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miR-154* and miR-379 in the DLK1-DIO3 microRNA mega-cluster regulate epithelial to mesenchymal transition and bone metastasis of prostate cancer

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Abstract

Purpose—MicroRNAs in the delta-like 1 homolog - deiodinase, iodothyronine 3 (DLK1-DIO3) cluster have been shown to be critical for embryonic development and epithelial to mesenchymal transition (EMT). DLK1-DIO3 cluster miRNAs are elevated in the serum of metastatic cancer patients. However, the biological functions of these miRNAs in the EMT and metastasis of cancer cells are poorly understood. We previously demonstrated the oncogenic and metastatic role of miR-409-3p/5p, a member of this cluster, in prostate cancer (PCa). In this study, we defined the role of miR-154* and miR-379, two key members of this cluster, in PCa progression and bone metastasis in both cell line models and clinical specimens.

Experimental design—Genetic manipulation of miR-154* and miR-379 was performed to determine their role in tumor growth, EMT and bone metastasis in mouse models. We determined the expression of miR-154* in prostate cancer clinical samples and bone metastasis samples using in situ hybridization and quantum dot labeling.

Results—Elevated expression of miR-154* and miR-379 was observed in bone metastatic PCa cell lines and tissues, and miR-379 expression correlated with PCa patient progression-free survival. Intracardiac inoculation (to mimic systemic dissemination) of miR-154* inhibitor-treated bone metastatic ARCaP_M PCa cells in mice led to decreased bone metastasis and increased survival.

Conclusion—miR-154* and miR-379 play important roles in PCa biology by facilitating tumor growth, EMT and bone metastasis. This finding has particular translational importance since miRNAs in the DLK1-DIO3 cluster can be attractive biomarkers and possible therapeutic targets to treat bone metastatic PCa.

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Keywords

miR-154*; miR-379; epithelial to mesenchymal transition; DLK1-DIO3 cluster

INTRODUCTION

Cancer cells often undergo epithelial to mesenchymal transition (EMT) prior to metastasizing to distant organs. Cancer cells activate embryonic programs and pathways that partially maintain stem cell identity, often referred as “stemness”. Recent studies suggest roles for microRNAs in both metastasis and cancer stem cell formation (1, 2). The microRNA members of the delta-like 1 homolog- deiodinase, iodothyronine 3 (DLK1-DIO3) clusters are activated in embryonic stem cells. During mouse embryogenesis, the microRNA (miRNA) members of the DLK1-DIO3 cluster have been shown to be elevated in the developing central nervous system, skeletal muscle, liver and lung (3). Similarly, several transgenic mouse models of prostate, liver and lung cancer also exhibit elevated levels of DLK1-DIO3 cluster miRNA members (4–8). The functions of many of these miRNAs within this imprinted region remain largely unexplored in cancer biology, specifically for EMT and cancer stemness.

Members of the DLK1-DIO3 cluster have been recently shown to be upregulated in the serum of prostate cancer (PCa) and lung cancer (LCa) patients. Members of this cluster, including miR-379, miR-154* and miR-409-3p, show increased levels in the circulating exosomes of patients with lung adenocarcinomas (9) and breast (10) and prostate (11) cancers. Our recent studies demonstrate the importance of miR-409-3p/5p in PCa growth, EMT and bone metastasis (12). In this study, we studied the role of miR-154* and miR-379, two key DLK1-DIO3 cluster miRNAs, in several EMT models of human PCa. We demonstrated that these miRNAs modulate EMT and drive metastasis. Thus, these miRNAs are promising therapeutic targets for aggressive PCa.

METHODS

Cell culture

Three human PCa bone metastatic progression models, ARCaP_E and ARCaP_M (13), LNCaP and C4-2 (14), were used in our study. PCa cells and 293T cells were cultured in T-medium (GibcoBRL) supplemented with 5% heat inactivated fetal bovine serum (Bio-Whittaker) with 5% FBS and 1% penicillin-streptomycin. All cells were tested for mycoplasma every three months and were negative. Small RNA preparations derived from embryonic stem cells and induced pluripotent stem cells (iPSC) were provided by Drs. Sareen and Svendsen of the Regenerative Medicine Institute at Cedars-Sinai Medical Center. Generation and characterization of these cells are described in the supplementary materials and methods section.

miRNA expression

Quantitative Real Time PCR (qRT-PCR)—Cells were trypsinized and total miRNA was extracted using a mirVana miRNA isolation kit (Ambion). miRNA expression analysis

by qRT-PCR was performed separately for each miRNA using specific primer sets (Applied Biosystems) as previously described (15). RNU6B was used for normalization.

mRNA analysis—Total RNA was isolated from confluent monolayers of cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was made using Superscript®III reverse transcriptase (Life Technologies, Grand island, NY). mRNA primers were designed and synthesized at Integrated DNA Technologies (Coralville, Iowa). mRNA expression levels were determined by qRT-PCR assays and SYBR Green Dye (Applied Biosystems). Samples were analyzed using the C_t method and normalized to 18S ribosomal RNA.

MSKCC database analysis

The dataset was published by the MSKCC team (16) and was obtained from cBioPortal (17). The expression levels of miR-154 and miR-379 were analyzed along with the survival data in the dataset. For the survival analysis of miR-379, the expression levels of miR-379 in patients with non-metastatic disease were compared with the median expression level of normal individuals. The disease-free survival of patients with miR-379 expression levels higher than normal individuals (n=29) was compared with those with lower miR-379 expression levels (n=78). A Kaplan-Meier survival curve was done by log-rank test between high and low expression groups. The expression levels of miR-154* were not available in the dataset. For the analysis of miR-154, the expression levels of normal healthy individuals (n=29) were compared with the expression levels of primary (n=99) and metastatic PCa patients (n=14). Two-tailed student's t tests were done between the normal group and two primary and metastatic groups for analysis of the differential expression of miR-154.

3' UTR assay

Stromal antigen 2 (STAG2) mutant luciferase activity—3' UTR STAG2 luciferase construct (Switchgear genomics) was used as the wild type (WT) construct and it was further mutated as described below. miR-154* mimic and control miRNA were transiently transfected along with the WT or mutant (STAG2) construct into these 293T cells and luciferase activity was determined 24 h later using the Lightswitch luciferase assay system (Switchgear Genomics).

3' UTR Mutant constructs—Mutated 3' UTR luciferase constructs were produced by sited-directed mutagenesis. Briefly, primer pairs with two sequential base pair mutations in the miRNA seed sequence of the 3' UTR were generated. Following polymerase chain reaction amplification, parental methylated template DNA was digested for 1 hour with *Dpn* I. 2 μ l of the reaction was then transformed into XL-10 Gold bacteria. 16 hours post-transformation, colonies were picked for liquid culture. Plasmid DNA was isolated by the Zippy Plasmid Miniprep Kit according to the manufacturer's directions (Zymo Research). Mutations were confirmed by sequencing before proceeding with luciferase assays.

