Regulatory role of miR-203 in prostate cancer progression and metastasis.

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TRANSLATIONAL RELEVANCE

Metastatic prostate cancer is a leading cause of cancer-related deaths in men. At present, this disease has limited therapeutic options and poor prognosis as the underlying molecular mechanisms driving prostate cancer metastasis are not completely understood. In this study, we have shown that miR-203 suppresses prostate cancer progression and metastasis via repression of a cohort of prometastatic targets. miR-203 expression is specifically attenuated in bone metastatic cell lines and clinical specimens supporting a fundamental ‘anti-metastatic’ role of this miRNA. Further, miR-203 regulates survivin expression which has prognostic significance and is associated with unfavorable outcome in prostate cancer. Also, our study suggests that miR-203 regulates the expression of several bone specific effectors including Runx2, a master regulator of bone metastasis. In view of these results, we propose that miR-203 may be an attractive target for designing of improved diagnostic and therapeutic strategies for advanced prostate cancer.

ABSTRACT

Purpose: Advanced metastatic prostate cancer is a fatal disease, with only palliative therapeutic options. Though almost 80% of cases of metastatic prostate cancer present bone metastasis, our current understanding of the molecular mechanisms that govern this metastatic dissemination remain fragmentary. The main objective of the present study was to identify microRNA genes that regulate metastatic prostate cancer.

Experimental Design: MicroRNA expression profiling was done in human prostate cell lines to identify dysregulated microRNA components of advanced prostate cancer. miR-203 expression was assessed in prostate carcinoma cell lines and clinical specimens by RT-PCR and in situ
hybridization. To assess the biological significance of miR-203, miR-203 was reexpressed in bone metastatic prostate cancer cell lines followed by *in vitro* and *in vivo* functional assays.

**Results:** miR-203 expression is specifically attenuated in bone metastatic prostate cancer suggesting a fundamental anti-metastatic role for this miRNA. Reintroduction of miR-203 in bone metastatic prostate cancer cell lines suppresses metastasis via inhibition of several critical steps of the metastatic cascade including epithelial-mesenchymal transition, invasion and motility. Ectopic miR-203 significantly attenuated the development of metastasis in a bone metastatic model of prostate cancer. Importantly, miR-203 regulates a cohort of pro-metastatic genes including ZEB2, Bmi, Survivin and bone specific effectors including Runx2, a master regulator of bone metastasis.

**Conclusions:** miR-203 is an ‘anti-metastatic’ microRNA in prostate cancer that acts at multiple steps of the prostate cancer metastatic cascade via repression of a cohort of prometastatic targets. miR-203 may be an attractive target for therapeutic intervention in advanced prostate cancer.

**INTRODUCTION**

Prostate cancer (PCa) is the most common male malignancy and the second leading cause of cancer death among men in the United States. Although significant gains have been made in the management of the early phases of prostate cancer, the evolution of prostate cancer to a hormone-independent stage invariably signals advanced metastatic disease, with limited therapeutic options and poor prognosis (1). Metastasis involves multiple sequential steps including the escape of neoplastic cells from a primary tumor (local invasion), intravasation into the systemic circulation, survival during transit through the vasculature, extravasation into distant tissues and finally, proliferation at a new site (2). The most critical step in invasion and...
metastases is attributed to the process known as epithelial-mesenchymal transition (EMT) (3), in which epithelial cells lose adhesion and cytoskeletal components concomitant with a gain of mesenchymal components and the initiation of a migratory phenotype. Advanced prostate cancer is mostly associated with metastatic dissemination, typically to the bones (4), causing both osteoblastic and osteolytic lesions (5). The mechanisms by which prostate cancer cells selectively metastasize to the bone remain largely unknown. Understanding molecular controls that signal prostate cancer progression and metastasis is a key to develop better therapeutic and diagnostic interventions for the disease.

