MiR-154-5p Inhibits Prostate Cancer Bone Metastasis by Inactivating PI3K/AKT Signaling

Dong Ren
Jiangmen Central Hospital
Xiangwei Yuan
Jiangmen Central Hospital
Jiazheng Cao
Jiangmen Central Hospital
Bin Wang
Jiangmen Central Hospital
Ruixiao Li
Tangdu Hospital Fourth Military Medical University: Air Force Medical University Tangdu Hospital
Rui Zhang
Jiangmen Central Hospital
Baoyi Liu
Jiangmen Central Hospital
Meimei Wu
Jiangmen Central Hospital
Liangliang Ren
Jiangmen Central Hospital
Zijie Meng
Jiangmen Central Hospital
Wanting Wu
Jiangmen Central Hospital
Xingxing Chai
Guangdong Medical University
Lili Li
Jiangmen Central Hospital
Ronggang Li
Jiangmen Central Hospital
Xiufang Huang
Jiangmen Central Hospital
Jinhua Wu
Jiangmen Central Hospital
Yan Zheng

Jiangmen Central Hospital

Jincheng Zeng

Guangdong Medical University

Xin Zhang (zhangxin_toby@hotmail.com)

Jiangmen Central Hospital, Affiliated Jiangmen Hospital of Sun Yat-sen University

https://orcid.org/0000-0001-6036-2685

Junjiu Huang

Jiangmen Central Hospital

Zhonglin Xue

Jiangmen Central Hospital

Yuming Li

Jiangmen Central Hospital

Research

Keywords: miR-154-5p, miR-154-3p, cytokine receptor, PI3K/AKT signaling pathway, bone metastasis, prostate cancer.

Posted Date: September 1st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-840996/v1

Abstract

Background: Generally, both strands of a single pre-miRNA have been demonstrated to play a similar role in the same tumor type. However, there are no available literatures yet so far clarifying the opposite roles of both strands froma single miRNAin one tumor type. The purpose of this study is to investigate the functional role of both strands of miR-154 in bone metastasis of prostate cancer (PCa).

Methods: miR-154-5p expression was examinedin 285 clinicalPCa tissuesby in situ hybridization. The clinical correlation ofmiR-154-5p expression with clinicopathological features,and overall and bone metastasis-free survival inPCa patients was evaluated by Kaplan-Meier survival and statisticalanalysis. The biological roles of miR-154-3p and miR-154-5p in the bone metastasis of PCa were investigated both *in vitro*and *in vivo*.Bioinformatics analysis, western blot and luciferase reporter analysis were used to determinethe potential targets of miR-154-5p.Luciferase assay and Western blotting were performed to clarify the underlying pathway implicated in the role of miR-154-5p in bone metastasis of PCa.

Results: Contrary to the well established pro-bone metastatic role of miR-154-3p in PCa, we found that miR-154-5p expression was reduced in PCa tissues with bone metastasis andbone metastatic PCa cell lines. Downexpression of miR-154-5p was positively associated with bone metastasis status, and predicted poorer bone metastasis-free survival in PCa patients. Gain of function experiments showed that upregulating miR-154-5p repressed, while silencing miR-154-5p promoted invasion, migration and proliferation capacities of PCa cells *in vitro*. Conversely, miR-154-3p yielded an opposite effect oninvasion and migration capacities of PCa cells. Importantly, administration of agomir-154-5p effectivelyinhibited bone metastasis of PCa cells*in vivo*. Mechanistic dissection further demonstrated miR-154-5p inhibited invasion, migration and proliferation by targeting EGFR and FGFR1, leading to inactivation of PI3K/AKT signaling. However, the autocrine levels of corresponding ligands in the supernantant of PCa cells were not affected by the changed expression of miR-154-5p.

Conclusion: Our results for the first time reveal the different role of both strands froma single miRNA in bone metastasis of PCa, which will facilitate the development of anti-bone metastatic therapeutic strategy in PCa.

Background

Bone is the most preferential metastatic site of prostate cancer (PCa), with a high incidence of 65–80% in advanced PCa [1].Once cancer cells disseminate to bone, they significantly disrupt normal bone remodeling, resulting in bone fractures, nerve compression, pain, and hypercalcemia [2].Despite great progresses in the treatment of primary PCa in the past decades, distant bone metastasis remains the priming issue responsible for the poor survival time of PCa patients [3]. Indeed, the 5-year survival rate of PCa patients with distant bone metastasis is approximately 30% compared with 90% around in primary PCa patients [4]. Thus, prevention of bone metastasis is a major goal of treatment, and elucidation of the

crucial factors or validation of the molecular mechanism underlying the high bone metastatic propensity of PCa is of paramount importance.