Primers

miR-154* Stag2 1	aactagaactgctgagaggactgtatatacaattttaaacctaagttgattttttctc
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miR-154* Stag2 2	gagaaaaaaatcaacttaggtttaaattgtatatacagtcctctcagcagttctagt
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Lentiviral transduction

ARCaP_E PCa cell lines were transduced with lentivirus expressing control or miRZip-154* (miR-154*i) or miRZip-379 (miR-379i) (System Biosciences) or cluster overexpression plasmid (custom made, miR-154*, miR-379, miR-409-3p/-5p) with green fluorescent protein (GFP) or control GFP plasmid. ARCaP_M PCa cell lines were transduced with lentivirus expressing cluster inhibitor plasmid (custom made) with GFP. Lentiviral preparation and transduction of cell lines were performed per the manufacturer's instructions (System Biosciences, Mountain View, CA). GFP positive cells were FACS sorted and cultured *in vitro* before experiments were performed.

Cell viability assay and invasion assays

Cell viability assays were performed using trypan blue staining. Cancer cell invasion was assayed in Companion 24-well plates (Becton Dickinson Labware) with 8 µm porosity polycarbonate filter membranes as described previously (18).

Western analysis

Whole cell lysates from cell lines were prepared using a modified RIPA lysis buffer (50 mM Tris HCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 10% glycerol) supplemented with 1:100 dilution of the protease inhibitor cocktail and the tyrosine phosphatase inhibitor (Sigma). Proteins were then separated on 4–20% or 10% acrylamide gels and transferred to nitrocellulose membrane. Membranes were probed with STAG2 (Cell Signaling Technology) antibody. β-actin (Sigma) was used as the normalization control.

In situ hybridization (ISH)-Quantum dots (QD)

Mouse tibia was formalin-fixed and paraffin-embedded. The miRNA ISH protocol was followed per the manufacturer's instructions (Exiqon, MA). The scramble and miR154* probes were 5'-biotin labeled. The probes were linked to streptavidin-conjugated QD. Tissue sections were deparaffinized, treated with proteinase-K and dehydrated. ISH was performed for 1 h at 55°C, followed by washes and streptavidin blocking and a reaction with streptavidin-conjugated QD at a specified wavelength. The QD staining procedure was followed as previously described (19). Single QD labeling was performed and scramble or miR-154* probes were labeled with 625 nm QDs. Images were taken at 40×. H&E staining was performed on subsequent tissue sections.

Human tissue array—A Gleason score tissue array was obtained from Vancouver Prostate Center. The use of tissue specimens was approved by the institutional review board of the Cedars-Sinai Medical Center (IRB# Pro21228). The tissues consisted of BPH (N=4), Gleason score 6 (N=12) and Gleason 7 (N=7). Each tissue had two sample cores. The tissue array was stained for H&E and graded by a pathologist to confirm the Gleason score. Single QD labeling was performed as previously mentioned (19). miR-154* was labelled with 625

nm QD and signals were quantified as previously mentioned (19). The QD fluorescence intensity of each tissue section was determined and analyzed. Human prostate cancer bone tissues were stained following the same procedure, except multiplexed ISH-QD was performed, where miR-154* (red) was stained first followed by miR-409-3p (green) or miR-409-5p (green) which was labeled with 565 nm QD.

In vivo metastasis study—All animal experiments were IACUC approved and done in accordance with institutional guidelines. Luciferase tagged ARCaP_M control and ARCaP_M-154*ⁱ cells were injected intra-cardially as previously described (20) in SCID/beige mice (Charles River Laboratories) (N=5). Mice were imaged using X-ray and bioluminescence via the IVIS® Lumina Imaging system. Mice were given NIR dye (IR783) 48 h before euthanasia. The tumor-specific NIR dye was used to detect metastatic tumors in the mice.

Statistical analysis

Values were expressed as means ± standard deviation. All experiments were done in triplicates at least two independent times. Statistical analysis was performed using Student's t-test or ANOVA. Values of p<0.05 were considered to be statistically significant.

RESULTS

miR-154* and miR-379 of the DLK1-DIO3 cluster are overexpressed in bone metastatic EMT models of human PCa

We determined the levels of miR-154/154* and miR-379 in two bone metastatic models of PCa, ARCaP and LNCaP. Mesenchymal-type ARCaP_M cells upon intracardiac inoculation or orthotopic implantation have 100% bone metastatic capability compared to their isogenic epithelial-type counterpart ARCaP_E cells (13). Similarly, C4-2 PCa cells have a high metastatic ability compared to their lineage-related LNCaP cells (21). Both miR-154* and miR-379 were elevated in ARCaP_M cells compared to ARCaP_E cells, and in C4-2 cells compared to LNCaP cells (Figure 1A). We determined the expression of miR-154* in intra-tibial prostate cancer bone metastasis tissues by ISH-QD and observed higher expression in the metastatic prostate cancer cells (Figure 1B). Previous studies demonstrated that several members of the DLK1-DIO3 miRNA cluster are elevated in human embryonic stem cells and induced pluripotent cells (iPSCs) (22, 23). We measured the relative levels of these miRNAs in H9 embryonic stem cells and in iPSCs. Both H9 human embryonic stem cells and patient-derived iPSC cells had elevated expression of miR-154* (Figure 1C and 1D) but not miR-379 (23). We previously demonstrated that one of the predominant signaling pathways activated in PCa bone metastasis is the β 2-microglobulin/HFE pathway (20, 24). Inhibition of either of these proteins results in reversal of EMT (20). We observed that ARCaP_M HFE knockdown cells (ARCaP_M KD^{HFE1} cells) had significantly decreased expression of miR-154* and miR-379 similar to the epithelial ARCaP_E PCa cells (Figure 1E). Additionally, other members, such as miR-409-3p/-5p of the DLK1-DIO3 cluster, were also decreased in ARCaP_M HFE knockdown cells. These data together demonstrate that miR-154* and miR-379 are elevated in PCa EMT and bone metastatic models.