MicroRNAs (miRNAs) constitute an evolutionarily conserved class of small RNAs that suppress gene expression posttranscriptionally via sequence-specific interactions with the 3’- untranslated regions (UTRs) of cognate mRNA targets (6). In mammalian cells, miRNAs effect gene silencing via both translational inhibition and mRNA degradation; an individual miRNA is capable of regulating dozens of distinct mRNAs. It has been estimated that miRNAs regulate ~30% of the human genome (6). Dysregulation of miRNA expression has been identified in various cancers and accumulating data suggests that miRNAs function as classical oncogenes or tumor suppressor genes (7). In addition, recently microRNAs have been discovered to have a role in progression and metastasis of human cancers (8, 9). More than 20 miRNAs have been shown to impact critical steps in the metastatic cascade, such as epithelial-mesenchymal transition (EMT), apoptosis, and angiogenesis by acting on multiple signaling pathways and targeting various proteins that are major players in this process. Furthermore, several clinical studies have identified correlations between miRNA expression and recurrence, development of metastases and/or survival (8, 9).
An expansive body of literature shows that miRNAs are significantly altered in prostate cancer suggesting that miRNAs act as key regulators of prostate carcinogenesis (10). Several studies have been conducted to identify the PCa-specific miRNA signature. However, no consensus has been reached on which miRNAs are relevant for development and progression of this malignancy. In this context, the main objective of the present study was to identify miRNAs that regulate prostate carcinogenesis. Towards this, we performed microRNA expression profiling in human prostate cell lines. In this effort, we identified that miR-203 is progressively lost in advanced metastatic prostate cancer. miR-203 has been reported to be epigenetically silenced in hematopoietic malignancies and hepatocellular carcinomas (11, 12). However, there is no study demonstrating the role of this microRNA in prostate cancer. Here we provide evidence that miR-203 is a crucial component of the complex circuitry that regulates prostate cancer progression and metastasis. To our knowledge, this is the first report that directly demonstrates a role of miR-203 in prostate cancer disease progression.

RESULTS

miR-203 expression is attenuated in bone metastatic prostate cancer

To evaluate the role of miR-203 in prostate cancer, miR-203 expression was assayed in human prostate cell lines, which included normal epithelial prostate cell lines (RWPE-1 and PWR-1E) and prostate carcinoma cell lines (LNCaP, Du145, PC3, VCaP, MDA-PCa-2b). It was consistently observed that relative miR-203 expression is specifically attenuated in prostate cancer cell lines derived from bone metastasis (PC3, VCaP and MDA-PCa-2b) (Fig. 1A). We extended our analysis to clinical malignancies by assessing miR-203 levels by in situ hybridization analysis of a prostate adenocarcinoma tissue microarray with normal adjacent
tissues and metastatic bone tissues (Fig. 1B-1C). miR-203 expression was significantly attenuated in bone metastatic tissues compared to normal tissues.

**miR-203 expression is progressively lost in primary tumors**

We examined the expression levels of miR-203 in laser capture microdissected (LCM) prostate cancer tissues (n=22) and matched adjacent normal regions by real-time PCR. Relative to matched normal tissues, 11/22 (50%) tissues showed lower miR-203 levels (Fig. 2A). Furthermore, *in situ* hybridization (ISH) was performed on prostate adenocarcinoma tissue microarray with 36 cases of prostate cancer-8 of which had matched normal adjacent tissue. This analysis showed that miR-203 expression is downregulated progressively in tumor tissues compared to adjacent normal tissue (Fig. 2B-2D). Collectively, the data suggests that average expression of miR-203 was attenuated in tumor tissues compared to normal prostatic tissues. We further assessed if miR-203 expression in clinical tissues correlated with clinicopathological characteristics such as pathological stage and gleason score (Fig. 2D). It was observed that cases with advanced prostatic disease had decreased miR-203 expression compared to adjacent normal tissues.

**Reexpression of miR-203 suppresses metastasis-relevant traits in vitro**

In view of the observed inverse correlations between miR-203 expression and bone metastasis in cell lines and clinical malignancies, we assessed the potential for an anti-metastatic role of miR-203. Thus, miR-203 was reintroduced in metastatic PC3 human prostate cancer cells followed by functional assays. miR-203 transfection resulted in miR-203 levels comparable to those in non-malignant prostate epithelial cell lines (Fig. 3A). Reexpression of miR-203 led to marked morphological changes suggesting transition from a fibroblast- to epithelial-like phenotype (Fig. 3B). Specifically, a pronounced decrease in the fraction of cells with an elongated, spindle shape
was paralleled by an increase in that of rounded cells. Wound healing assay, transwell migration and invasion assays were carried out to evaluate the effects of miR-203 on the migratory and invasive behavior of prostate cancer cells (Fig. 3C-E). miR-203 over-expressing cells were less proficient than equivalent control miR (miR-CON) transfected cells at closing an artificial wound created over a confluent monolayer (Fig. 3C). Also, transwell assays showed that miR-203 reintroduction decreases the migration (Fig. 3D) and invasion (Fig. 3E) by ~50% suggesting that miR-203 reexpression suppresses invasiveness and motility of prostate cancer cells.

**miR-203 reexpression induces mesenchymal-to-epithelial transition (MET) in metastatic prostate cancer cells**

Since the phenotype of miR-203-transfected cells, including change in morphology, reduced migration/invasion was reminiscent of a reverse transition from a mesenchymal to an epithelial state (MET), we asked whether miR-203 introduction could influence marker expression in a way consistent with MET induction. Immunoblotting of PC-3 cells showed that ectopic miR-203 expression resulted in a net increase in epithelial marker E-cadherin with a concomitant decrease in mesenchymal markers Vimentin and Fibronectin (Fig. 3F, upper panels). Immunofluorescent staining of cells for E-cadherin revealed expression was induced and E-cadherin localized to the plasma membrane, typical of the pattern observed in epithelial cells (Fig. 3F, lower panels).