So far, several cellular signaling pathways have been confirmed by a large amount of literature to be closely related to bone metastases of PCa, including TGF-β [5], Wnt [6], NF-kB [7], EGFR [8] and PI3K/AKT signaling [9]. Compared with other well-established pro-bone metastatic signaling in PCa, accumulating attention has been made regarding the role of PI3K/AKT signaling in bone metastasis of PCa. Since discovered, phosphoinositide 3-kinase (PI3K)/Akt signaling has been involved in diverse cellular processes, including cell growth, proliferation and survival [10, 11]. The PI3K/AKT signaling initiates after binding multiple extracellular stimuli, including EGF [12], IGF-1 [13], insulin [14] and FGF [15]. Then, the activated PI3K phosphorylates phosphatidylinositol-3,4-bisphosphate [PI(3,4)P2] and phosphatidylinositol – 3,4,5-trisphosphate [PI(3,4,5)P3], at the 3'-hydroxyl group of the inositol ring of phosphatidylinositol, and further recruits Akt and phosphoinositide-dependent kinases to the plasma membrane, which ultimately results in activation of Akt signaling [16, 17]. The activated Akt further phosphorylates a variety of downstream effectors, leading to unlimited proliferation and growth of cells [18–20]. Constitutive activation of the PI3K/Akt pathway not only play an important role in the tumorigenesis of many types of cancer [21], but also contributes to the progression and metastasis in various types of cancer [22–24]. Notably, a study from Li and colleagues has reported that PI3K/Akt signaling -mediated stabilization of histone methyltransferase WHSC1 promoted bone metastasis and osteolytic bone lesions in PCa [9]. However, the underlying mechanism responsible for activation of PI3K/Akt signaling in bone metastasis of PCa is still further clarified.

MicroRNAs (miRNAs), a class of non-coding RNAs, function as negative regulators of targeted genes [25]. Being able to coordinately regulate repertoires of target genes, miRNAs can potentially modulate multiple steps of cancer development and progression [26–28]. The aberrant expression of miRNAs in cancers is widely reported, which has shown a correlation of miRNAs and metastatic tumors [29, 30]. Furthermore, several miRNAs have been identified as crucial mediators in the bone metastasis of PCa [7, 31]. As one of the originally identified miRNAs, miR-154-5p has been reported to be frequently downregulated in multiple cancer types [32, 33]. In PCa, Gururajan and colleagues have reported that miR-154-3p was elevated in bone metastatic prostate cancer cell lines and tissues, and silencing miR-154-3p led to decreased bone metastasis and increased survival [34]. Strikingly, a study from Formosa has shown that miR-154-5p expression was dramatically downregulated in metastatic PCa cell lines as compared with normal prostatic epithelial cells (PrEC) through miRNAs microarray [35]. These findings suggested that both strands from a single miRNA may play the opposite roles in the bone metastasis of PCa. However, the clinical significance and biological role of miR-154-5p in the bone metastasis of PCa, as well as the underlying molecular mechanisms by which miR-154-5p regulates bone metastasis of PCa have not been reported yet.

In this study, our results found that miR-154-5p expression was decreased in PCa tissues with bone metastasis compared with PCa tissues without bone metastasis, but was no significant difference of miR-154-5p expression between PCa tissues and adjacent normal tissues. Low level of miR-154-5p

predicted poor bone metastasis-free survival in PCa patients. Moreover, upregulating miR-154-5p inhibited, while silencing miR-154 increased proliferation, invasion and migration abilities of PCa cells *in vitro*. By contrast, miR-154-3p played an opposite role in PCa cells. Importantly, agomir-154-5p injection through tail vein dramatically suppressed the bone metastasis of PCa cells *in vivo*. Our results further demonstrated that miR-154-5p repressed activity of PI3K/AKT signaling by targeting EGFR and FGFR1, which further inhibited bone metastasis of PCa. Therefore, our results unveil a novel mechanism responsible for constitutive activation of PI3K/AKT signaling in bone metastasis of PCa.

Methods

Cell lines and cell culture

The human PCa cell lines 22RV1, PC-3, VCaP, DU145, LNCaP and normal prostate epithelial cells RWPE-1 were obtained from Procell (Wuhan, China). RWPE-1 cells were grown in defined keratinocyte-SFM (1×) (Invitrogen). PC-3, LNCaP and 22Rv1 cells were cultured in RPMI-1640 medium (Life Technologies, Carlsbad, CA, US) supplemented with penicillin G (100 U/ml), streptomycin (100 mg/ml) and 10% fetal bovine serum (FBS, Life Technologies). DU145 and VCaP cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS. The C4-2B cell line was purchased from the MD Anderson Cancer Center and maintained in T-medium (Invitrogen) supplemented with 10 % FBS. All cell lines were grown under a humidified atmosphere of 5 % CO2 at 37°C.

Plasmids, Transfection And Generation Of Stable Cell Lines

The human MIR154 gene was PCR-amplified from genomic DNA and cloned into a Ubi-MCS-SV40-EGFP-IRES-puromycin lentiviral vector (GV369, Genechem, Shanghai, China). The 3'UTR of IGF1R, EGFR and FGFR1 were PCR-amplified from genomic DNA and cloned into pmirGLO vectors (Promega, USA), and the list of primers used in cloning reactions is shown in Additional file 1- Table S1. Agomir-154-3p, agomir-154-5p, antagomir-154-3p, antagomir-154-59, the siRNA of EGFR and FGFR1 were purchased from RIBOBIO Company (Guangzhou, China). Cells were treated with MK-2206 (Selleck Chemicals, Houston, TX, USA) at the concentrations (1 μM). Transfection of miRNA, siRNAs, and plasmids was performed as previously described [36].

Rna Extraction, Reverse Transcription, And Real-time Rt-pcr

Total RNA from tissues or cells was extracted as described previously [37]. Messenger RNA (mRNA) and miRNA were reverse transcribed from total mRNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, USA) according to the manufacturer's protocol. Complementary DNA (cDNA) was amplified and quantified on the CFX96 system (BIO-RAD, USA) using iQ SYBR Green (BIO-RAD, USA). The primers are provided in Additional file 2- Table S2. Real-time PCR was performed according to a standard method, as described previously [38]. Primers for U6, miR-154-3p and miR-154-5p were synthesized and

purified by RiboBio (Guangzhou, China). U6 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous controls. Relative fold expressions were calculated with the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method.