Inhibition of miR-154* or miR-379 results in reversal of EMT (MET) of PCa cells

To test the hypothesis whether miR-154* and miR-379 is involved in PCa EMT, we transiently depleted miR-154* and miR-379 in ARCaP_M and C4-2 cells using siRNA and determined the cell viability by the trypan blue exclusion test. Both miR-154* knockdown cells and miR-379 inhibited cells underwent increased cell death compared to control transfected cells in both of the PCa cell line models studied (Figure 2A). Next, we introduced a shRNA in the mesenchymal-type ARCaP_M PCa cells to generate miR-154* knockdown (ARCaP_M-154*i) or miR-379 knockdown (ARCaP_M-379i) cells. In addition we used a scrambled shRNA to generate a control shRNA vector (ARCaP_M-C). Reduced expression of miR-154* was detected in the ARCaP_M-154*i cells by qRT-PCR analysis compared to the shRNA control vector ARCaP_M-C. Reduced expression of miR-379 was also observed in the ARCaP_M-379i cells by qPCR analysis compared to the ARCaP_M-C cells (Figure 2B). RNU6B was used for normalization. The miR-154* target gene, stromal antigen 2 (STAG2), was measured in the knockdown cells (TargetScan v6.2). STAG2 is a tumor suppressor protein (25). The mRNA and protein expression of STAG2 was increased in the ARCaP_M-154*i cells compared to the control ARCaP_M cells (Figure 2C). We further tested if miR-154* directly binds to the 3' UTR of STAG2 using a luciferase reporter assay. Compared to control miRNA treated cells, miR-154* mimic-treated cells had reduced basal luciferase activity (Figure 2D). We mutated the 3' UTR of STAG2 at the miR-154* binding site and demonstrated restoration of luciferase activity in response to the miR-154* mimic (Figure 2D). Interestingly, inhibition of miR-154* or miR-379 led to reversion of mesenchymal ARCaP_M cells to an epithelial phenotype (Figure 2E) accompanied by increases in E-cadherin mRNA expression in ARCaP_M-154*i and ARCaP_M-379i cells but not in ARCaP_M-C cells (Figure 2F). To determine if functional reversal of EMT (MET) occurred in addition to morphological changes, we performed invasion assays on these cell lines. ARCaP_M-154*i and ARCaP_M-379i cells had significantly decreased invasive capacity compared to ARCaP_M-C cells (Figure 2G). Taken together, these results demonstrate that miR-154* and miR-379 play an important role in the EMT and invasive capacity of PCa cells.

Inhibition of miR-154* results in decreased bone and soft tissue metastasis of PCa cells

Cancer cells gain their metastatic potential by undergoing EMT. Previous studies from our laboratory using the ARCaP model demonstrate the close association between EMT and PCa bone metastasis (26). Since miR-154* levels are elevated in metastatic cancer cells, we determined whether inhibition of miR-154* would lead to decreased metastasis *in vivo*. Consistent with our hypothesis, we found that inhibition of miR-154* resulted in increased cell death, mesenchymal to epithelial transition (MET) and decreased invasion *in vitro*. To determine if miR-154* plays a role in cancer metastasis *in vivo*, we inoculated luciferase-tagged ARCaP_M-C control cells or ARCaP_M-154*i (miR-154* inhibited) cells via the intracardiac route into SCID/Beige mice (N=5/group) to mimic *in vivo* metastasis. Tumor growth was monitored by luciferase imaging. Mice that received ARCaP_M-154*i cells had a significantly decreased incidence of metastasis (0/5) compared to mice that received ARCaP_M-C control cells (4/5) at 15 weeks post-inoculation. X-ray and luciferase imaging of representative mice from both groups are shown in Figure 3A. The tumors were detected by

IR783 (near infrared dye) in all mice (Figure 3A). The control mice developed metastatic tumors at 1–5 sites in the body, while ARCaP_M-154*_i injected mice did not develop any tumors. Bone tumors sites included the tibia, femur, humerus and mandible, and had mixed osteoblastic and osteolytic lesions (27). Mice inoculated with ARCaP_M-C control cells had decreased survival compared to those inoculated with ARCaP_M-154*_i cells as shown in the Kaplan Meier survival curve (Figure 3B). Taken together, these studies demonstrate that miR-154* is essential for the development of bone metastasis of human PCa cells and that knockdown of miR-154* reduces bone metastasis and increases the survival of mice.

Elevated expression of miR-154* and miR-379 in human PCa clinical samples

We next determined the expression of miR-154* in human PCa tissues using in situ hybridization and quantum dot analysis. miR-154* probes were biotin-labeled (Exiqon) and further labeled to a streptavidin conjugated QD at a specified wavelength. The tissues were separated into three groups, benign prostatic hyperplasia (BPH) (N=4), Gleason 6 (N=12) and Gleason 7 (N=7). Each tissue sample has two sections. Gleason 7 tumor tissues had significantly higher staining of miR-154* compared to BPH (Figure 3C). A representative image of Gleason 7 demonstrates higher staining in the tumor tissues (Figure 3C). We also determined the expression of miR-154* in other cancers and found increased expression in other urological cancers (Supplementary Figure S1). Using the publicly available database (MSKCC), we demonstrated that elevated expression of miR-379 is associated with progression-free survival and miR-154 (the opposite strand of miR-154*) is downregulated in both primary and metastatic PCa tissues compared with normal individuals (Figure 3D & E) (28–30). These results are consistent with previous studies demonstrating that miR-154 is decreased in prostate cancer cell lines and clinical samples (28–30). These databases did not have information on miR-154*. Nevertheless, our observations in bone metastatic PCa cell line models are clinically relevant and our experimental observations correlated well with tumor progression. These results collectively demonstrate that miR-154* and miR-379 are highly expressed in PCa and miR-379 expression correlates with progression-free survival in patients.

Overexpression of miRNA members of the DLK1-DIO3 cluster promotes EMT in PCa cells

Previously published studies by our lab demonstrate that miR-409-3p/5p, two key miRNAs in the DLK1-DIO3 cluster, are elevated in PCa cells (12). Since all four members (miR-409-3p/5p, miR-154* and miR-379) of the DLK1-DIO3 cluster are elevated in PCa bone metastatic cells, we stably transduced ARCaP_E cells, a marginally metastatic epithelial cell line, using a lentivirus that carries a GFP control vector (ARCaP_E-C) or a lentiviral overexpression vector that carries GFP and a combination of all four miRNAs- miR-409-3p, -5p, miR-154* and miR-379 (ARCaP_E-cluster overexpression). The miRNA expression of the members of the DLK1-DIO3 cluster was determined by qRT-PCR assay (Figure 4A). In the ARCaP_E-cluster overexpression cells, miR-409-5p and miR-154* were highly expressed (100 fold) followed by moderate expression of miR-379 and miR-409-3p (5–10 fold) when compared to ARCaP_E-C PCa cells (Figure 4A). Next, we measured the expression levels of the target genes of these miRNAs by qRT-PCR. Several of the target genes of these four miRNAs include tumor suppressors that are shared by these miRNAs (Prediction software: TargetsScan v6.2 June 2012 and Pictar). Overexpression of these miRNAs resulted in a

decrease in mRNA levels of several of the target genes that are commonly shared by these miRNAs. *STAG2* (target gene of miR-154* and miR-409-5p) and Ras suppressor protein 1 (*RSU1*) (target gene of miR-409-3p and miR-409-5p) were significantly decreased in the ARCaP_E-cluster overexpression cells compared to control (Figure 4B). miR-409-5p targets including *STAG2*, retinoblastoma-like 2 (*RBL2*), nitrogen permease regulator-like 2 (*NPRL2*) and *RSU1* were decreased in the cluster overexpression cells (Figure 4B). Targets of miR-409-3p including *RSU1*, polyhomeotic homolog 3 (*PHC3*) and tumor suppressor candidate 1 (*TUSC1*) were decreased in cluster overexpression cells (Figure 4B). These results demonstrate that overexpression of miRNA members of the DLK1-DIO3 microRNA mega-cluster results in decreased expression of several tumor suppressor genes. We also observed EMT changes in ARCaP_E-cluster overexpression PCa cells. The cells appeared spindle shaped and had decreased expression of E-cadherin and increased expression of vimentin compared to ARCaP_E-C cells (Figure 4C). To evaluate the combinatorial effects of knockdown of all four miRNAs (miR-409-3p, miR-409-5p, miR-379 and miR-154*) in metastatic ARCaP_M prostate cancer cells, we introduced shRNA that targets all four miRNAs and found that successful shRNA-mediated knockdown of miR-154* and miR-409-3p (the knockdown of miR-409-5p and miR-379 was not effective in this experiment) led to reversal of EMT. ARCaP_M cells underwent morphological, biochemical and functional EMT changes upon knockdown of miR-154* and miR-409-3p (Supplementary Fig. S2, A–D).

