miR-409-3p/-5p Promotes Tumorigenesis, Epithelial-to-Mesenchymal Transition, and Bone Metastasis of Human Prostate Cancer

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Abstract

Purpose: miR-409-3p/-5p is a miRNA expressed by embryonic stem cells, and its role in cancer biology and metastasis is unknown. Our pilot studies demonstrated elevated miR-409-3p/-5p expression in human prostate cancer bone metastatic cell lines; therefore, we defined the biologic impact of manipulation of miR-409-3p/-5p on prostate cancer progression and correlated the levels of its expression with clinical human prostate cancer bone metastatic specimens.

Experimental Design: miRNA profiling of a prostate cancer bone metastatic epithelial-to-mesenchymal transition (EMT) cell line model was performed. A Gleason score human tissue array was probed for validation of specific miRNAs. In addition, genetic manipulation of miR-409-3p/-5p was performed to determine its role in tumor growth, EMT, and bone metastasis in mouse models.

Results: Elevated expression of miR-409-3p/-5p was observed in bone metastatic prostate cancer cell lines and human prostate cancer tissues with higher Gleason scores. Elevated miR-409-3p-5p expression levels correlated with progression-free survival of patients with prostate cancer. Orthotopic delivery of miR-409-3p/-5p in the murine prostate gland induced tumors where the tumors expressed EMT and stemness markers. Intracardiac inoculation (to mimic systemic dissemination) of miR-409-5p inhibitor–treated bone metastatic ARCaPM prostate cancer cells in mice led to decreased bone metastasis and increased survival compared with control vehicle–treated cells.

Conclusion: miR-409-3p/-5p plays an important role in prostate cancer biology by facilitating tumor growth, EMT, and bone metastasis. This finding bears particular translational importance as miR-409-3p/-5p appears to be an attractive biomarker and/or possibly a therapeutic target to treat bone metastatic prostate cancer.
Translational Relevance

Currently, there are limited options for targeted treatment or biomarkers of cancer bone metastasis. In this study, we have identified a novel role for miR-409-3p/-5p in prostate tumor growth, epithelial-to-mesenchymal transition, stemness, and bone metastasis. miR-409-3p/-5p is located in an embryonically regulated cluster and appears to be activated during metastasis. We demonstrate that both miR-409-3p and miR-409-5p are elevated in tumor tissues of prostate cancer patients with high Gleason scores. Using a publicly available database, we observed that elevated miR-409-3p levels correlate with patient disease-free survival. Overexpression of miR-409-3p/-5p in mouse prostate induces tumor growth, and inhibition of miR-409-5p results in decreased bone metastasis in experimental models. Thus, miR-409-3p/-5p, implicated in embryonic development, has an unexpected oncogenic role in prostate cancer bone metastasis, and thus could serve both as a biomarker and as a therapeutic target.

with high-risk prostate cancer compared to patients with low-risk prostate cancer (12). miR379 expression was increased in the tissues of metastatic prostate cancer compared with localized prostate cancer (13). Also, miR379 and miR154 have been shown to be increased in circulating exosomes of patients with lung adenocarcinomas versus healthy smokers (14). In this study, we manipulated miR-409-3p/-5p expression in adult normal prostate and in prostate cancer cells and report a surprising and novel discovery of transforming effects of this miRNA conferring prostate epithelium to undergo EMT, and expressing stemness and tumorigenic phenotypes in mice. Inhibition of miR-409-5p in a human prostate cancer cell line resulted in decreased bone metastasis in vivo.