Patients And Tumor Tissues

A total of 285 PCa tissues, including 203 PCa tissues without bone metastasis and 82 PCa tissues with bone metastasis, and 46 benign prostate hyperplasia tissues (BPH) were obtained during surgery or needle biopsy between January 2014 and December 2018 at the Jiangmen Key Laboratory of Clinical Biobanks and Translational Research, The Affiliated Jiangmen Hospital of Sun Yat-sen University (Guangdong, China). Patients were diagnosed based on clinical and pathological evidence, and the specimens were immediately snap-frozen and stored in liquid nitrogen tanks. For the use of these clinical materials for research purposes, prior patient' consents and approval from the Institutional Research Ethics Committee were obtained. The clinicopathological features of the patients are summarized in Additional file 3- Table S3. The median of miR-154-5p expression in PCa tissues was used to stratify high and low expression of miR-154-5p.

Mirna Immunoprecipitation

Cells were co-transfected with HA-Ago2, followed by HA-Ago2 immunoprecipitation using anti-HA-antibody. Real-time PCR analysis of the IP material was performed to test the association of the mRNA of EGFR, FGFR1, IGF1R and MET with the RISC complex. The specific processes were performed as previously described [39].

Western Blot

Western blot was performed according to a standard method, as previously described [40]. Antibodies against EGFR, FGFR1, IGF1R, p-AKT (S473), p-AKT (T308) and AKT were purchased from Cell Signaling Technology. As a loading control, membranes were stripped and reprobed with an anti-α-tubulin antibody (Sigma-Aldrich, USA).

Luciferase Reporter Assay

Cells (4×10^4) were seeded in triplicate in 24-well plates and cultured for 24 h and performed as previously described [41]. Luciferase and Renilla signals were measured 36 h after transfection using a Dual Luciferase Reporter Assay Kit (Promega).

Akt Activity Assay

To measure Akt kinase activities of in cells or tumor tissues, Akt activity assay was performed as previous described [42]. The immune complexes were then incubated with a biotinylated peptide substrate that became phosphorylated in the presence of activated Akt. The phosphorylated substrates, which reflected the activity of Akt kinase in the extract, was then quantified with the K-LISA Akt Activity Kit (Calbiochem, Darmstadt, Germany) that comprises a primary antibody recognizing the phosphorylated substrate peptides.

Animal Study

All mouse experiments were approved by the Institutional Animal Care and Use Committee of Guangdong Medical University, and the approval-No. was GDY2102160. For the bone metastasis study, BALB/c-nu mice ((5–6 weeks old, 18–20 g)) were anaesthetized and inoculated into the left cardiac ventricle with 1×10^5 PC-3 cells in 100 μl of PBS. After two days of cell injection, animals were injected with 100 μl agomir scramble or agomir-154-5p through the lateral tail vein every three days for 4 weeks. Bone metastases were monitored by bioluminescent imaging (BLI) as previously described [43]. Osteolytic lesions were identified on radiographs as radiolucent lesions in the bone. The area of the osteolytic lesions was measured using the Metamorph image analysis system and software (Universal Imaging Corporation), and the total extent of bone destruction per animal was expressed in square millimeters. Each bone metastasis was scored based on the following criteria: 0, no metastasis; 1, bone lesion covering < 1/4 of the bone width; 2, bone lesion involving 1/4 ~ 1/2 of the bone width; 3, bone lesion across 1/2 ~ 3/4 of the bone width; and 4, bone lesion > 3/4 of the bone width. The bone metastasis score for each mouse was the sum of the scores of all bone lesions from four limbs. For survival studies, mice were monitored daily for signs of discomfort, and were either euthanized all at one time or individually when presenting signs of distress, such as a 10% loss of body weight, paralysis, or head tilting.

In Situ Hybridization

In situ hybridization (ISH) was performed on PDAC tumors using locked nucleic acid (LNA) probes for miR-154-5p (Exiqon, Vedbaek, Denmark). Briefly, Paraffin-embedded tumors were deparaffinized, treated with proteinase K, and fixed in paraformaldehyde. The digoxigeninlabeled LNA probe was hybridized overnight. Slides were rinsed and incubated with anti-digoxigenin, a horseradish peroxidase (HRP)-linked antibody (Zsbio, China), for 2 hr. The detection reaction was performed using the DAB Ready-to-Use Kit (ZLI-9018, Zsbio, China). For each sample, the whole fields of each slide were analyzed by optical microscope. The ISH scores given by the two independent investigators were averaged for further comparative evaluation of the miR-154-5p expression. Tumor cell proportion was scored as follows: 0 (no positive tumor cells), 1 (< 10% positive tumor cells), 2 (10–35% positive tumor cells), 3 (35–70% positive tumor cells), and 4 (> 70% positive tumor cells). The staining intensity was graded according to the following criteria: 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellow brown), and 3 (strong staining, brown). The ISH score was calculated as the product of staining intensity score and

the proportion of positive tumor cells. Using this method of assessment, we evaluated miR-154-5p expression in pancreatic cancer samples by determining the staining intensity (SI), with scores of 0, 1, 2, 3, 4, 6, 8, 9, or 12. ISH score 4 was the median of all sample tissue SI scores. High and low expression of miR-154-5p were stratified by the follow criteria. An ISH score of 4 was used to define tumors with high expression of miR-154-5p and SI < 4 as tumors with low expression of iR-154-5p.