Analyses of the target pathways regulated by these miRNAs include oncogenic pathways such as E2F signaling, the Ras pathway, hypoxia inducible factor signaling, and the WNT and transforming growth factor- β (TGF- β) pathways. These pathways also activate EMT and the cancer stem cell phenotype (Figure 5). miR-154* targets *STAG2* and *SMAD7*. *STAG2* is known to induce aneuploidy (25, 31). *SMAD7* is an inhibitor of the TGF- β pathway (32). miR-379 is predicted to inhibit forkhead box F2 (*FOXF2*) which has been shown to inhibit the WNT pathway in colon cancer development (33). We previously demonstrated that miR-409-3p and miR-409-5p inhibit *RSU1*, which is a known inhibitor of the oncogenic Ras pathway (12, 34, 35). Other targets of miR-409-3p include von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase (*VHL*) and *PHC3*. *VHL* is a tumor suppressor and degrades HIF-1 α (36). Inhibition of *VHL* results in the stabilization of HIF-1 α , which is known to induce resistance to radiation and chemotherapeutic agents (37). *PHC3* is a tumor suppressor protein and is lost in osteosarcoma (38). *PHC3* and Ephrin receptors have been predicted to exhibit protein-protein interactions (www.biograph.be/). miR-409-5p has been shown to target *STAG2*, *RSU1*, *NPRL2* and *RBL2* (12). *NPRL2* activates AKT pathway (39, 40) and *RBL2* activates the E2F pathway (41). Thus, these miRNA activate oncogenic proteins by targeting tumor suppressors. Since these miRNAs are elevated in PCa bone metastatic models, we determined the levels of these miRNAs in human PCa bone metastatic samples using multiplexed ISH-QD labeling. We observed increased miR-154*, miR-409-3p and miR-409-5p staining in human metastatic tumor tissues in the bone (Figure 6). Taken together, these results demonstrate that miR-154*, miR-409-3p/-5p and miR-379 induce EMT in PCa cells, show upregulated expression in human PCa bone metastasis tissues and correlate with progression-free survival in PCa patients.

DISCUSSION

To understand the biology of the miRNAs of the DLK1-DIO3 mega cluster in EMT and cancer bone metastasis and to identify novel biomarkers and/or therapeutic targets, we probed for specific miRNAs of this megacluster in unique EMT models of human PCa developed in our laboratory. Specifically, the miRNAs miR-154* and miR-379, located within the DLK1-DIO3 cluster, were highly upregulated in two PCa cell lines with a mesenchymal phenotype and with bone metastatic potential (Figure 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 uncovered a surprising role for miR-154* and miR-379, which is expressed by embryonic stem cells and pluripotent stem cells, promoting PCa development and metastasis. Specifically, we show that 1) miR-154* and miR-379 are elevated in human PCa tumor tissues and miR-379 expression correlate with PCa patient progression-free survival, 2) miR-154* and miR-379 can promote EMT of PCa cells *in vitro*, and 3) inhibition of miR-154* results in decreased bone metastatic tumor growth and increased survival. Thus, miR-154* and miR-379 appear to be promising new biomarkers for cancer detection and attractive new therapeutic targets for PCa treatment.

The primary functions of miR-154* are to repress tumor suppressors and modulate the expression of EMT and stemness to mediate downstream convergent signal axes. Inhibition of miR-154* resulted in increased E-cadherin and decreased invasion *in vitro*. miR-154*-depleted PCa cells had decreased bone metastasis and increased survival. miR-154* mediates its effects by downregulating its target gene, STAG2, which is a tumor suppressor. STAG2 plays a critical role in the cohesion complex and a decrease in STAG2 has been correlated with aneuploidy and cancer (25, 31). Other miR-154* target genes may be involved such as SMAD7, which inhibits the TGF- β pathways (32) involved in EMT (42). Interestingly, the sense strand of miR-154* is undetectable or decreased in PCa models and in PCa patients (28, 29, 30). miR-154* is upregulated in both lung squamous cell carcinoma and lung adenocarcinoma patients (9). miR-379, another member of the DLK1-DIO3 cluster, located upstream of the miR-154 gene cluster, is moderately elevated in PCa cells (Figure 1). Inhibition of miR-379 also resulted in reversal of EMT (MET), increased E-cadherin and decreased invasion. miR-379 expression in clinical specimens correlates with disease-free survival of prostate cancer patients. Consistent with our observations, previous reports demonstrate that the miRNAs of the DLK1-DIO3 cluster are elevated in the serum of cancer patients compared to healthy patients (9, 11). Consistent with our studies, it was shown that miR-379 is also elevated in metastatic PCa patients compared to patients with localized disease (43). Interestingly, other members of the DLK1-DIO3 megacluster share similar mRNA target genes. STAG2 is targeted by both miR-154* and miR-409-5p, whereas RSU1 is targeted by both miR-409-5p and miR-409-3p. Thus the members of the cluster work synergistically and are elevated in the mesenchymal type PCa cells to promote EMT and metastasis (Figure 5). We conclude that the miRNA members of the DLK1-DIO3 cluster promote EMT, stemness, and bone metastasis in PCa. These findings have potential clinical implications in both the biomarker field and the therapeutic arena.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational relevance

Currently there are no good biomarkers or therapy for prostate cancer (PCa) bone metastasis. MicroRNAs in the delta-like 1 homolog - deiodinase, iodothyronine 3 (DLK1-DIO3) cluster located in human chromosome 14 have been shown to be critical for embryonic development and epithelial to mesenchymal transition (EMT) of embryonic cells. In this study, we identified a novel role for miR-154* and miR-379, two members of the DLK1-DIO3 cluster, in prostate tumor growth, epithelial to mesenchymal transition (EMT), stemness and bone metastasis. Further, we demonstrate that miR-154* and other miRNA members are elevated in cell lines and bone metastasis tissues from prostate cancer patients. Overexpression of miR-154* and miR-379 in benign PCa cells promotes EMT, and inhibition of miR-154* and miR-379 results in decreased EMT and bone metastasis in experimental models. Thus miR-154* and miR-379, already implicated in embryonic development, have a role in PCa metastasis and could serve both as a novel biomarker and as a therapeutic target.