**miR-203 reexpression suppresses prostate cancer metastasis in vivo**

We next asked if ectopic miR-203 could inhibit prostate cancer metastasis in vivo. Bioluminescent human prostate carcinoma cell line PC-3M-luc was stably transfected with control miR or miR-203 (Fig. S1). As an experimental bone metastasis model, stable clones were injected into the left ventricle of the heart followed by periodic monitoring of the development of tumors in vivo by bioluminescent imaging (Fig. 3G). A successful intracardiac injection was
indicated by day 0 images showing a systemic bioluminescence distributed throughout the animal. Representative bioluminescence images of mice from the two experimental groups, taken at days 16, 23 and 30 after intracardiac injection of PC-3M-luc cells indicated that miR-203 reexpression decreases the metastatic lesions. Quantification of whole-animal bioluminescence (Fig. 3H) on day 30 showed that miR-203 reexpression had a negative effect on the development of metastases.

**miR-203 influences cellular proliferation and apoptosis in prostate cancer cells**

Cell proliferation, survival and migration are among the common functions required by tumor cells for metastatic progression in target microenvironments. Restoration of miR-203 in metastatic prostate cancer cell line PC3 also led to decreased cell growth (Fig. 4A), clonability (Fig. 4B) and induced cell cycle arrest at the G0-G1 phase of the cell cycle (Fig. 4C). Along with the growth arrest, miR-203 induced a senescent-like phenotype in PC-3 cells, as determined by senescence-associated β-galactosidase activity (Fig. S2). Also, the apoptotic cell fractions (Early apoptotic + Apoptotic) were significantly increased upon miR-203 reexpression (19%+5%) compared to control cells (3%+1%) with a concomitant decrease in the viable cell population (Fig. 4D). miR-203, therefore, suppressed tumorigenesis and metastasis, in part, through an inhibition of proliferation and induction of apoptosis.

**Survivin is a direct target of miR-203**

The ability of miR-203 to impede multiple steps in the progression and metastasis of prostate cancer might derive from its ability to pleiotropically regulate genes involved in diverse aspects of metastatic dissemination. To identify effectors of miR-203, we used two algorithms that predict the mRNA targets of a miRNA, miRANDA (13) and TargetScan (14). Guided by the target prediction algorithms, we found that miR-203 regulates a cohort of genes which play a
role in cellular proliferation, survival, invasion and metastasis (Fig. 5 and 6). Of significance, we found that miR-203 targets survivin/BIRC5, a small inhibitor of apoptosis (IAP) protein that is differentially expressed in cancer (15) and plays a pivotal role in prostate cancer progression and metastasis (16, 17). miR-203 significantly reduced the endogenous protein (Fig.5A) and mRNA level (Fig. 5B) of survivin in PC3 cells. The 3’-UTR of survivin mRNA has a highly conserved putative miR-203 binding site (Fig. 5C). Luciferase reporter assays with increasing concentrations of the survivin 3’-UTR construct in miR-203/miR-CON expressing or mock transfected PC3 cells (Fig. 5C) showed that miR-203 represses survivin directly. Survivin is a regulator of cellular homeostasis, with functions in modulation of cell death and survival, regulation of cell cycle and microtubule dynamics (15). Immunofluorescence staining for survivin confirmed that miR-203 expression dramatically downregulates the expression of survivin and co-staining with α-tubulin in the transfectants revealed a disorganized tubulin cytoskeleton in the miR-203 expressing cells compared to control cells (Fig. 5D).

**miR-203 regulates a cohort of metastatic genes**

We performed Western blot analysis for other putative miR-203 targets in PC3 cells that were either mock transfected or transfected with miR-203 or miR-CON (Fig. 6A). Interestingly, miR-203 repressed the protein levels of E-box-binding transcription factor ZEB2, also known as Smad-interacting protein 1 (SIP1), a transcriptional repressor that regulates the expression of E-cadherin and EMT (18, 19). Also, miR-203 inhibited the expression of polycomb repressor, Bmi1. Importantly, ectopic miR-203 repressed the expression of bone specific transcriptional regulators, Runx2 and Dlx5, that are involved in homing of prostate cancer cells to bone. In addition, we found that Smad4, a central mediator of TGF-β intracellular signaling, is regulated by miR-203. Also, miR-203 repressed endogenous levels of transcription factor E2F1, which has
been reported to be elevated in metastatic hormone-resistant prostate cancer (20). In agreement with the inhibitory effects of miR-203 on migration and invasion, we found decreased MMP10 expression in cells overexpressing miR-203. We cloned the 3’- UTRs of the targets that are potential mediators of the effects of miR-203 on prostate cancer progression and metastasis, namely, ZEB2, Runx2, Dlx5 and Smad4 into a luciferase construct. Reporter assays with PC3 cells revealed that miR-203 repressed these four validated targets directly (Fig. 6B-E).