Materials and Methods

Cell culture

Human androgen-refractory prostate cancer cells (ARCaP_E and ARCaP_0) and LNCaP(ADO) and LNCaP(RANKL) prostate cancer cells (15–17) were used. Prostate cancer cells and 293T cells were cultured in T-medium (GibcoBRL) supplemented with 5% heat-inactivated FBS (Bio-Whittaker), as previously mentioned (18). All cells were tested for mycoplasma every 3 months and were negative. The embryonic stem cells and iPSC-derived small RNA preparations were provided by Drs. Sareen and Clive Svendsen. Derivation of these cells is included in the Supplementary Materials and Methods and Supplementary figure legends.

miRNA expression

Quantitative real-time PCR. miRNA expression analysis by quantitative real-time PCR (qRT-PCR) was performed separately for each miRNA using specific primer sets (Applied Biosystems) as previously described (19). RNU6B was used for normalization.

mRNA analysis. Total RNA was isolated using the RNeasy Mini Kit (Qiagen). cDNA was made using SuperScriptIII Reverse Transcriptase (Life Technologies). mRNA primers were designed and synthesized at Integrated DNA Technologies. mRNA expression levels were determined by qRT-PCR assays and SYBR Green Dye (Applied Biosystems).

Long noncoding RNA analysis

miRNA was extracted as described above. LncRNA expression levels were determined as per manufacturer’s instructions (System Biosciences) using real-time PCR. Relative levels of MEG3 were plotted normalized to GAPDH.

Cytoscape analysis

Cytoscape image was created using miR-409-5p and miR-409-3p target genes from Targetscan v12 software analysis and Genecard website (STRING: functional protein association networks).

In situ hybridization–quantum dots

Human Gleason tissue array. A Gleason score tissue array was obtained from Vancouver Prostate Center. The use of specimens in research was approved by the institutional review board of the Cedars-Sinai Medical Center (IRB# Pro21228). The tissues consisted of benign prostatic hyperplasia (BPH; N = 14), Gleason 6 (N = 26), and Gleason ≥7 (N = 35). Each tissue had two cores in the array. These specimens had no treatment. The tissue array was stained for hematoxylin and eosin (H&E) and graded by a pathologist. Information on Gleason score of the cancer and miR-409 intensity is included in Supplementary Fig. S1. The control scramble and miR-409-5p and -3p probes were 5'-biotin labeled. The probes were linked to streptavidin-conjugated quantum dot (QD). Multiplex QD labeling (mQDL) was performed as previously described (16). miR-409-5p was labeled with 625 nm QD (red) followed by miR-409-3p (green) which was labeled with 565 nm QD (16). The QD fluorescence intensity of each tissue section was determined and analyzed. Statistical analysis was performed on the dataset using a Kruskal–Wallis one-way ANOVA and post hoc Tukey method for multiple comparisons between groups. Data distribution was depicted as box plots.

In vivo animal studies. Mouse tumor and tumor xenografts were formalin-fixed and paraffin-embedded. miRNA in situ hybridization (ISH) protocol was followed as per the manufacturer’s instructions (Exiqon). Single QD labeling was performed as previously mentioned (16). Scramble, miR-409-5p, or miR-409-3p probes were labeled with 625 nm QDs (16). Images were taken at ×40 magnification. H&E staining was performed on subsequent tissue sections.

MSKCC dataset analysis

The dataset was published by Memorial Sloan-Kettering Cancer Center team (MSKCC, New York, NY; ref. 20) and was obtained from cBioPortal (21). miR-409-3p but not miR-409-5p was analyzed in the dataset. For the analysis of miR-409-3p with different Gleason scores, patients with
Gleason score 6 or 7 (n = 86) were grouped together to compare with those with Gleason score 8 or 9 (n = 12). A Student t test was done between the two groups for analysis of differential expression of miR-409-3p between two cohorts. For the survival analysis, the expression levels of miR-409-3p in patients were compared with the median expression level of normal individuals. The disease-free survival of patients with miR-409-3p expression levels higher than normal individual (n = 29) was compared with that with lower miR-409-3p expression levels (n = 78). The Kaplan–Meier survival curve was done by the log-rank test between high- and low-expression groups.