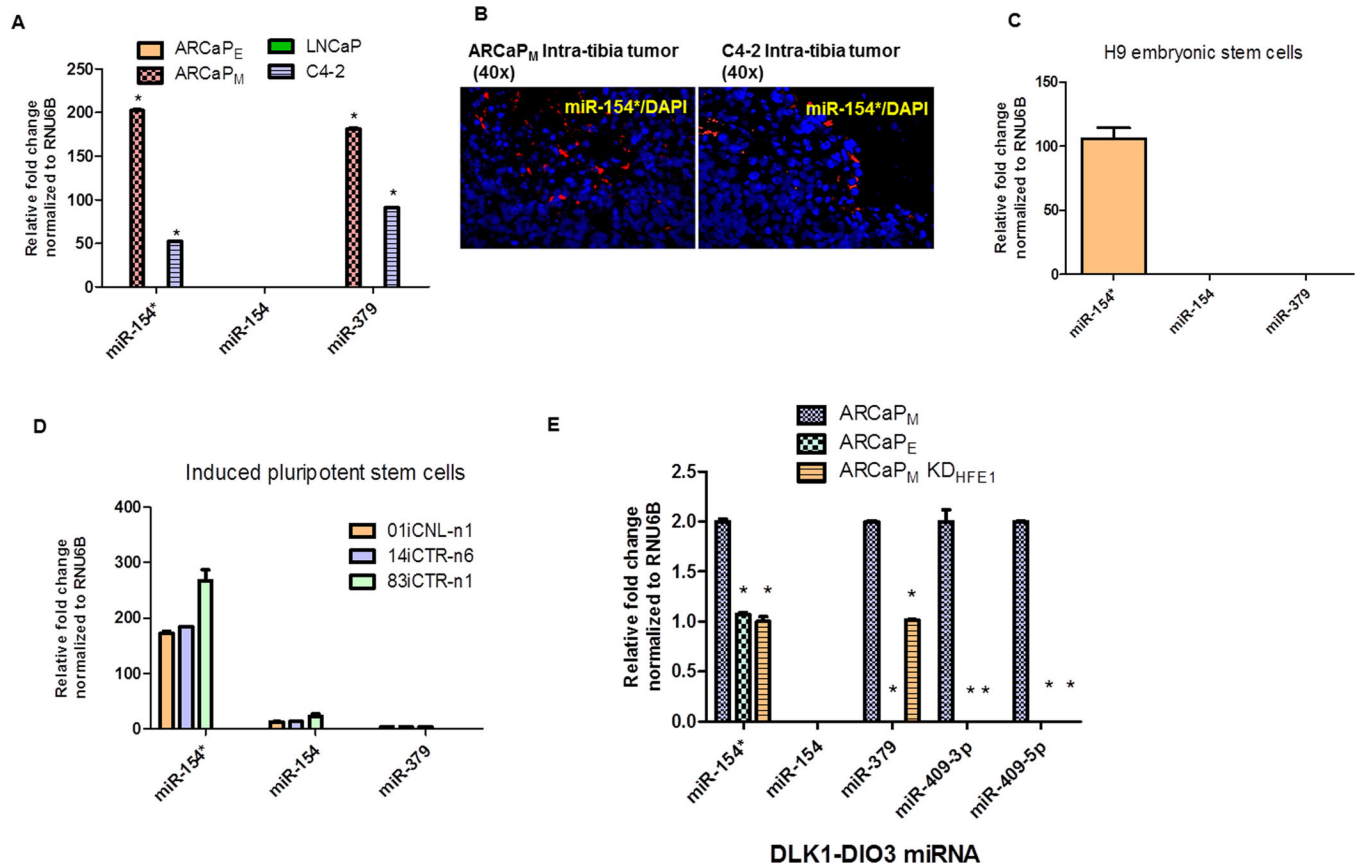


Figure 1. Members of the DLK1-DIO3 miRNA cluster (miR-154/154* and miR-379) are overexpressed in aggressive bone metastatic EMT models of human PCa (A) miRNA expression of miR-154/154* and miR-379 in PCa models (mesenchymal cells ARCaP_M compared to ARCaP_E and LNCaP versus C4-2 PCa cells) by qRT-PCR analysis. (B) miRNA stained or miR-154* stained PCa mouse bone metastatic models (C4-2 and ARCaP_M cells) assayed by ISH-QD analysis (Magnification 40X). miR-154* stained in red, nucleus stained with DAPI. (C), (D) miRNA expression of miR-154/154* and miR-379 in H9 embryonic stem cells and iPS cells by qRT-PCR. (E) miRNA expression in EMT models in PCa, mesenchymal cells-ARCaP_M, epithelial cells-ARCaP_E and ARCaP_M KD^{HFE1}, assayed by qRT-PCR. *: p<0.05 was considered to be statistically significant by one way ANOVA-Tukey analysis.