Invasion And Migration Assays

The invasion and migration assays were performed using Transwell chamber consisting of 8μm membrane filter inserts (Corning) with or without coated Matrigel (BD Biosciences) respectively, and was carried out as previously described [44]. Briefly, the cells were trypsinized and suspended in serum-free medium. Then, $1.5x10^5$ cells were added to the upper chamber, and lower chamber was filled with the culture medium supplemented with 10% FBS. After incubation for 24–48 h, cells passed through the coated membrane to the lower surface, where cells were fixed with 4% paraformaldehyde and stained with haematoxylin. The cell count was performed under a microscope (×100).

Mtt Assay

Cells were seeded into 96-well plates in triplicate at the initial density of 0.2×10^4 cells/well. At various time points, groups of cells were incubated with 100 μl of 0.5 mg/ml sterile MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide; Sigma] for 4 h at 37ºC. The culture medium was then removed, and 150 μl of DMSO (Sigma) was added. The absorbance values were measured at 570 nm using 655 nm as the reference wavelength.

Colony Formation Assay

Cells (0.2×10^3) were plated into six well plates and cultured for 10 days. Colonies were then fixed for 15 min with 10% formaldehyde and stained for 30s

with 1.0% crystal violet. Plating efficiency = number of colonies (≥ 50 cells per colony) per input cells x 100%. Different colony morphologies were captured under a light microscope (Olympus).

Statistical analysis

All values are presented as the mean ± standard deviation (SD). Significant differences were determined using the GraphPad 5.0 software (USA). One-way ANOVA was used to determine statistical differences between multiple testing and the post hoc test after ANOVA is Tukey. Unpaired or paired t-test was used to determine statistical differences between two groups. The chi-square test was used to analyze the relationship between miR-154-5p expression and clinicopathological characteristics. Survival curves were

plotted using the Kaplan Meier method and compared by log-rank test. $P < 0.05$ was considered statistical significant. All experiments were repeated three times.

Results

miR-154-5p is downregulated in PCa tissues with bone metastasis

To determine the clinical and prognostic significance of miR-154-5p in PCa, especially in bone metastatic PCa, we first examined miR-154-5p expression levels in 46 benign prostate hyperplasia tissues (BPH) and 285 PCa tissues with different TNM stages, International Society of Urological Pathology (ISUP) grades and bone metastatic status using in situ hybridization (ISH) (Fig. 1A). As shown in Fig. 1B, miR-154-5p was mainly detected in the cytoplasma of cells, and the case number of BPH and PCa tissues with different staining index (SI) was shown in Additional file 5-Figure S1A. First, differential SI of miR-154-5p between BPH and PCa tissues was not observed (Fig. 1C). Consistently, there was no significant difference of miR-154-5p expression level between in primary PCa tissues and in the adjacent normal tissues (ANT) by analyzing the miRNA sequencing dataset of PCa from The Cancer Genome Atlas (TCGA) (Additional file 5-Figure S1B and C). Further investigation showed that the SI of miR-154-5p was remarkably reduced in PCa tissues with bone metastasis (PCa/BM) compared with that in PCa tissues without bone metastasis (PCa/nBM) (Fig. 1D), which was further supported by TCGA dataset (Additional file 5-Figure S1D). Moreover, the percentage of low miR-154-5p expression in BM was obviously higher than that in nBM (Additional file 5-Figure S1E and F), but there was no statistical difference between BPH and tumor (Additional file 5-Figure S1G). We further examined the expression levels of miR-154-5p in 6 PCa cells and one normal prostate epithelial cells RWPE-1, and found that miR-154-5p expression were differentially downregulated compared with that in RWPE-1, particularly in bone metastatic PCa cell lines PC-3 (Additional file 5-Figure S1H). Collectively, these results suggest that low expression of miR-154-5p may be involved in the bone metastasis of PCa.

Low levels of miR-154-5p predicts poor bone metastasis-free survival in PCa patients

The clinical correlation of miR-154-5p expression levels with clinicopathological characteristics in PCa patients from TCGA was first analyzed. As shown in Additional file 6-Figure S2A-D, miR-154-5p expression had no significant correlation with T classification, N classification, M classification and ISUP grade in PCa patients. The results of statistical analysis revealed that low expression of miR-154-5p positively correlated with M classification and bone metastasis status in PCa patients (Additional file 4-Table S4). The difference of clinical correlation of miR-154-5p expression level with M classification between the dataset from our samples and TCGA may be explained that our PCa samples included more metastatic PCa tissues when analyzed, particularly bone metastatic PCa tissues. Kaplan-Meier survival analysis based on ISH staining index indicated that PCa patients with low miR-154-5p expression correlated with shorter bone metastasis-free and progression-free survival compared with those with high miR-154-5p expression (Fig. 1E and F), but had no effect on overall survival in PCa patients from our and

TCGA datasets (Additional file 7-Figure S3A and B). Interestingly, TCGA analysis showed no statistical significance of miR-154-5p expression with progression-free survival in PCa patients (Additional file 7-Figure S3C), which we speculated that compared with direct clinical detection technique through ISH, miR-154-5p levels examined by microarrays based on RNA extraction from sample tissues in TCGA dataset may be more likely to be influenced by technical factors. Taken together, these findings indicate that low levels of miR-154-5p strongly and positively correlates with poor bone metastasis-free survival in PCa patients.