It has been proposed that metastatic prostate cancer cells must be osteomimetic in order to metastasize, grow, and survive in the skeleton (21). In view of our present evidence suggesting the specific attenuation of miR-203 in bone metastatic prostate cancer cell lines and clinical specimens and the potential regulation of Runx2 and Dlx5, we asked if alteration of miR-203 levels affects the osteomimetic properties of prostate cancer cells. It has been suggested that altering the expression of certain critical transcription factors, such as Runx2 in prostate cancer cells, can confer profiles of gene expression, such as osteopontin (OP), osteocalcin (OC), that mimic that of osteoblasts (21). We analyzed the expression of OP and OC in miR-203 expressing PC3 cells (Fig. 6F) and found decreased expression of these two key osteoblastic genes suggesting that miR-203 may regulate the osteomimetic properties of prostate cancer cells.

DISCUSSION

Here we demonstrate that miR-203 expression is specifically attenuated in bone metastatic prostate cancer cell lines and clinical specimens suggesting a fundamental anti-metastatic role for this miRNA. Further, miR-203 expression profiling in archived primary tumors point to a progressive decline of this miRNA in advanced prostatic disease. However, due to the limited
follow-up data on these prostatic cases, we could not correlate miR-203 levels with metastatic recurrence.

To assess the biological significance of decreased miR-203 expression, we reexpressed miR-203 in bone metastatic prostate cancer cell line followed by \textit{in vitro} and \textit{in vivo} functional assays. Our results suggest that miR-203 reexpression decreased motility and invasiveness. Our \textit{in vivo} data suggests that ectopic miR-203 significantly attenuated the development of metastasis in a bone metastatic model of prostate cancer. Significantly, increasing miR-203 expression in metastatic prostate cancer cells induced morphological and molecular alterations consistent with mesenchymal-to-epithelial transition. miR-203 overexpression induced the expression of E-cadherin while concomitantly attenuated the expression of mesenchymal markers, Vimentin and Fibronectin. Altogether, our findings suggest a reversal of EMT and the acquisition of a less invasive phenotype upon miR-203 reexpression. The EMT process requires a complex genetic program coordinated in a large part by transcriptional repressors of E-cadherin, such as ZEB1 and ZEB2. We showed that ectopic expression of miR-203 in PCa cells reduced ZEB2 protein levels. Several recent studies have reported that the miR-200 family and miR-205 regulates EMT through direct targeting of ZEB1 and ZEB2 (22-24). Here we provide evidence that miR-203 is another miRNA that regulates EMT by directly targeting ZEB2, that in turn regulates invasion and metastasis of prostate cancer.

Metastasis reflects the acquisition of multiple molecular traits by tumor cells, including the ability to counteract anoikis (25), migrate, invade, survive (26) and proliferate in unrelated microenvironments. In the present study, we demonstrated that miR-203 plays pleiotropic roles where it affects multiple steps of the metastatic cascade including invasion, motility, cellular survival and proliferation that contribute to the metastatic dissemination of prostate cancer cells.
Since loss of miR-203 expression is a late event in prostate cancer progression, we propose that its effects on cellular proliferation and apoptosis may be relevant to the development of distant metastases.

Further, we demonstrate that miR-203 can pleiotropically regulate a cohort of metastatic effectors that includes apart from ZEB2, Bmi1 and Survivin. miR-203 represents a ‘stemness-inhibiting miRNA’ as it was shown to control the properties of skin stem cells through inhibition of the stem cell factor p63 (27, 28). Another recent study shows that ZEB1 regulates tumorigenesis by repressing stemness-inhibiting miRNAs, including miR-203 in pancreatic cancer (29). It has been suggested that the same miRNAs may be involved in invasion and metastasis of tumor cells and in maintenance of the cancer stem cell phenotype, possibly accounting for the thread of malignancy connecting primary to metastatic tumors (8). In view of our present results suggesting a role of miR-203 in metastasis and miR-203 mediated inhibition of stem cell factor, Bmi1, we hypothesize that miR-203 supports metastasis not only by promoting metastasis relevant traits; but also by maintaining a stem cell phenotype, which is necessary for the formation of metastasis from disseminated tumor cells.