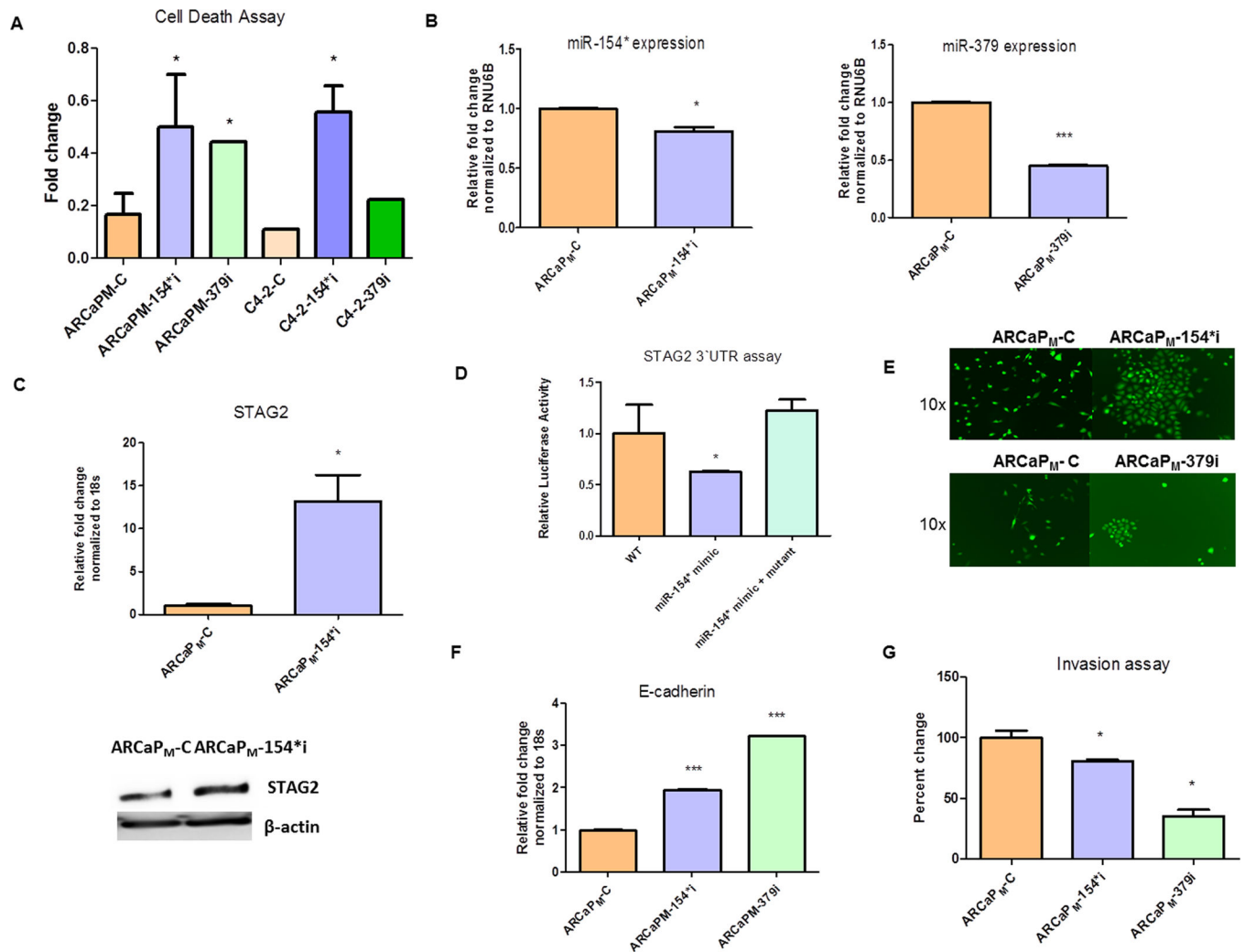


Figure 2. Inhibition of miR-154* or miR-379 results in mesenchymal to epithelial transition of PCa cells

(A) Cell death in ARCaPM PCa cells in response to a miR-154* and miR-379 inhibitor using trypan blue exclusion assay. (B) Expression of miR-154* and miR-379 assayed by qRT-PCR in ARCaPM-C control PCa cells and ARCaPM-154*i (miR-154* inhibitor transfected cells) and ARCaPM-379i (miR-379 inhibitor) transfected cells. Data is normalized to RNU6B. (C) STAG2 (miR-154* target) protein and mRNA expression in ARCaPM-C and ARCaPM-154*i PCa cells assayed by western analysis and qRT-PCR. (D) 3' UTR binding luciferase assay using wild type (WT) and mutant 3' UTR (STAG2) construct and miR-154* mimics in 293T cells. (E) Morphological changes in ARCaPM-154*i and ARCaPM-379i compared to ARCaPM-C control PCa cells. (F) E-cadherin mRNA assayed in ARCaPM-C, ARCaPM-154*i and ARCaPM-379i expressing PCa cells by qRT-PCR, normalized to 18s RNA. (G) Invasion assay of ARCaPM-C, ARCaPM-154*i and ARCaPM-379i expressing PCa cells. * $p < 0.05$, or *** $p < 0.001$ were considered to be statistically significant by t-test or ANOVA- Tukey test.

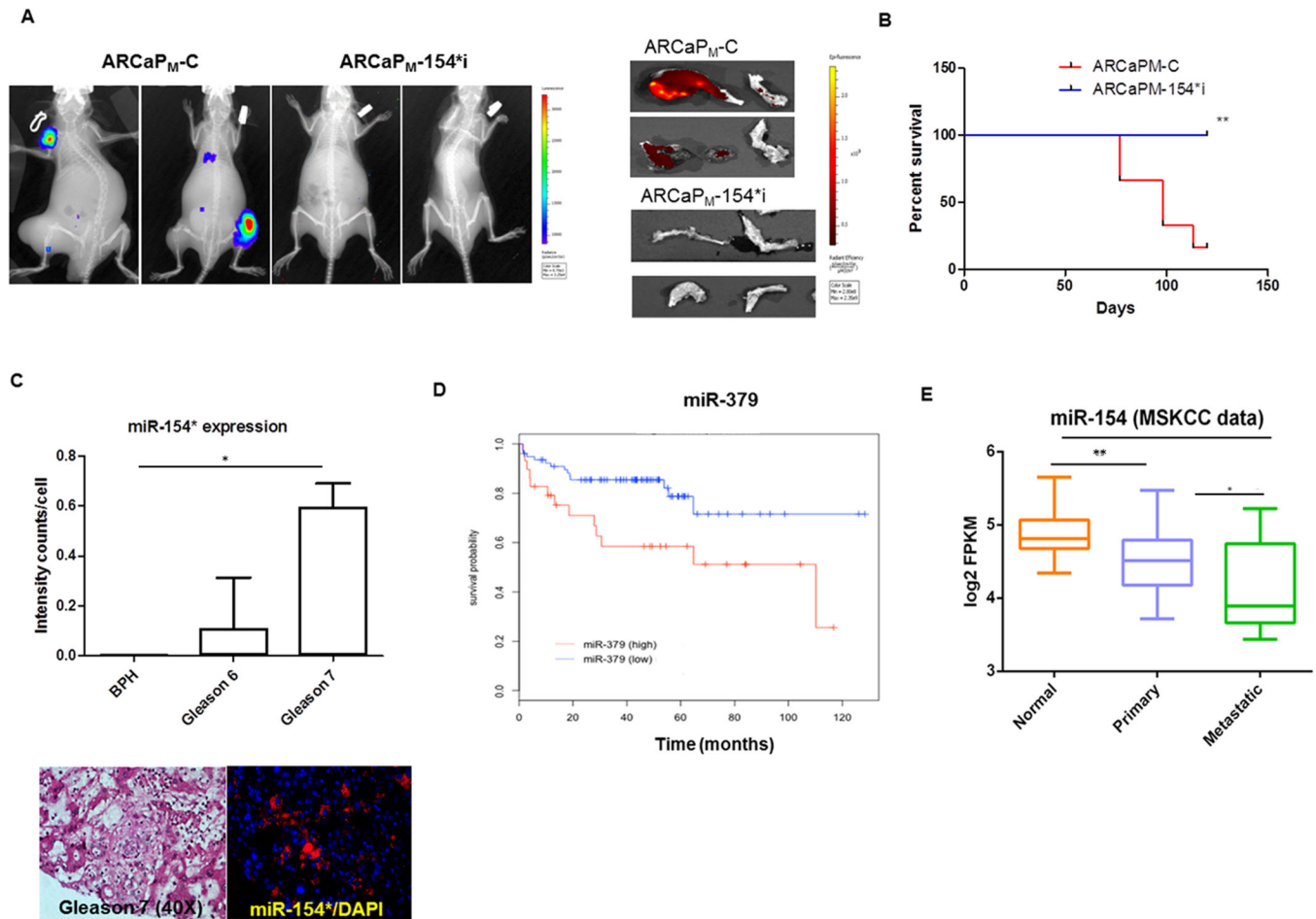


Figure 3. Inhibition of miR-154* results in decreased metastasis of bone and soft tissue of PCa cells