miR-154-3p and miR-154-5p play opposite roles in regulating invasion and migration abilities of PCa cells

To determine the effect of miR-154-5p on the bone metastasis of PCa, we first constructed miR-154-stably overexpressing three bone metastatic PCa cell lines, including PC-3, VCaP and C4-2B, and endogenously downregulated miR-154 in C4-2B cells. Real-time PCR results showed that miR-154-3p and miR-154-5p were simultaneously upregulated in miR-154-transfecting PCa cells, and reduced in miR-154-silenced C4-2B cells (Additional file 8-Figure S4A and B). Then, the effects of miR-154 overexpression or downregulation on invasion and migration abilities of PCa cells were further investigated. Surprisingly, neither upregulating nor silencing miR-154 had no significant effect on invasion and migration abilities of PCa cells (Additional file 8-Figure S4C and D), even though overexpressing miR-154-5p has been reported to inhibit invasion and migration abilities of PCa cells in several independent studies [35, 45, 46]. Therefore, we posed a hypothesis that miR-154-3p may play an opposite role in regulating invasion and migration abilities of PCa cells, which gave rise to the net results of miR-154 overexpression showing no significant difference on invasion and migration abilities of PCa cells compared with the control groups, because miR-154-3p and miR-154-5p were simultaneously upregulated in miR-154-transfecting PCa cells. To confirm this hypothesis, agomir-154-3p or agomir-154-5p at the concentrations (1 μM) was administered respectively. As shown in Fig. 2A and B, we found that agomir-154-5p reduced, while antagomir-154-5p enhanced invasion and migration abilities of PCa, which were consistent with the aforementioned studies [35, 45, 46]. Conversely, agomir-154-3p expectedly increased invasion and migration abilities of PCa cells; whereas antagomir-154-3p inhibited invasion and migration abilities of PCa cells (Additional file 8-Figure S4E and F). In fact, miR-154-3p has been previously demonstrated to play an oncogenic role in bone metastasis of PCa [34]. Therefore, our results in combination with other study indicate that both strands from a single miR-154 play opposite roles in regulating invasion and migration abilities of PCa cells.

Upregulating Mir-154-5p Represses Proliferation In Pca Cells

To further explore the biological functions of miR-154-5p in bone metastasis of PCa, Gene Set Enrichment Analysis (GSEA) based on miR-154-5p expression data from TCGA was performed. As shown in Additional file 9-Figure S5A-H and Additional file 10-Figure S6A and B, we found that low expression of miR-154-5p correlated with metastatic propensity and proliferation in multiple cancer types. Hence, MTT

assay was further performed to investigate the effects of miR-154-5p on proliferation ability of PCa cells. As shown in Fig. 2C, agomir-154-5p decreased, while antagomir-154-5p increased the proliferation ability of PCa cells. Colony formation assays revealed that agomir-154-5p attenuated, while antagomir-154-5p enhanced the colony forming ability of PCa cells (Fig. 2D). Therefore, our results demonstrate that upregulating miR-154-5p abrogates invasion, migration and proliferation abilities of PCa cells.

agomir154-5p inhibits bone metastasis of PCain vivo

To determine the effect of miR-154-5p on the bone metastasis of PCa *in vivo*, a mouse model of bone metastasis was used, where PC-3 cells were inoculated into the left cardiac ventricle of male nude mic. After two days of inoculation of PC-3 cells, the scramble or agomir-154-5p was injected through tail vein with three days interval for 4 weeks. As shown in Fig. 3A, agomir-154-5p decreased bone metastasis ability compared with the scramble group by X-rays. In addition, agomir-154-5p dramatically reduced the tumor burden in bone by H&E staining (Fig. 3B). Importantly, agomir-154-5p not only decreased bone metastatic score and osteolytic area of metastatic tumors (Fig. 3C and D), but also prolonged bone metastasis-free survival (Fig. 3E). Collectively, our results demonstrate that upregulating miR-154-5p represses the bone metastasis of PCa *in vivo*.

Mir-154-5p Inhibits Pi3k/akt Signaling Pathway

The results of GSEA analysis revealed that miR-154-5p expression significantly correlated with activity of PI3K/AKT signaling pathway that has been demonstrated to play an important role in bone metastasis of PCa [9, 47] (Additional file 11-Figure S7A and B). Thus, we further investigated whether miR-154-5p has an influence on activity of PI3K/Akt signaling in PCa cells. AKT activity was first examined in PCa cells by luciferase reporter assays, and the results revealed that AKT activity was decreased in agomir-154-5p-treated cells and increased in antagomir-154-5p-treated cells (Fig. 4A).Western blotting analysis showed that agomir-154-5p decreased the phosphorylation levels of AKT at S473 and T308, whereas antagomir-154-5p increased their expression (Fig. 4B). Therefore, these results indicate that miR-154-5p inhibits AKT signaling activity in bone metastatic PCa cells.