miR-203 mediated regulation of survivin is a highly significant finding as this nodal protein orchestrates extensive, tumor specific signaling networks and is an attractive drug target (30). Recent studies suggest a direct role of survivin in cancer progression and metastasis (16, 17). In prostate cancer, survivin is a significant contributor to the development of hormone resistance in prostate cancer and is associated with unfavorable outcome. It has been hypothesized that targeting survivin and blocking it would enhance prostate cancer cell susceptibility to anti-androgen therapy (31).
In addition, miR-203 regulates bone-specific effectors including Runx2, Dlx5 and Smad4. Considerable effort has been devoted to map the requirements of bone lesions in prostate tumors. It has been proposed that prostate cancer cells are osteomimetic in their ability to thrive and grow in the bone milieu (21). In concordance with this idea, prostate cancer cells that metastasize to bone express several classes of bone matrix and signaling proteins involved in adhesion and migration (32). Runx2, a transcription factor essential for osteogenesis, becomes highly activated in prostate cancer cells that metastasize to bone, and is detected in human and mouse prostate cancer tissue, but not normal prostate, in vivo (33, 34). Non-metastatic cells exhibit low levels of Runx2 (32, 34, 35). Recent studies have identified Runx2 as a crucial regulator of prostate bone metastasis (36). Runx2 has been associated with the osteomimetic properties of bone metastatic prostate cancer cells, via transcription of genes implicated in osteoblastic lesions such as bone matrix proteins (OC, OP) and enzymes involved in bone turnover (32, 35). Also, Runx2 mediates the response of cells to signaling pathways hyperactive in tumors, including BMP/TGFβ signaling. Interestingly, ectopic miR-203 repressed the expression of Runx2 and also decreased the expression of the osteoblastic genes, OC and OP, suggesting that miR-203 is a critical determinant of the osteomimetic properties of prostate cancer cells. OC and OP have been implicated in the selective affinity of cancer cells for bone and have prognostic significance (37-39). We also observed miR-203 mediated repression of a homeodomain protein, Dlx5, an activator of Runx2, which is an important regulator of bone formation (40). In light of these results, it seems reasonable to suggest that downregulation of miR-203 in bone metastatic prostate cancer leads to an augmentation of expression of Runx2 and Dlx5 and hence, increased prostate bone metastasis. We also observed repression of Smad4, a central mediator of TGF-β signaling, by miR-203 in bone metastatic prostate cancer cell line. TGF-β plays a dual role in
prostate cancer, being a tumor suppressor in early stages and a tumor promoter in advanced metastatic prostate cancer (41). Smad4 is required for TGF-ß-induced EMT and bone metastasis of breast cancer cells (42, 43). In view of the observed effects of miR-203 on Runx2, Dlx5 and Smad4 along with effects on invasion-metastasis of prostate cancer, it can be envisaged that communication of prostate cancer cells with the bone microenvironment, which is needed for optimal tumor cell growth and metastasis, may be affected in tumors that display attenuated miR-203 expression.

Collectively, the findings of the present study suggest that miR-203 is an ‘anti-metastatic’ miRNA and this miRNA acts at multiple steps of the prostate cancer progression and metastasis cascade via repression of a cohort of prometastatic targets. Advanced metastatic prostate cancer is a fatal phase of the disease, with only palliative therapeutic options, and an area in urgent need of new diagnostic and therapeutic strategies. With the realization of the extreme molecular complexity of advanced prostate cancer, therapeutic strategies are being envisioned to disable multiple networks of tumor maintenance, rather than individual signaling pathways (32). In our present study we identified a miR-203 regulatory network that plays a pivotal role in the progression and metastasis of prostate cancer by affecting an array of effectors. miR-203 potentially targets pleiotropic signaling pathways via these effectors and therefore, we envision that miR-203 may be an attractive target for therapeutic intervention.

**EXPERIMENTAL PROCEDURES**

**Cell culture and miRNA transfection** Non-malignant epithelial prostate cell lines (RWPE-1 and PWR-1E) and prostate carcinoma cell lines (LNCaP, Du145, PC3, VCaP, MDA-PCa-2b) were obtained from the American Type Culture Collection. Cell lines RWPE-1 and PWR-1E...
were cultured in keratinocyte growth medium supplemented with 5 ng/mL human recombinant epidermal growth factor, 0.05 mg/mL bovine pituitary extract (Invitrogen). Prostate cancer cell lines LNCaP, Du145, PC3 were maintained in RPMI 1640 media (UCSF cell culture facility) and VCaP cells were cultured in DMEM media, each supplemented with 10% fetal bovine serum (FBS) (Atlanta biologicals) and 1% penicillin/streptomycin (UCSF cell culture facility). MDA-PCa-2b cells were maintained in BRFF-HPCI (AthenaES), 20% FBS, and 1% penicillin/streptomycin. Cell lines were maintained in an incubator with a humidified atmosphere of 95% air and 5% CO2 at 37°C. For miRNA transfections, cells (early passages) were plated in growth medium without antibiotics ~24hrs before transfections. MicroRNA precursors were purchased from Ambion and transient transfection of miR-203 precursor (PM10152) or negative control (miR-CON) (AM17110) was carried out by using Lipofectamine 2000 (Invitrogen) according to the manufacturers’s protocol. All miRNA transfections were for 72hrs.