(A) Representative metastatic lesions observed by X-ray/luciferase imaging of ARCaP_M-C cell and ARCaP_M-154*i* cell tumors in SCID/Beige mice (n=5/group) following intra-cardial injection. Images of bone with tumor in the ARCaP_M-C cells and ARCaP_M-154*i* injected mice using near infra-red dye (IR783). (B) Kaplan Meier's curve of ARCaP_M-C cells and ARCaP_M-154*i* injected SCID/Beige mice (n =5/group). (C) miR-154* expression in PCa clinical samples from BPH, Gleason 6 and 7 tissues assayed by ISH-QD labeling. Data were plotted as intensity counts/cell in tissues. Representative image of Gleason 7 tissue with miR-154* staining in red (magnification 40×). Nuclei stained by DAPI. (D) Kaplan-Meier disease free survival (DFS) curves for the PCa patients, based on miR-379 expression in the MSKCC dataset. The y-axis is disease free survival probability, and the x-axis is survival in months. Blue line represents the DFS of patients with miR-379 lower than the median of the normal individuals (n=78). Red line represents the DFS of patients with miR-379 higher than the median of the normal individuals (n=29). Data was analyzed using log-rank test (p=0.0117). (E) miR-154 expression in normal, primary and metastatic PCa patients. Significant differential expression of miR-154 was noted between normal individual (n=29), primary (n=99) and metastatic (n=14) PCa patients. *: p<0.05, or ** p < 0.005 were considered to be statistically significant by t-test or ANOVA- Tukey test.

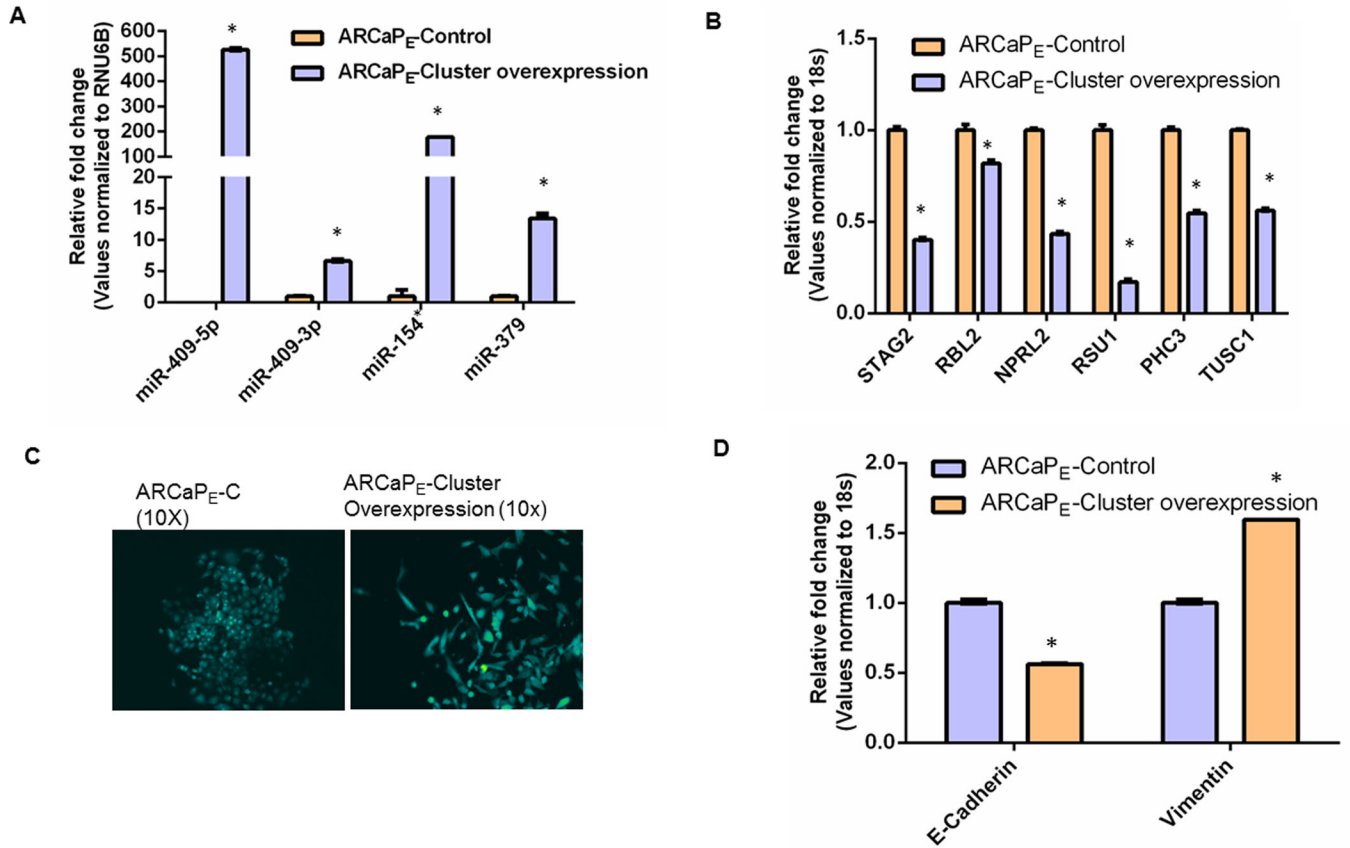


Figure 4. Overexpression of miR-154*, miR-409-3p/-5p and miR-379 induces EMT in PCa cells (A) Expression of miR-409-5p/-3p, miR-154* and miR-379 assayed by real time PCR in ARCaPE-C control PCa cells and ARCaPE-cluster overexpressing cells normalized to RNU6B. (B) RNA expression of miR-409-5p/-3p and miR-154* targets in ARCaPE-M PCa cells assayed by qRT-PCR. (miR-154* mRNA targets: STAG2, miR-409-5p mRNA targets: STAG2, RBL2, NPRL2 and RSU1, miR-409-3p mRNA targets: RSU1, PHC3 and TUSC1). (C) Morphological EMT changes in ARCaPE-C control PCa cells and ARCaPE-cluster overexpressing cells; magnification 10× (D) RNA expression of EMT markers, E-cadherin and vimentin in ARCaPE-C control PCa cells and ARCaPE-cluster overexpressing cells assayed by qRT-PCR. *: $p < 0.05$ was considered to be statistically significant by t-test.