AKT signaling is essential for the tumor-promoting role of antagomir-154-5p in PCa

We further explored the functional significance of AKT signaling in the pro-metastasis role of miR-154-5p downregulation in PCa cells using an allosteric AKT inhibitor MK-2206. As shown in Additional file 12-Figure S8A, MK-2206 showed gradient inhibition of the AKT activity in a dose-dependent manner in PCa cells. Notably, the stimulatory effect of antagomir-154-5p on AKT activity was abrogated by MK-2206 (Additional file 12-Figure S8B). In addition, inhibition of AKT activity by MK-2206 attenuated the invasion and migration abilities in antagomir-154-5p-treated C4-2B cells (Fig. 4C and D). Consistently, inhibition of AKT kinase activity decreased the proliferation and colony formation abilities stimulated by antagomir-154-5p in C4-2B cells (Fig. 4E and F). These findings indicate that silencing miR-154-5p promotes the invasion, migration and proliferation via activating the AKT signaling pathway in PCa cells.

Mir-154-5p Targets Several Cytokine Receptors

By analyzing several available algorithms TargetScan, miRanda and miRWalk, we found that several cytokine receptors, including EGFR, FGFR1, FGFR2, IGF1R, ERBB4 and INSR, may be potential target of miR-154-5p (Fig. 5A and Additional file 13-Figure S9A). Numerous literatures have reported that several cytokine signaling, including EGF/EFGR [12], IGF-1/IFG1R [13], insulin/INSR [14] and FGF/FGFR [15], have been reported to play important roles in activation of PI3K/Akt pathway. Real-time PCR analysis showed that agomir-154-5p reduced, while antagomir-154-5p increased the mRNA expression levels of EGFR, FGFR1 and IGF1R, but not of FGFR2, ERBB4 and INSR (Fig. 5B, and Additional file 13-Figure S9B and C). Western blot was further performed to examine the effect of miR-154-5p on protein expression levels of EGFR, FGFR1 and IGF1R, and the results showed that agomir-154-5p reduced, while antagomir-154-5p enhanced the protein expression levels of EGFR and FGFR1, but not for IGF1R in PCa cells (Fig. 5C). This finding indicated that miR-154-5p inhibits the expression of EGFR and FGFR1 via inducing mRNA degradation, and IGF1R via translational inhibition in PCa cells. Luciferase assay revealed that agomir-154-5p decreased, while antagomir-154-5p increased the reporter activity of the 3'UTRs of EGFR and FGFR1 transcripts, but had no effect on mutant reporter activity (Fig. 5D, and Additional file 13-Figure S9D and E). RNA immunoprecipitation (IP) assay demonstrated a selective association of miR-154-5p with EGFR and FGFR1 transcripts (Fig. 5E-G). These findings indicate that EGFR and FGFR1 are direct targets of miR-154-5p in PCa cells.

miR-154-5p inhibits invasion, migration and proliferation of PCa cells by targeting EGFR and FGFR1

Subsequent rescue experiments showed that individual silencing EGFR or FGFR1 rescued the AKT activity enhanced by antagomir-154-5p in PCa cells (Fig. 6A). Consistently, silencing EGFR or FGFR1 partially reversed the stimulatory effect of antagomir-154-5p on invasion, migration and colony formation abilities in PCa cells (Fig. 6B-D). ELISA assays showed that upregulation or downregulation of miR-154-5p had no significant effect on EGF, bFGF, IGF1 and IGF2 concentration in the supernantant of PCa cells (Additional file 14-Figure S10A-C). Collectively, these results indicate that miR-154-5p represses AKT signaling activity via inhibiting cytokine receptors rather than cytokines expression, which further inhibits proliferation and mobility of PCa cells.

Discussion

The critical findings of the current study present novel insights into the inhibitory role of miR-154-5p in the activation of PI3K/AKT signaling, which further inhibits bone metastasis of PCa. Here, we reported that miR-154-5p expression was dramatically decreased in bone metastatic PCa tissues, which positively correlated with PSA levels and bone metastasis status in PCa patients, and more importantly predicted poor bone metastasis-free survival in PCa patients. Our results further demonstrated that miR-154-5p repressed PI3K/AKT signaling in PCa cells via directly targeting EGFR and FGFR1, finally inhibiting the development of bone metastasis in PCa. Therefore, our results uncover a novel mechanism by which

miR-154-5p inhibits bone metastasis of PCa, further determining the tumor-suppressive role of miR-154-5p in bone metastasis of PCa.

miR-154-5p has been extensively demonstrated to be downregulated in a variety of cancer types, and function as an important tumor suppressor [33, 35, 48, 49]. Strikingly, several studies have reported that miR-154-5p was upregulated in certain types of cancer, including squamous cell carcinoma of tongue [50], glioblastoma [51] and medullary thyroid carcinoma [52]. These findings suggest that the pro- and anti-cancer role of miR-154-5p is tumor type dependent. In PCa, several lines of evidence have showed that miR-154-5p was downregulated in PCa tissues [45, 53]. Importantly, miR-154-5p expression has been identified to be dramatically downregulated in metastatic cell lines as compared with normal prostatic epithelial cells (PrEC) through miRNAs microarray [35], suggesting that low level of miR-154-5p was implicated in the metastatic phenotype of PCa. However, the clinical significance of miR-154-5p in the progression and bone metastasis of PCa, as well as the biological role of miR-154-5p and its molecular mechanisms underlying bone metastasis of PCa have not been elucidated. In this study, our results demonstrated that miR-154-5p was downregulated in bone metastatic PCa tissues, and low levels of miR-154-5p positively correlated with poor bone metastasis-free survival in PCa patients. Furthermore, our results revealed that miR-154-5p inhibited PI3K/AKT signaling by simultaneously targeting EGFR and FGFR1, which further repressed bone metastasis of PCa. Therefore, our results indicate that miR-154-5p plays a tumor-suppressive role in bone metastasis of PCa. Notably, Gururajan and colleagues have reported that miR-154-3p was elevated in bone metastatic prostate cancer cell lines and tissues, and silencing miR-154-3p led to decreased bone metastasis and increased survival [34]. Our results in combination with this finding elucidate that different spliced products of a pre-miRNAs may play different, even opposite roles in the same biological process.