Lentiviral transduction

ARCaPE or LNCaP prostate cancer cell lines were transduced with miR-409 lentivirus expressing GFP or control GFP lentivirus and ARCaPm prostate cancer cell lines were transduced with miR-409-5p lentivirus expressing GFP or control GFP lentivirus. Lentiviral preparation and transduction of cell lines were performed as per the manufacturer’s instructions (System Biosciences). GFP-positive cells were FACSorted and cultured in vitro.

Growth assay, invasion, and migration assays

ARCaPM-C and ARCaPM-409-5pi cells were grown and counted for a week. Cell viability assay was performed using MTS assay as previously mentioned (18). Cancer cell invasion and migration were assayed in Companion 24-well plates (Becton Dickinson Labware) as described previously (22).

Western blotting analysis

Western analysis was performed as previously described (22). The membranes were incubated with mouse monoclonal antibody against STAG2 (Cell Signaling Technology), RSU1 (Proteintech Group), β-actin (Sigma-Aldrich), respectively, at 4 °C overnight.

Xenograft studies

All animal experiments were Institutional Animal Care and Use Committee approved and done in accordance with the institutional guidelines.

Orthotopic study. Preparation of grafts: 293T cells were transduced with either miR-409 expressing lentiviral vector carrying GFP or control vector carrying a GFP plasmid (System Biosciences) viral particles. 293T cells were incubated for 24 hours and the cells were trypsinized. Cell grafts were made by mixing 3 parts of rat-tail collagen and 1.2 parts of setting solution. The mixture was added to the 293T cells. The mixture (6 × 10^5 293T cells) was orthotopically injected into 4-week-old male nude mice (NCRNU, Tacoxnic) prostates (N = 5/group). The control or miR-409 GFP plasmids were expected to be released from the 293T cells and enter the adjacent epithelium and stroma of the mouse prostate. The 293T cells were lysed when the viruses were released. Mice were monitored for miR-409 expression by detecting GFP fluorescence and for tumor growth using near-infrared (NIR) dye (IR783; ref. 23) using the IVIS Lumina Imaging System. Tumors developed from 2 to 6 months in the miR-409 group. Mice were euthanized, and tumors sections were stained for specific markers.

Immunohistochemistry

Immunohistochemical (IHC) staining was performed as previously described (22). The following primary antibodies were used: Ki67 (Abcam), STAG2, p-AKT (Cell Signaling Technologies), RSU1 (ProteinTech Group), Vimentin (V9), Nanog, Oct-3/4, Cytokeratin 5 (Santa Cruz Biotechnology), Cytokeratin 8 (Covance, Inc.) were used. Additional information attached in the Supplementary Materials and Methods and Supplementary Figure legends.

In vivo metastasis study

Luciferase-tagged ARCaPM control and ARCaPM-409-5pi cells were injected intracardially as previously mentioned (24) in male SCID/beige mice (Charles River Laboratories; N = 5/group). Mice were imaged for bioluminescence and X-ray detection using IVIS Lumina Imaging System. Mice were euthanized when they produced large tumors. Mice were given NIR dye (IR783) 48 hours before euthanasia; the tumor-specific NIR dye was used to detect metastatic tumor in the mice.

Statistical analysis

Values were expressed as means ± SD. All experiments were done in triplicate at least two independent times. Statistical analysis was performed using the Student t test. For tissue Gleason score array, the difference between the groups were tested by Kruskal–Wallis one-way ANOVA. A post hoc Tukey method was used to enable multiple comparisons between groups. Values of P < 0.05 were considered to be statistically significant.

