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.
for prostate cancer–suppressing 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, hydrocor- tisone, 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 (pAb, 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 complementary binding sites inserted into pEGFP-N1 downstream of GFP, was kindly provided by Dr. F.G. Wulczyn (Institute of Cell Biology and Neurobiology, Charité/C19). For BMI-1 and NANOG, fragments containing the pre-miR128 UTR, respectively. pcDNA-CW-CAT BMI-1 (BMI-1) lacking BMI-1 3' UTR and its parent pcDNA-CW-CAT (Ctrl) were cotransfected with miR128 mimic for rescue experiments. These BMI-1–related vectors were courtesy of Dr. R. Vibhakar (Children’s Hospital Colorado and University of Colorado, Denver, CO; ref. 26).

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 × B27 (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-REPORTer 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 Histopaque-1077 gradient followed by removing murine cells LAPC9, and other human prostate cancer xenografts in Tumor transplantation experiments primary implantation. Transplantation was performed using strategies similar to the inverted epi stained for hematoxylin and eosin, Ki67, and active caspase-fluorescence, 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 HCl, 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+ 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 epifluorescence 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 purified 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 retransfected 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 suppresses prostate cancer—suppressive effects.

We performed immunohistochemical staining of Ki67 and activate 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).

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 2+ PC3 tumor transplantations, 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 apoptotic caspase-3 in endpoint tumor tissues. Original magnification, ×200. C, quantitation of Ki67+ (top) and active caspase-3+ (bottom) cells in endpoint tumors. Bars, mean ± SD. *, P < 0.05; **, P < 0.01.
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−/C0 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

Figure 2. miR128 overexpression suppresses prostate cancer cell proliferation and invasion in vitro. A, effect of miR128 on prostate cancer cell growth. Cells as indicated and transfected with NC or miR128 mimic (30 nmol/L) were plated in triplicate in 6-well plate (50,000 cells/well) on day 0. Cells were harvested and counted on day 1, 3, and 5. Shown are the mean ± SD. B, effect of miR128 on BrdUrd incorporation. Shown are representative micrographs (left) and quantification (right) of BrdUrd staining in PC3 and Du145 cells transfected with miR-NC or miR128 (30 nmol/L, 72 hours). C, effect of miR128 on prostate cancer cell cycle. PC3 and Du145 cells transfected with miR128 or NC mimic for 72 hours were used in cell-cycle analysis upon propidium iodide staining. Shown are representative histograms (left) and quantification of the S-phase cells (right; mean ± SD). D, effect of miR128 on prostate cancer cell invasion. Shown are representative images (left) and quantification (right) of cell invasion in PC3, PPC-1, Du145, and LNCaP C4-2 cells as determined by Transwell assay. The invasive activity detected in NC-transfected cells was used as the calibrator (assigned a value of “1,” indicated by red dotted line). Error bars represent the mean ± SEM of three independent experiments, with each conducted in duplicate. In all experiments, *P < 0.05 and **P < 0.01.
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).
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 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 antiproliferative 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, 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⁻⁰⁷ 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 EGF, 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.

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 multiform (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-lo 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 NANOG in prostate cancer cells upregulates molecules such as c-Myc, CXXCR4, CD133, and ALDH1 and reprograms bulk prostate cancer cells to CSCs (42). In this study, we also find that miR128 can directly target the \textit{NANOG} mRNA in prostate cancer cells. Whether this will lead to reduced NANOG protein levels and whether NANOG (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 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\(^+\) prostate cancer cells suppresses whereas anti-miR128 expression in CD44\(^+\) 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 NANOG. In support, silencing BMI-1 in CD44\(^+\) Du145 cells significantly inhibits their CSC properties, whereas BMI-1 overexpression in CD44\(^-\) cells confers an oncogenic effect \textit{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 NANOG also represents a functionally relevant target of miR128 in prostate stem/cancer/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**

\textbf{Conception and design:} M. Jin, C. Liu, M.A. Badeaux, D.G. Tang

\textbf{Development of methodology:} M. Jin, C. Liu, C. Jeter, D.G. Tang

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

\textbf{Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):} M. Jin, C. Liu, D.G. Tang

\textbf{Writing, review, and/or revision of the manuscript:} M. Jin, C. Liu, R. Liu, C. Jeter, A.V. Vlassov, D.G. Tang

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

\textbf{Study supervision:} T. Zhang, D.G. Tang

**Acknowledgments**

The authors thank Dr. F.G. Wulczyn for providing the miR128 sensor vectors and Dr. B. Vihakkar for sharing the BMI-1 plasmids. The authors also thank Ms. P. Whitney for assistance in flow sorting, Dr. J.J. Shen and his group for qRT-PCR analysis, and the other lab members for advice and discussions.

**Grant Support**

This project was supported, in part, by grants from NIH (R01-CA155693), Department of Defense (W81XWH-13-1-0552 and PC130483), CPRIT (RP120380), and the MDACC Research Center Laura & John Arnold Foundation grant (D.G. Tang). C. Jeter was supported, in part, by CPRIT RP120394. C. Liu was supported, in part, by DOD postdoctoral fellowship (PC121553) and NSFC grant 81302208. R. Liu was supported, in part, by NSFC grant 81302208. Shanghai Municipal Natural Science Fund 13ZR145990, and the Fundamental Research Funds for Central Universities 2012KJ041. M. Jin was supported by the China Scholar Council (CSC) as a joint-training Ph.D. student from Tongji Medical College of Huazhong University of Science and Technology, China, and the Department of Molecular Carcinogenesis of the University of Texas MD Anderson Cancer Center, Texas.

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

Received February 12, 2014; revised May 21, 2014; accepted May 28, 2014; published OnlineFirst June 5, 2014.

**References**

miR128 Suppressing Prostate Cancer


www.aacrjournals.org Cancer Res; 74(15) August 1, 2014 4195

Published OnlineFirst June 5, 2014; DOI: 10.1158/0008-5472.CAN-14-0404

Downloaded from cancerres.aacrjournals.org on December 31, 2017. © 2014 American Association for Cancer Research.
miRNA-128 Suppresses Prostate Cancer by Inhibiting BMI-1 to Inhibit Tumor-Initiating Cells

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


Updated version
Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-14-0404

Supplementary Material
Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2014/06/03/0008-5472.CAN-14-0404.DC1

Cited articles
This article cites 45 articles, 14 of which you can access for free at: http://cancerres.aacrjournals.org/content/74/15/4183.full#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/74/15/4183.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/74/15/4183.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.