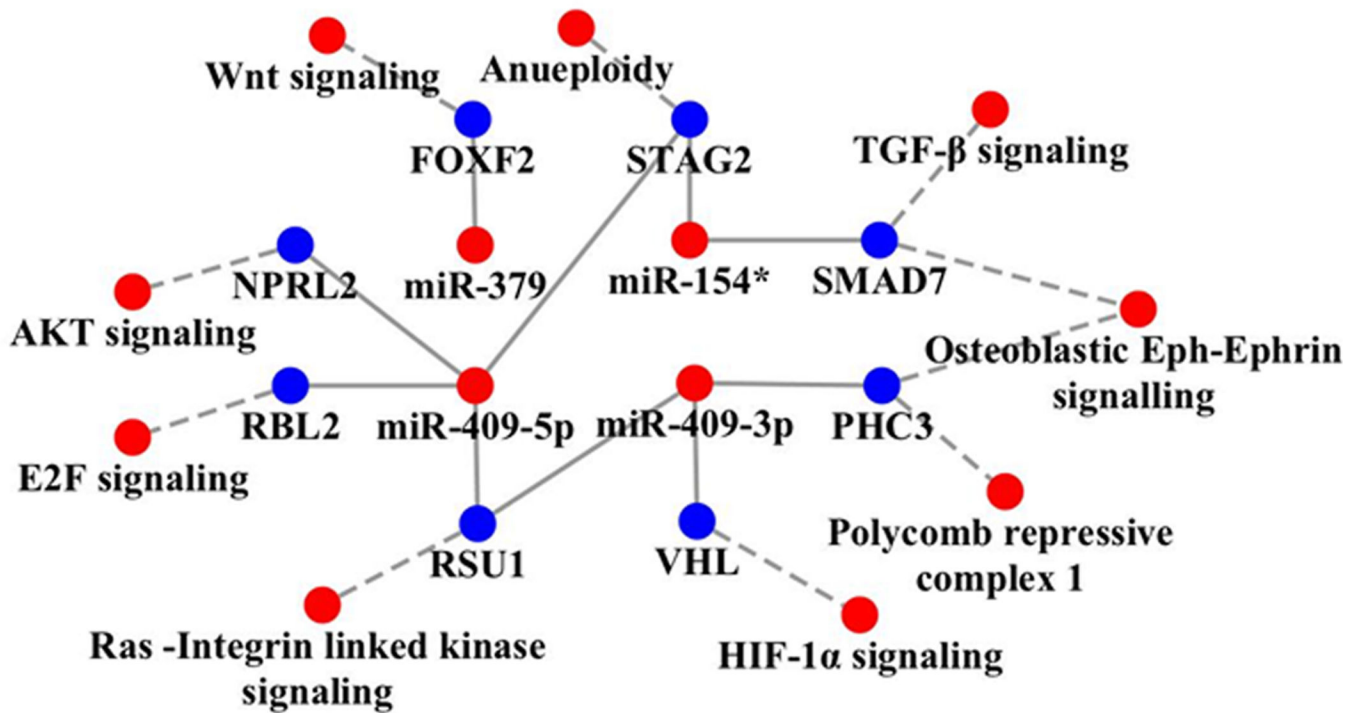


Figure 5.

Cytoscape analysis of target genes and signaling pathways altered by miR-409-3p/5p, miR-154* and miR-379 in the DLK1-DIO3 cluster. The miRNA in this cluster target tumor suppressors which block several pathways in human cancer. Red dots represent activation of miRNA and oncogenic pathways and blue dots represent inhibition of tumor suppressor genes.

Human prostate cancer bone metastatic tissues (40X)

Tissue # 1

Tissue # 2

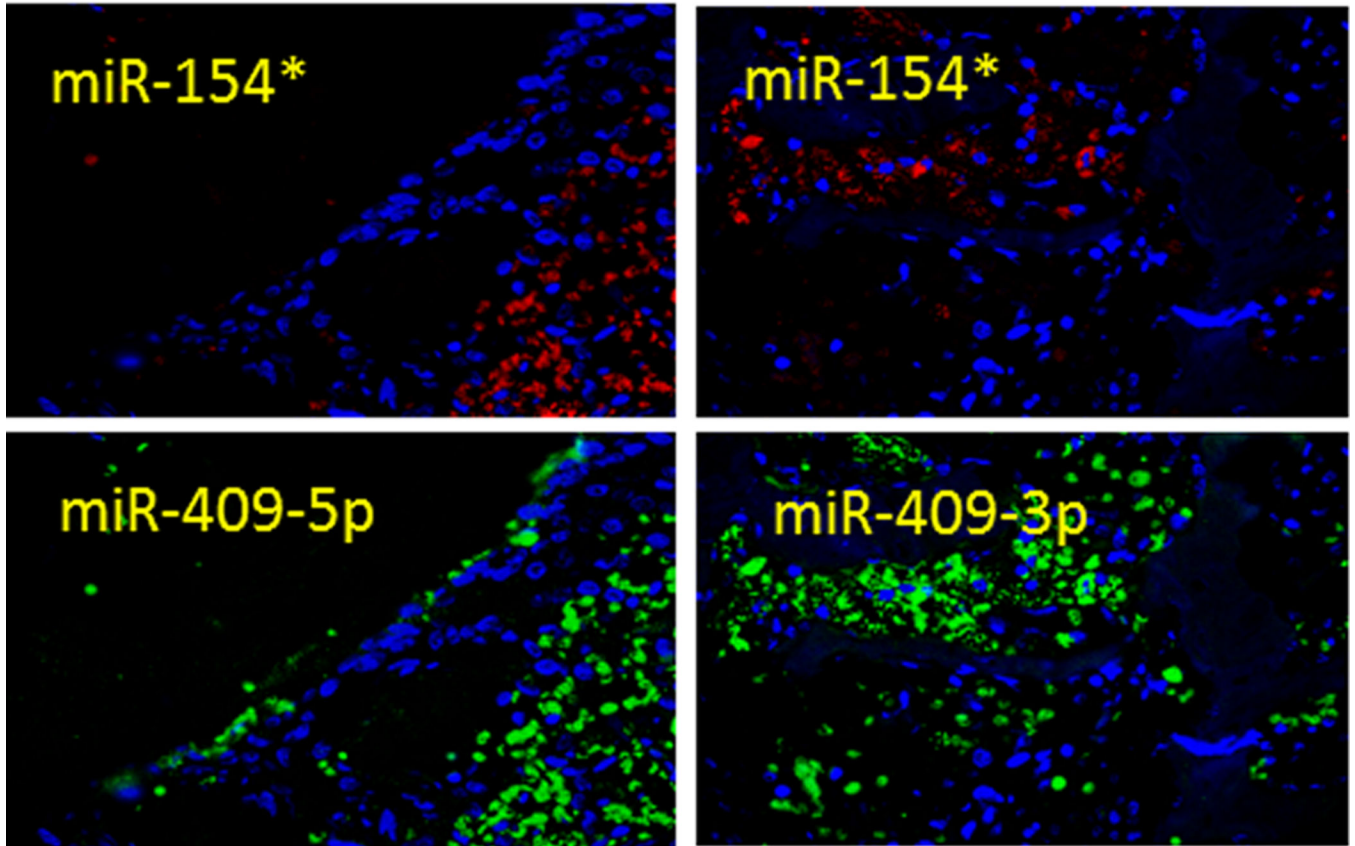


Figure 6. miR-154* (red), miR-409-3p (green) and miR-409-5p (green) staining of human prostate bone metastatic tissue using multiplexed ISH-QD labeling. Two human prostate bone metastatic tissues were used, and multiplexed for probes against miR-154* and miR-409-3p or miR-409-5p.