Results

miR-409-3p/-5p is overexpressed in bone metastatic EMT models of human prostate cancer

To understand the regulatory role of miRNAs in EMT and prostate cancer bone metastasis, we performed miRNA profiling of two lineage-related, differentially bone metastatic human prostate cancer cell lines, ARCaPm (non-metastatic line) and ARCaPM (metastatic line), denoting, respectively, their epithelial (ARCaPm) and mesenchymal (ARCaPM) phenotype (refs. 15, 25; Supplementary Tables S2 and S3). The differential miRNA expression of the non-metastatic (ARCaPm) and metastatic prostate cancer cells (ARCaPM) are represented in a Supplementary Table S3. We observed markedly upregulated miR-409-3p/-5p expression in the bone metastatic ARCaPM variant (Fig. 1A). miR-409-3p and -5p miRNAs were in the top five of the differentially expressed miRNAs between ARCaPm and ARCaPM prostate cancer cells. We observed a similar increase in miR-409-3p/-5p expression in the LNCaP<sup>NEO</sup> versus LNCaP<sup>ANKL</sup> (16, 17) bone metastasis prostate cancer model (Fig. 1A). Thus, in two different prostate cancer bone metastatic EMT models, we observed an increase in miR-409-3p/-5p. miR-409-3p and -5p are generated from an immature transcript and transcribed from the 5' end of the pre-miRNA. miR-409 is located in a region that overlaps the long non-coding RNA MEG9 (26). The expression levels
of MEG9 lncRNAs hence were elevated in the metastatic ARCaPM prostate cancer cells compared to nonmetastatic ARCaPE prostate cancer cells (Fig. 1B). In addition to bone metastatic human prostate cancer cells, human embryonic stem cells and induced pluripotent cells also notably expressed elevated levels of miR-409-3p/-5p (Fig. 1C and D). Thus, we demonstrate that miR-409-3p/-5p is upregulated in two aggressive, bone metastatic EMT prostate cancer models and in human embryonic stem cells and iPSCs.

**miR-409-3p-5p inhibits tumor suppressor genes in prostate cancer**

Targetscan 6.2 (June 2012) software analysis revealed putative miR-409-5p targets that include tumor suppressor genes like stromal antigen 2 (STAG2), ras suppressor protein 1 (RSU1), retinoblastoma-like 2 (RBL2) and nitrogen permease regulator-like 2 (NPRL2). Predicted mRNA targets of miR-409-3p include polyhometic 3 (PHC3), RSU1, and tumor suppressor candidate 1 (TUSC1). The miR-409-5p and -3p targets were validated by qRT-PCR and were found to be downregulated in metastatic ARCaPM cells that express elevated levels of miR-409-3p/5p compared with ARCaPE cells that express lower levels of miR-409-3p/5p (Fig. 2A). We demonstrated that miR-409-5p binds the 3’UTR of STAG2 and RSU1 (Supplementary Fig. S1B and S1C). In addition, the binding sites of miR-409-5p and miR-409-3p on RSU1 3’UTR are indicated in Supplementary Fig. S1A. Using gene cards and string interactions, we created a cytoscape map of the possible human cancer pathways regulated by miR-409-5p and miR-409-3p that would account for its activity in cells. miR-409-3p is predicted to activate the Ras signaling pathway and the hypoxia-inducible factor-1α pathway, and regulate polycomb group proteins and osteoblastic pathways (Fig. 2C). miR-409-5p is predicted to activate the E2F pathway, Akt pathway, and aneuploidy (Fig. 2D). Taken together, we demonstrate that miR-409-3p-5p is elevated in the bone metastatic EMT cell models and it functions by repressing several tumor suppressor genes.