Tissue samples

Formalin-fixed, paraffin-embedded (FFPE) prostate cancer samples were obtained from the Veterans Affairs Medical Center (San Francisco). All slides were reviewed by a board certified pathologist for the identification of prostate cancer foci as well as adjacent normal glandular epithelium. Also, prostate adenocarcinoma tissue microarray (US Biomax) consisting of human normal adjacent prostate tissue samples (n =15) and primary prostate cancer tissues of various pathological stages (pT2, n =14; pT3, n=52; pT4, n=6) and metastatic (n =8) carcinomas was used for miR-203 ISH analysis.

Experimental metastasis model
To generate metastases, groups of six male nude mice (strain BALB/c nu/nu; Charles River Laboratories), 4-5 weeks old, received intracardiac injections of $1 \times 10^6$ PC-3M-luc cells overexpressing control miR or miR-203 in a volume of 100 μl. Successful intra-cardiac injections were confirmed by immediate whole body bioluminescence imaging. Development of metastases was monitored via bioluminescence imaging at regular intervals.

**Luciferase Assays**

Control constructs and various 3’-UTR reporter constructs (0.2 ug) were cotransfected into PC3 cells cultured in 24-well plates along with 50nM miR-203 or miR-CON (Ambion) using Lipofectamine 2000 (Invitrogen). 48 hr post-transfection, firefly and renilla luciferase activities were measured by using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. Firefly luciferase was normalized to Renilla luciferase activity.

**Statistical analyses**

Data are represented as mean ± S.E.M. A two-tailed Student’s t-test was used for comparisons, with p < .05 considered significant.

**Supplemental data**

The supplemental data includes supplemental methods and two supplemental figures.

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**REFERENCES**


Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
FIGURE LEGENDS

Fig. 1 miR-203 expression is attenuated in bone metastatic prostate cancer

(A) Quantitative RT-PCR analysis of relative miR-203 expression levels in human prostate cancer and non-malignant cell lines. Data were normalized to the RNA control RNU48 and are represented as mean ± SEM.

(B) In-situ hybridization (ISH) analysis of relative miR-203 expression levels in bone metastatic prostate cancer tissues and normal prostate tissues. Tissues were hybridized with DIG-labeled locked nucleic acid (LNA)-based probes for mir-203 and U6 (control). miR-203 expression was scored and normalized to U6 scores. The average value for each group is represented by the horizontal line.

(C) Representative examples of ISH analysis of miR-203 expression in normal prostate and bone metastatic tissues (left panels). U6 staining confirmed the preservation of intact small RNAs in the same cases (middle panels); H & E stained sections allowed the identification of tumors (right panels).

Fig. 2 miR-203 expression is progressively lost in primary tumors

(A) Quantitative RT-PCR analysis of relative miR-203 expression in LCM-prostate cancer tissues (n=22) and the matched adjacent normal regions.

(B) In-situ hybridization (ISH) analysis of relative miR-203 expression levels in primary prostate cancer and normal prostate tissues. Horizontal line represents the average value for each group.
(C) Representative examples of ISH analysis of miR-203 (left panels) and U6 expression (middle panels) along with H & E stained sections (right panels) in matched normal and primary prostate cancer tissues.

(D) Correlation of miR-203 expression with clinicopathological stage (left panel) and Gleason score (right panel) of prostate cancer tissues used for miR-203 expression analysis.

**Fig. 3** **Reexpression of miR-203 suppresses prostate cancer metastasis**

(A) PC3 cells were mock-transfected or transfected with a control miR (miR-CON) or miR-203 at 50nM concentration for 72 hrs followed by various functional assays. Relative miR-203 expression levels in PC3 cells after transfection with miR-203 precursor as determined by real time PCR.

(B) Morphological alterations in PC3 cells upon miR-203 restoration assessed by phase-contrast microscopy. miR-203 transfected cells display a more rounded, epithelial morphology (right panel) compared to control cells (left panel).

(C) Wound-healing assay after transfection of PC3 cells with miR-CON (left panels) or miR-203 (right panels). Cells were examined by light microscopy at indicated time points.

(D) Transwell-migration assay and (E) Invasion assay after indicated transfections showing that miR-203 reexpression impairs the migratory and invasive behavior of PC3 cells.

(F) Immunoblotting of PC3 cells for epithelial (E-cadherin) and mesenchymal markers (Vimentin, Fibronectin) after the indicated transfections (upper panels). E-cadherin immunostaining (green) and DAPI counterstain (blue) in PC3 cells after transfections with miR-CON or miR-203. Scale bar 10 um (lower panels). miR-203 reexpression causes a reversal of EMT.
(G) miR-203 reexpression suppresses prostate cancer metastasis in vivo. Following intracardiac injection of control miR or miR-203 expressing PC3M-luc cells in nude mice, metastasis was evaluated at the indicated time points by in vivo bioluminescent imaging. Representative bioluminescence images from the two groups are shown. The scale bar on the right is the relative intensity of bioluminescence.