The PI3K/Akt signaling cascade can be initiated by several stimuli, including G-protein-coupled receptors, receptor tyrosine kinases and cytokine receptors, [10, 11], where cytokine-induced regulatory mechanism has been regarded as a primary way in the activation of PI3K/Akt signaling. In this scenario, activation of PI3K/Akt signaling ignites with the binding of various types of cytokines to the corresponding receptor, such as EGF [12], IGF-1 [13], insulin [14] and FGF [15]. Numerous studies have reported that upregulation of the cytokine receptors, or aberrant secretion of cytokines in an autocrine or paracrine manner contributes to the sustained activity of PI3K/Akt signaling [54–57]. However, how these cytokines and the receptors are concomitantly disrupted in cancers, giving rise to the constitutive activation of PI3K/AKT signaling, remains largely unknown. In the current study, we found that several cytokine receptors, including EGFR, ERBB4, FGFR1, FGFR2, IGF1R and INSR, may be potential targets of miR-154-5p through analyzing publicly available algorithms. Notably, only protein expression levels of EGFR and FGFR1 were decreased in agomir-154-5p-treated PCa cells, and upregulated in antagomir-154-5p-treated PCa cells. Importantly, autocrine levels of the corresponding cytokines were not affected by changed expression of miR-154-5p in PCa cells. Therefore, our results indicate that miR-154-5p inhibits AKT signaling via directly targeting EGFR and FGFR1 in PCa cells.

In metastatic PCa tumor tissues, loss-of-function mutations or genomic alterations of the core components of the PI3K/AKT signaling, such as phosphatase and tensin homolog (PTEN), contributes to 70% of aberrant activation of PI3K/AKT signaling in cancer [58, 59], elucidating the crucial roles of PI3K/AKT signaling in metastatic phenotype of PCa. Moreover, epigenetic regulations has seized more attention as important contributing factors for the unrestrained activation of AKT signaling [60], particularly that dysregulation of miRNAs situates in a compelling component in epigenome. miR-508 has been reported to upregulated in oesophageal squamous cell carcinoma tissues, which further sustained the activation of PI3K/Akt signaling via simultaneously targeted multiple phosphatases, including INPP4A, INPP5J and PTEN, leading to the aggressive phenotype of oesophageal squamous cell carcinoma [42]. In PCa, muitiple miRNAs, including miR-16, miR-106b, miR-148a, miR-4534 and miR-195, have been reported to be implication in the activation of the PI3K/Akt signaling pathway [61–63]. In the current study, our results demonstrated that miR-154-5p concomitantly targeted EGFR and FGFR1 in PCa cells, and antagomir-154-5p dramatically augmented the activity of PI3K/Akt signaling in PCa cells. Therefore, our results unravel a novel mechanism responsive for constitutive activation of PI3K/Akt signaling pathway in the bone metastasis of PCa, supporting a functional and clinical significance of epigenetic events in bone metastasis of PCa.

It has been widely documented that miRNA could serve as a potential non-invasive biomarker for the diagnosis and prognosis of cancer. Recently, the involvement of circulating miR-154-5p as a potential biomarker for diagnosis and prognosis of cancer are becoming increasingly appreciated. In rectal adenocarcinoma, serum miR-154-5p has been identified as a non-invasive predictive biomarker of the chemoradiotherapy responsiveness in patients with rectal adenocarcinoma [64]. Furthermore, low level of serum miR-154-5p was significantly associated with smoking-related lung cancer [65]. However, the potential applicable values of circulating miR-154-5p in PCa as well as its bone metastatic phenotypes remain scanty. In this study, our results demonstrated that low expression of miR-154-5p strongly correlated with bone metastasis-free survival in PCa patients, suggesting that miR-154-5p may serve as a potential bone metastasis diagnostic marker in PCa patients. However, whether serum levels of miR-154-5p in PCa patients can serve as a potential non-invasive marker to predict bone metastasis of PCa will be further confirmed in the following work.

Conclusion

In summary, our results demonstrate that miR-154-5p inhibits PI3K/AKT signaling by targeting EGFR and FGFR1, which further represses the bone metastasis of PCa. Thus, in-depth of understanding the functional role of miR-154-5p in the pathogenesis of PCa bone metastasis will facilitates the development of novel anti-bone metastatic therapeutic methods against PCa.

Abbreviations

BPH: benign prostate hyperplasia; Non-BM:non bone metastasis; BM:bone metastasis; ISH:in situ hybridization; ISUP:International Society of Urological Pathology; miRNAs:MicroRNAs; EGFR:Epidermal

Growth Factor Receptor; FGFR1:Fibroblast Growth Factor Receptor 1; FGFR2:Fibroblast Growth Factor Receptor 2; IGF1R:Insulin Like Growth Factor 1 Receptor; ERBB4:Erb-B2 Receptor Tyrosine Kinase 4; INSR:Insulin Receptor; PCR:Polymerase Chain Reaction; TCGA:The Cancer Genome Atlas; H&E:Hematoxylin and Eosin Stain.