**Human prostatic tissues with higher Gleason score and prostate cancer bone metastasis tissues express elevated levels of miR-409**

To validate our findings in clinical samples, we determined the levels of miR-409-3p-5p in human prostate tissues with various Gleason scores using ISH and multiplexed QD labeling. The miRNA probes were biotin-labeled (Exiqon) and further labeled to a streptavidin-conjugated...
QD at a specified wavelength. miR-409-3p/-5p was detected both in the tumor tissues. The tissues were separated into three groups, BPH (N = 14), Gleason 6 (N = 26), and Gleason ≥7 (N = 35). Tumors with higher Gleason ≥7 had significantly higher miR-409-3p and miR-409-5p staining in the tumor areas compared with the tissues with BPH. miR-409-3p was significantly higher in the Gleason ≥7 compared with Gleason 6 (Fig. 3A), as analyzed by Kruskal–Wallis one-way ANOVA-Tukey method. A representative image of Gleason 8 shows increased staining of miR-409-3p (green) and -5p (red) in prostate cancer tissues (Fig. 3B). We used a dataset published by MSKCC (20) to determine the miR-409-3p expression in different Gleason score tissues in Fig 3C. The miR-409-3p expression levels were compared between Gleason_low (Gleason 6, 7; n = 86) and Gleason_high (Gleason 8, 9; n = 12) groups (Fig. 3C). miR-409-3p expression was significantly elevated in higher Gleason tissues compared with low Gleason tissues, consistent with our own staining data (P = 0.0151). The miR-409-5p expression was not provided in this dataset. Furthermore, we analyzed the survival of this patient cohort based on their miR-409-3p expression level (Fig. 3D). The patients were separated into two groups based on their miR-409-3p expression levels relative to the normal samples. We found that the patients with higher miR-409-3p than normal sample were correlated with poor progression-free survival (P = 4.32 × 10⁻⁵). This suggests the miR-409-3p is clinically relevant in prostate cancer.

Figure 2. miR-409 inhibits tumor suppressor genes in prostate cancer. A, mRNA targets of miR-409-5p: STAG2, RBL2, RSU1, and NPRL2 and mRNA targets of miR-409-3p: RSU1, PHC3, and TUSC1, assayed by triplicate wells in qRT-PCR of ARCaPE and ARCaPM cells. The representative RT-PCR is shown. The experiment was repeated twice, *P < 0.05 was considered statistically significant by t test; ***, P < 0.0001. B, Western blot analysis of STAG2 and RSU1 in ARCaPE and ARCaPM prostate cancer cells. C and D, cytoscape images of the miR-409-3p and miR-409-5p signaling pathways.
Collectively, these results demonstrate that miR-409 expression correlated with higher Gleason score in prostatic tissues and progression-free survival of patients, possibly linking miR-409 expression with tumor progression.

**Ectopic expression of miR-409-3p/-5p leads to increased invasiveness and aggressiveness of prostate cancer cells, and conversely, inhibition of miR-409-3p/-5p results in increased cell death in prostate cancer cells**

To determine the effects of miR-409-3p/-5p action in prostate cancer, we ectopically introduced this miRNA in less aggressive epithelial-type ARCaPE cells and LNCaP cells. A significant increase in miR-409-3p/-5p expression was confirmed using qRT-PCR (Fig. 4A and Supplementary Fig. 2A). The mRNA expression of target genes of miR-409-3p/-5p was determined using qRT-PCR. We report that miR-409-5p target mRNAs (STAG2, RSU1, RBL2, and NPRL2) were decreased in ARCaPE cells that overexpress miR-409 (ARCAPC-409) compared with the control miRNA-treated cells (Fig. 4B). Two of the three mRNA targets of miR-409-3p were also decreased in ARCAPC-409 cells compared with control (RSU1 and TUSC1), but not PHC3 (Fig. 4B). Moreover, ARCAPC-409 cells showed increased migratory and invasive capacity compared with control prostate cancer cells (Fig. 4C).