(H) Quantitation of bioluminescence emitted from whole body of mice on day 30. Data represents the mean of each group ±SD.

Fig. 4 miR-203 influences cellular proliferation and apoptosis in prostate cancer cells

(A) Cellular viability assay showing that miR-203 reexpression significantly decreases the viability of PC3 cells (* P< .05).

(B) miR-203 overexpression significantly inhibits the colony formation ability of PC3 cells.

(C) Cell cycle assay in PC3 cells after mock (left panel), miR-CON (middle panel) or miR-203 (right panel) treatments showing induction of G0/G1 cell cycle arrest by miR-203.

(D) Apoptosis assay in PC3 cells showing induction of apoptosis by miR-203 overexpression. The biparametric histogram shows cells in early (lower right quadrant) and late apoptotic states (upper right quadrant). Viable cells are double negative (lower left quadrant).

Fig. 5 Survivin is a direct target of miR-203

(A) Immunoblot for endogenous survivin in PC3 cells that were mock-transfected or transfected with miR-Con or miR-203 for 72 hrs. GAPDH was used a loading control.

(B) Real time PCR showing miR-203 expression decreases survivin mRNA.

(C) Schematic representation of survivin 3’-UTR showing putative miR-203 target site and luciferase activity assay with increasing concentrations of survivin 3’ UTR construct or control luciferase construct contransfected with mock, miR-CON/miR-203. Firefly luciferase values were...
normalized to renilla luciferase activity and plotted as relative luciferase activity (* P< .05 as compared to miR-CON).

(D) Immunofluorescence staining for survivin (red) and α-tubulin (green) in PC3 cells expressing miR-CON (upper panels) or miR-203 (lower panels). Nuclei were counterstained with DAPI (blue). Panels on the right indicate the merged survivin, tubulin and DAPI-stained images.

**Fig. 6 miR-203 regulates a cohort of metastatic genes**

(A) Immunoblot analysis showing that miR-203 introduction leads to decreased protein levels of endogenous Zeb2, Bmi1, Runx2, Dlx5, Smad4, MMP10 and E2F1 protein in PC3 cells. GAPDH was used a loading control.

(B)-(E) Schematic representation of 3’-UTRs of ZEB2, Runx2, Dlx5, Smad4 showing putative miR-203 target site/sites and luciferase activity assays with the indicated 3’ UTR construct or control luciferase construct contranfected with mock, miR-CON/miR-203. Firefly luciferase values were normalized to renilla luciferase activity and plotted as relative luciferase activity (* P< .05 as compared to miR-CON).

(F) RT-PCR analysis of Osteopontin (OP) and Osteocalcin (OC) expression in miR-CON or miR-203 transfected PC3 cells.
Fig. 1  miR-203 expression is attenuated in bone metastatic prostate cancer

(A) Relative miR-203 expression scores of various prostate cancer cell lines. RWPE-1, PWR-1E, PC3, MDA-MB-231, VCaP, DU145, and LNCaP. Significant differences are indicated by asterisks (*) (P < 0.05).

(B) Box plots showing the relative miR-203 expression scores in normal and bone metastatic prostate samples. The mean relative miR-203 expression score is significantly lower in the bone metastatic samples compared to the normal samples (P < 0.0002).

(C) Representative images of miR-203 expression (left), U6 expression (middle), and H&E staining (right) in normal and bone metastatic prostate samples. The miR-203 expression is significantly attenuated in the bone metastatic samples, while the U6 expression remains unchanged.

P=0.0002

Research.
**Fig. 2.** miR-203 expression is progressively lost in primary tumors.

**A**

![Graph showing relative miR-203 expression](image)

**B**

![Box plot showing relative miR-203 expression scores](image)

**C**

- **miR-203**
  - Normal
  - Tumor

- **U6**
  - Normal
  - Tumor

- **H&E**
  - Normal
  - Tumor

**D**

<table>
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<td>pT2c</td>
<td>13 (59)</td>
<td>7/13 (54%) 6/13 (46%)</td>
<td>8</td>
<td>1 (5) 1/1 (100%)</td>
</tr>
<tr>
<td>pT3b</td>
<td>3 (14)</td>
<td>2/3 (67%) 1/3 (33%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissues for in situ hybridization analysis (n=38)</td>
<td></td>
<td></td>
<td>6</td>
<td>1 (3) - 1/1 (100%)</td>
</tr>
<tr>
<td>pT2</td>
<td>7 (19.4)</td>
<td>4/7 (57%) 3/7 (43%)</td>
<td>7</td>
<td>13 (36) 9/13 (69%) 4/13 (31%)</td>
</tr>
<tr>
<td>pT3</td>
<td>21 (56.4)</td>
<td>18/21 (86%) 3/21 (14%)</td>
<td>8</td>
<td>6 (17) 6/6 (100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9</td>
<td>14 (39) 13/14 (93%) 1/14 (7%)</td>
</tr>
</tbody>
</table>
Fig. 3 Reexpression of miR-203 suppresses prostate cancer metastasis

