 cells to determine how that would affect their respective biological behaviors in vitro. As expected, the lentiviral shBMI-1 downregulated BMI-1 mRNA and...
protein in CD44+/ Du145 cells (Fig. 8A) and resulted in reduced proliferation (Supplementary Fig. S6A), holoclone establishment (Fig. 8B), and sphere formation (Fig. 8C). BMI-1 downregulation also inhibited proliferation of bulk PC3 and PPC-1 cells (Supplementary Fig. S6A). In contrast, BMI-1 overexpression (Fig. 8D) promoted proliferation (Supplementary Fig. S6B) and holoclone (Fig. 8E) and sphere (Fig. 8F) formation in CD44+/C0 Du145 cells, as well as the proliferation of bulk PC3 and PPC-1 cells (Supplementary Fig. S6B).

Considering the reciprocal expression patterns of miR128 and BMI-1 in CD44+/Du145 cells, the above results suggest that the PCSC-inhibitory effects of miR128 are mediated, at least in part, via downregulating BMI-1.

Discussion

Our present study has revealed the following novel findings: (i) exogenously overexpressed miR128 suppresses tumor regeneration in 3 prostate cancer xenograft models and inhibits cell proliferation in vitro and in vivo; (ii) miR128 overexpression inhibits CSC-associated properties, including holoclone and sphere formation; (iii) prostate cancer cells that express low levels of endogenous miR128 possess high clonal, clonogenic, and tumorigenic properties; (iv) miR128 targets several stem cell regulatory factors in prostate cancer cells, including BMI-1, NANOG, and TGFBR1; (v) miR128 and its targets exhibit reciprocal expression patterns in several PCSC populations; (vi) BMI-1 represents a direct and functionally relevant target of miR128 in prostate cancer cells; and (vii) miR128 and BMI-1 are reciprocally expressed and also exhibit opposite biological functions in the CD44+/Du145 prostate cancer cells.

miR128 was originally identified as a "brain-specific" miRNA whose expression profile is associated with brain development: it is present mainly in mature, terminally differentiated neurons but absent in neural stem cells...
implicating miR128 in neuronal differentiation. Aberrant expression of miR128 has been observed in some malignancies and several studies have lent credence to the notion that miR128 functions as a tumor suppressor in glioblastoma multiforme (34) and medulloblastoma (26). However, upregulation of miR128 has also been reported in acute lymphoblastic leukemia (35) and in letrozole-resistant breast cancer cell lines (36). These findings imply miR128 as either a tumor-suppressive or oncogenic miRNA, probably depending on specific cancer types. Recently, miR128 was found to be downregulated in prostate cancer compared with the normal/benign prostate tissues, and even more so in metastases in comparison to primary tumors (23, 24). However, a causal role of miR128 in prostate cancer has not been reported. Here, for the first time, we thoroughly characterize the biological activities of miR128 and provide direct evidence for its tumor-suppressive functions in prostate cancer.

Exhaustive overexpression studies by introducing exogenous miR128 mimic reveal its tumor-inhibitory effects in 3 prostate cancer cell models. Complementary studies using the miR128-reporting sensor demonstrate that the miR128-ko prostate cancer cells possess higher clonal, clonogenic, and tumorigenic activities than the isogenic miR128-hi prostate cancer cells. These latter observations highlight the cellular heterogeneity of prostate cancer cells (3, 37) in that the cells expressing different levels of a single miRNA can manifest intrinsic differences in many fundamental tumor-associated biological traits. Along the same line, the miR128 sensor construct, when used as a decoy to neutralize the endogenous miR128 in bulk prostate cancer cells, behaves opposite to miR128 mimic and promotes prostate cancer cell clonal and clonogenic growth.

We explored the potential mechanisms underlying the prostate cancer-inhibitory effects of miR128 by focusing on molecular and cellular targets. At the molecular level, miR128 seems to target, in a cell type-dependent manner, several stem cell-related genes, including BMI-1, NANOG, and TGFBR1. BMI-1, a component of the PRC2 polycomb repressor complex, is crucial for self-renewal and malignant transformation of prostate stem cells (38). BMI-1 is frequently overexpressed in cancer, including prostate cancer, and is a potential biomarker for the diagnosis and prognosis of prostate cancer (39, 40). In prostate cancer cells, we find that miR128 directly regulates BMI-1 through its 3’-UTR. BMI-1 represents a bona fide as well as a functional target of miR128, as expression of a BMI-1 cDNA lacking the miR128 binding site at the 3’-UTR partly rescues the proliferation and sphere-forming defects in miR128 overexpressing prostate cancer cells. These observations suggest that BMI-1 is an important downstream target for miR128 to exert its tumor inhibitory functions. miR128 likely also targets other important stem cell regulators such as NANOG, which has previously been shown by our lab to be enriched in cancer.
stem/progenitor cells and to play important positive roles in promoting tumorigenicity (41). Inducible expression of NANO G in prostate cancer cells upregulates molecules such as c-Myc, CXC4R4, CD133, and ALDH1 and reprograms bulk prostate cancer cells to CSCs (42). In this study, we also find that miR128 can directly target the NANO G mRNA in prostate cancer cells. Whether this will lead to reduced NANO G protein levels and whether NANO G (protein and/or RNA) represents a functionally relevant target of miR128 in prostate cancer cells awaits further investigations.

At the cellular level, miR128 seems to negatively regulate prostate cancer stem/progenitor cells. miR128 overexpression inhibits the holoclone and sphere-forming activities of bulk prostate cancer cells. Vice versa, miR128−lo prostate cancer cells possess high clonal and sphere-forming capacity. Furthermore, miR128 is underexpressed whereas its targets are overexpressed in several prostate cancer stem/progenitor cell populations. Importantly, miR128 overexpression in CD44+/CD133− prostate cancer cells suppresses whereas anti-miR128 expression in CD44+/CD133+ prostate cancer cells promotes tumor regeneration. The inhibitory effects of miR128 on prostate cancer-initiating cells are likely mediated through its targeting critical stem cell molecules such as BMI-1 and NANO G. In support, silencing BMI-1 in CD44+/CD133− Du145 cells significantly inhibits their CSC properties, whereas BMI-1 overexpression in CD44+/CD133+ cells confers an oncogenic effect in vitro similar to the phenotype of miR128 inhibition. Consistent with our results, BMI-1 has been shown to be a relevant target of miR128 in regulating glioblastoma (43) and breast (44) CSCs. Future work will determine whether NANO G also represents a functionally relevant target of miR128 in prostate cancer stem/progenitor cells. Our present results on miR128, together with our recent data on miR34a (21) and let-7 (22), reinforce the concept that multiple key tumor-suppressive miRNAs function, concertedly and coordinately, to negatively regulate tumor-initiating cells (45). In principle, these tumor-suppressive miRNAs can be developed into CSC-targeting "replacement" therapeutics (21, 45).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

**Conception and design:** M. Jin, C. Liu, M.A. Badeaux, D.G. Tang

**Development of methodology:** C. Liu, B. Liu, C. Jeter, D.G. Tang

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** M.A. Badeaux, D.G. Tang

**Analysis and interpretation of data (e.g., statistical analysis, biosciences, computational analysis):** M. Jin, C. Liu, D.G. Tang

**Writing, review, and/or revision of the manuscript:** M. Jin, C. Liu, B. Liu, C. Jeter, A.V. Vlassov, D.G. Tang

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** X. Chen, A.V. Vlassov, D.G. Tang

**Study supervision:** T. Zhang, D.G. Tang

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**References**

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Min Jin, Tao Zhang, Can Liu, et al.


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