On the contrary, inhibition of miR-409-3p in ARCAPM prostate cancer cells using a shRNA inhibitor resulted in cell death of prostate cancer cells and hence further experiments could not be carried out due to complete lethality of the cells in vitro. Inhibition of miR-409-5p using shRNA resulted in cell death of aggressive metastatic prostate cancer cells (Fig. 4D) compared with the control scramble miRNA-expressing cells. We generated stable lentiviral...
clones of ARCaPM prostate cancer cells expressing miR-409-5p inhibitor (ARCaPM-409-5pi). ARCaPM-409-5pi prostate cancer cells had a decreased growth rate compared with ARCaPM-C cells (Fig. 4D). ARCaPM-409-5pi cells had decreased miR-409-5p levels compared with ARCaPM-C cells (Fig. 4E). Next, we measured the levels of mRNA targets of miR-409-5p, which include STAG2, RPL2, NPRL2, and RSU1 (miR-409-5p mRNA targets: RSU1, PH3C, and TUSC1). C, invasion and migration assay of ARCaPM-C and ARCaPM-409-expressing prostate cancer cells. D, cell viability in ARCaPM prostate cancer cells in response to a miR-409-5p inhibitor. Growth curve of ARCaPM-C and ARCaPM-409-5pi prostate cancer cells. E, expression of miR-409-5p assayed by qRT-PCR in ARCaPM-C control prostate cancer and ARCaPM-409-5pi (miR-409-5p inhibitor-transfected cells). F, RNA expression of miR-409-5p targets in ARCaPM-C control and ARCaPM-409-5pi cells assayed by qRT-PCR (miR-409-5p mRNA targets: NPRL2 and STAG2). G, protein expression of STAG2 and RSU1 in ARCaPM-C cells and ARCaPM-409-5pi cells.

Role of miR-409 in Prostate Cancer Bone Metastasis

Ectopic expression of miR-409 leads to increased invasiveness and aggressiveness of prostate cancer cells, and conversely, inhibition of miR-409 results in increased cell death in prostate cancer cells. A, miR-409-5p and -3p expression by qRT-PCR in ARCaPM-C and ARCaPM-409-expressing prostate cancer cells. B, RNA expression of miR-409-5p/-3p targets in ARCaPM-C and ARCaPM-409-expressing prostate cancer cells assayed by real-time PCR. (miR-409-5p mRNA targets: STAG2, RPL2, NPRL2, and RSU1; miR-409-3p mRNA targets: RSU1, PH3C, and TUSC1). C, invasion and migration assay of ARCaPM-C and ARCaPM-409-expressing prostate cancer cells. D, cell viability in ARCaPM prostate cancer cells in response to a miR-409-5p inhibitor. Growth curve of ARCaPM-C and ARCaPM-409-5pi prostate cancer cells. E, expression of miR-409-5p assayed by qRT-PCR in ARCaPM-C control prostate cancer and ARCaPM-409-5pi (miR-409-5p inhibitor-transfected cells). F, RNA expression of miR-409-5p targets in ARCaPM-C control and ARCaPM-409-5pi cells assayed by qRT-PCR (miR-409-5p mRNA targets: NPRL2 and STAG2). G, protein expression of STAG2 and RSU1 in ARCaPM-C cells and ARCaPM-409-5pi cells.