A

Relative miR-203 expression

0
200
400
600
800
1000
1200
1400
1600
1800

Mock miR-CON miR-203

B

miR-CON miR-203

0 hr

miR-CON miR-203

24 hr

48 hr

C

D

P=0.036

E

P=0.005

F

E-cadherin

Vimentin

Fibronectin

GAPDH

G

Days   0           16          23     30

H

Luciferase units

P<0.05

P=0.005

miR-CON miR-203

miR-CON miR-203
Fig. 4. miR-203 influences cellular proliferation and apoptosis in prostate cancer cell lines.

A. Graph showing relative viability (%) over days 0 to 4 for Mock, miR-CON, and miR-203.

B. Images comparing colony formation for Mock, miR-CON, and miR-203, with a graph of average colony number and statistical significance (P=0.006).

C. Table showing cell cycle distribution for Mock, miR-CON, and miR-203:

<table>
<thead>
<tr>
<th></th>
<th>G0/G1</th>
<th>S phase</th>
<th>G2/M</th>
<th>Sub-dip</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>66%</td>
<td>9%</td>
<td>24%</td>
<td>1%</td>
</tr>
<tr>
<td>miR-CON</td>
<td>64%</td>
<td>8%</td>
<td>27%</td>
<td>1%</td>
</tr>
<tr>
<td>miR-203</td>
<td>76%</td>
<td>4%</td>
<td>19%</td>
<td>1%</td>
</tr>
</tbody>
</table>

D. Table showing cell death distribution for Mock, miR-CON, and miR-203:

<table>
<thead>
<tr>
<th></th>
<th>Apoptotic</th>
<th>Early apoptotic</th>
<th>Viables</th>
<th>Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>3%</td>
<td>1%</td>
<td>93%</td>
<td>3%</td>
</tr>
<tr>
<td>miR-CON</td>
<td>3%</td>
<td>1%</td>
<td>93%</td>
<td>3%</td>
</tr>
<tr>
<td>miR-203</td>
<td>19%</td>
<td>5%</td>
<td>72%</td>
<td>4%</td>
</tr>
</tbody>
</table>
Fig. 5: Survivin is a direct target of miR-203

(A) Mock miR-CON miR-203
Survivin
GAPDH

(B) Relative survivin expression

(C) Survivin 3'UTR

(D) miR-CON miR-203
Survivin Tubulin Merge
DAPI
Tubulin Survivin Merge
P=0.01
miR-203 regulates a cohort of metastatic genes

**Fig. 6** miR-203 regulates a cohort of metastatic genes.

**A**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mock</th>
<th>miR-CON</th>
<th>miR-203</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZEB2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bmi1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RUNX2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DLX5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMAD4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2F1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

**Site 1**

5'...CAGUGUCUGGUUAACA... CAUUUCAA... 3'

3' GAUCACCAGGAU-UUGAAAGUG 5'

Runx2 3' UTR

**Site 2**

5'...UGUGUUUGAU-UUGAUAAG... 3'

3' GAUCACCAGGAU-UUGAAAGUG 5'

Runx2 3' UTR

**Site 3**

5'...AGCCACAU-UUAUAAUCCA-UUACAG... 3'

3' GAUCACCAGGAU-UUGAAAGUG 5'

Runx2 3' UTR

**C**

**RUNX2 3' UTR**

<table>
<thead>
<tr>
<th>Site 1</th>
<th>Site 2</th>
<th>Site 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2345-2351)</td>
<td>(2756-2762)</td>
<td>(2893-2899)</td>
</tr>
</tbody>
</table>

**D**

**Site 2**

5'...GCAUGUAAGUUAUUGCAUUUCAA... 3'

3' GAUCACCAGGAU-UUGAAAGUG 5'

Dlx5 3' UTR

**E**

**Site 3**

5'...UAUAAUGGAUAGUAUUUCAC... 3'

3' GAUCACCAGGAU-UUGAAAGUG 5'

Smad4 3' UTR

**F**

**Relative Luciferase Activity**

**Control**  | **ZEB2**  | **Dlx5**  | **Smad4**

**Relative Expression Levels**

**Osteopontin** | **Osteocalcin**

**P=0.006**  | **P=0.007**
Clinical Cancer Research

Regulatory role of miR-203 in prostate cancer progression and metastasis

Sharanjot Saini, Shahana Majid, Soichiro Yamamura, et al.

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