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producer cells, transfected with the miR-409-expressing lentiviral vector carrying GFP or control vector carrying a GFP plasmid, orthotopically into the prostate gland of athymic nude mice (N = 5/group). Tumor development was monitored using the tumor-specific NIR dye (IR783; ref. 23). The rationale behind this procedure is that the lentivirus will be secreted by the producer cells (293T) and infect prostate epithelial and/or stromal cells in vivo. Strikingly, prostate tumors developed in 2 to 5 months in 3 of 5 mice that received the producer cells transfected with miR-409 (Fig. 5A). Mice that were implanted with producer cells expressing control lentiviral plasmid did not develop any tumors in the prostate. The tumors had green fluorescence and showed tumor-specific NIR dye uptake (IR783; Fig. 5A). H&E staining of tissue sections revealed tumors ranging from prostatic interstitial neoplasia, basal cell hyperplasia, and adenocarcinoma in the miR-409 prostates (Fig. 5B). The tissue sections were also analyzed for miR-409-3p/-5p levels using ISH-QD labeling. miR-409-3p and miR-409-5p expression was observed only in miR-409-expressing prostates in tumor cells but not in control prostates (Fig. 5B). Levels of miR-409 expression appear to correlate with the overall size of the tumors. IHC staining revealed elevated expression of tumor proliferation markers such as Ki67 and oncogenic kinases like p-AKT (Fig. 5C), downregulated expression of STAG2 and RSU1, and upregulated expression of mesenchymal markers, such as vimentin, when compared with the control prostate gland (Fig. 5C). IHC staining of orthotopic tumors revealed positive staining of Oct-3/4 (strong nuclear staining) and Nanog (weak nuclear staining), both of which are stem cell markers, in both the epithelial and the stromal compartment of miR-409-expressing neoplastic prostates (Supplementary Fig. S3). Strikingly, in the epithelial compartment, both the basal and luminal cells in the prostate underwent proliferation, as exhibited by strong Ki67 staining, in response to uptake of miR-409-3p/-5p, with cytokeratin 5, representing the basal cell marker and cytokeratin 8, representing the luminal cell marker (Supplementary Fig. S3). Taken together, these studies suggest that, miR-409-3p/-5p is oncogenic and its expression is sufficient to drive tumorigenesis of the adult normal prostate gland.
Inhibition of miR-409-5p results in decreased bone metastasis of aggressive prostate cancer in vivo

Because the inhibition of miR-409-3p using a shRNA inhibitor resulted in complete cell lethality, further experiments could not be carried out. Inhibition of miR-409-5p in ARCaPM cells resulted in reversal of EMT (MET, Fig. 6A), accompanied by an increase in E-cadherin expression and a decrease in N-cadherin expression and epithelial morphologic changes (Fig. 6A). Inversely, overexpression of miR-409 in ARCaPM and LNCaP resulted in decreased E-cadherin expression (Supplementary Fig. S2C). Knocking down miR-409-5p also resulted in moderate decrease in migration and invasion of cancer cells (Fig. 6A). To determine whether miR-409 plays a role in cancer metastasis, we inoculated viable ARCaPM-C control cells or viable ARCaPM-409-5pi cells via the intracardiac route into SCID/Beige mice (N = 5/group) to mimic in vivo metastasis. Mice that received ARCaPM-C cells had 100% incidence of bone metastasis, whereas mice that received ARCaPM-409-5pi cells did not develop any metastasis at 15 weeks. The luciferase-tagged cancer cells were imaged by luciferase imaging (Fig. 6B). The survival of the ARCaPM-C and ARCaPM-409-5pi injected mice is depicted as a Kaplan–Meier curve, where majority (4 of 5) of control mice died by 15 weeks but not ARCaPM-409-5pi–injected mice (Fig. 6C). Using X-ray imaging, we observed bone metastatic tumor sites in tibia, femur, mandible, and humerus (Fig. 6D). Each mouse developed 1 to 5 metastatic tumors in the control group, detected by IR783 imaging and confirmed by luciferase imaging (Fig. 6E). X-ray imaging of mice inoculated with ARCaPM-409-5pi revealed no evidence of bone lesions consistent with the lack of luciferase signals (Fig. 6B and data not shown). Thus, inhibition of miR-409-5p induced MET and significantly abrogates the metastatic potential of
metastatic prostate cancer cells in vivo. Taken together, these studies demonstrate that miR-409 is associated with bone metastasis of human prostate cancer cells in mouse models.

Discussion

To understand the biology of noncoding RNAs in EMT and cancer bone metastasis and to identify novel biomarkers and/or therapeutic targets, we profiled miRNAs in unique EMT models of human prostate cancer developed in our laboratory. miR-409-3p/-5p, located within the DLK1-DIO3 cluster was highly upregulated in two prostate cancer cell lines with mesenchymal phenotype and with bone metastatic potential (Fig. 1). The miRNA members of the DLK1-DIO3 cluster have been shown to be important for totipotency during embryogenesis and induced pluripotent stem cell formation. We report an unexpected discovery of the oncogenic role of miR-409-3p/-5p, which is expressed by embryonic stem cells and pluripotent stem cells, to promote prostate cancer development and metastasis. Specifically, we showed that (i) miR-409-3p/-5p is elevated in human prostate cancer tumor tissues and correlates with prostate cancer patients progression-free survival, (ii) miR-409-3p/-5p can transform normal mouse prostate epithelium to exhibit tumorigenic phenotype and promote the growth and invasion of human prostate cancer cells by downregulating tumor suppressor genes in vitro and in vivo, (iii) miR-409-3p/-5p can promote EMT and stemness of prostate epithelium in vivo, and (iv) inhibition of miR-409-5p results in decreased bone metastatic tumor growth and increase in survival. Thus, miR-409 appears to be a promising new biomarker for cancer detection and an attractive new therapeutic target for prostate cancer treatment.

Because inhibition of miR-409-3p resulted in cell lethality, further studies in the future will require the use of inducible systems. miR-409 appears to mediate its tumorigenic effects through targeting of tumor suppressor genes (Figs. 2, 4, and 5). One such target gene of miR-409-3p and -5p is RSU1. Previous studies have shown that RSU1 protein blocks the oncogenic Ras/MAPK pathway and the integrin-linked kinase (ILK) pathway in prostate cancer (27–29). Another target gene for miR-409-5p appears to be STAG2. In the tumor cells, STAG2 is a part of the cohesion complex, where deregulation of the cohesion complex is thought to cause aneuploidy, cancer initiation, and progression (30, 31). miR-409-5p also appears to target NPR2, a tumor suppressor protein decreased in solid tumors (32–34). There are differences in the genes targeted by miR-409-3p and miR-409-5p. At the same time, they do share some similar targets. Thus, miR-409-3p and miR-409-5p could be considered as distinct miRNAs with some shared functions.

Orthotopic delivery of miR-409-3p/-5p in mouse prostate resulted in adenocarcinoma as well as prostatic hyperplasia. This dual phenotype could be attributed to difference in uptake of levels of miR-409-3p/-5p by the mouse prostate. miR-409-3p was found to be elevated in the serum of patients with prostate cancer with high Gleason score (12). Consistently, we found that the metastatic ARCaPM cells secrete higher levels of miR-409 and inhibition of miR-409-5p in these cells decreases this process (Supplementary Fig. S4). Our metastatic model involves injection of cells into the blood stream and hence sites of tumor formation could be sites that permit tumor growth, and in our study it is the bone. Hence, future studies will require implantation of ARCaPM-409-5p cells in the prostate and study their bone metastatic ability.

Our data suggest that miR-409-3p and -5p are elevated in the tumor tissues of prostate cancer and can predict poor prognosis and prostate cancer patient progression-free survival. It was also observed that miR-409-3p and miR-409-5p colocalized with higher Gleason score compared with low Gleason score (data not shown). Thus, both the miRNAs are active in more aggressive cancer and together induce tumorigenesis. Inhibition of miR-409-5p in vitro resulted in decreased growth and MET and, this was extended in the in vivo setting in which miR-409-5p cells did not grow, thus inhibiting the metastatic ability of highly aggressive bone metastatic prostate cancer cells in vivo (Fig. 6).

In summary, our study demonstrates the oncogenic roles of miR-409-3p/-5p, which is capable of promoting the malignant transformation of prostate epithelium in mice, including EMT, stemness, and bone metastasis. Therefore, miR-409-3p/-5p may be a new biomarker and a therapeutic target for the treatment of prostate cancer bone metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Josson, M. Gururajan, P. Hu, C. Shao, G.-C.-Y. Chu, H.E. Zhu, C. Liu, J. Lichterman, E.M. Posadas, D. Sareen


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