Declarations

Ethical Approval

The ethics approval statements for animal work were provided by theInstitutional Animal Care and Use Committee ofGuangdong Medical University. The ethics approval number for animal work was GDY2102160.

Consent for publication

Not applicable.

Availability of supporting data

The datasets generated and analysed during the current study are available

in the TCGA (https:// https://cancergenome.nih.gov/). Gene Set Enrichment Analysis (GSEA)was performed using GSEA 3.0(http://www.gsea-msigdb.org/gsea/index.jsp), and gene set was performed by Molecular Signatures Database v5.2 (http://

software.broadinstitute.org/gsea/msigdb). Heat map wasperformed by MeV4.9 software (http://mev.tm4.org/).

Competing financial interests

No conflicts of interest were declared.

Funding

This study was supported by the grants from the National Natural Science Foundation of China (81802918; 81902941; 82173284), the China Postdoctoral Science Foundation Grant (2019M660206; 2019M653218), the Science and Technology Project of Guangdong Province (2015A030313835, 2019A1515011565, 2018A030310007; 2019A1515011246), the Medical Science Foundation of Guangdong Province (B2020104, A2018205), the Science and Technology Project of Jiangmen (2020030103140008978, 2019030102430012905), and the Medical Science Foundation of Jiangmen Central Hospital (J202001).

Authors' contributions

Xin Zhang, ZhonglinXue and Yuming Li developed ideas and drafted the manuscript. Dong Ren, Xiangwei Yuan and Jiazheng Cao conducted the experiments and contributed to the analysis of data. Bin Wang, Ruixiao Li andRui Zhangcontributed to the analysis of data. Baoyi Liu,Meimei Wu,Liangliang Ren,Zijie Meng, Wanting Wu, Xingxing Chai andLili Li conducted the experiments. Ronggang Li andXiufang Huang examined the miR-154-5p expression in clinical PCa tissues and performed clinical analysis.Jinhua Wu, Yan ZhengJincheng Zeng andJunjiu Huang contributed to the analysis of data and revised the manuscript. All authors contributed to revise the manuscript and approved the final version for publication.

***Dong Ren, Xiangwei Yuan and Jiazheng Caocontributed equally to this work.**

Acknowledgements

Not applicable

References

1. Weilbaecher KN, Guise TA, McCauley LK: **Cancer to bone: a fatal attraction**. *Nature reviews Cancer* 2011, **11**(6):411-425.
2. Roodman GD: **Mechanisms of bone metastasis**. *The New England journal of medicine* 2004, **350**(16):1655-1664.
3. Bubendorf L, Schopfer A, Wagner U, Sauter G, Moch H, Willi N, Gasser TC, Mihatsch MJ: **Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients**. *Human pathology* 2000, **31**(5):578-583.
4. Siegel RL, Miller KD, Jemal A: **Cancer statistics, 2015**. *CA: a cancer journal for clinicians* 2015, **65**(1):5-29.
5. Dai Y, Ren D, Yang Q, Cui Y, Guo W, Lai Y, Du H, Lin C, Li J, Song L *et al*: **The TGF-beta signalling negative regulator PICK1 represses prostate cancer metastasis to bone**. *British journal of cancer* 2017.
6. Ren D, Dai Y, Yang Q, Zhang X, Guo W, Ye L, Huang S, Chen X, Lai Y, Du H *et al*: **Wnt5a induces and maintains prostate cancer cells dormancy in bone**. *The Journal of experimental medicine* 2019, **216**(2):428-449.
7. Ren D, Yang Q, Dai Y, Guo W, Du H, Song L, Peng X: **Oncogenic miR-210-3p promotes prostate cancer cell EMT and bone metastasis via NF-kappaB signaling pathway**. *Molecular cancer* 2017, **16**(1):117.
8. Chang YS, Chen WY, Yin JJ, Sheppard-Tillman H, Huang J, Liu YN: **EGF Receptor Promotes Prostate Cancer Bone Metastasis by Downregulating miR-1 and Activating TWIST1**. *Cancer research* 2015, **75**(15):3077-3086.
9. Li N, Xue W, Yuan H, Dong B, Ding Y, Liu Y, Jiang M, Kan S, Sun T, Ren J *et al*: **AKT-mediated stabilization of histone methyltransferase WHSC1 promotes prostate cancer metastasis**. *The Journal of clinical investigation* 2017, **127**(4):1284-1302.

10. Cantley LC: **The phosphoinositide 3-kinase pathway**. *Science* 2002, **296**(5573):1655-1657.
11. Chang F, Lee JT, Navolanic PM, Steelman LS, Shelton JG, Blalock WL, Franklin RA, McCubrey JA: **Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy**. *Leukemia* 2003, **17**(3):590-603.
12. Ojeda L, Gao J, Hooten KG, Wang E, Thonhoff JR, Dunn TJ, Gao T, Wu P: **Critical role of PI3K/Akt/GSK3beta in motoneuron specification from human neural stem cells in response to FGF2 and EGF**. *PloS one* 2011, **6**(8):e23414.
13. Peltier J, O'Neill A, Schaffer DV: **PI3K/Akt and CREB regulate adult neural hippocampal progenitor proliferation and differentiation**. *Developmental neurobiology* 2007, **67**(10):1348-1361.
14. Rafalski VA, Brunet A: **Energy metabolism in adult neural stem cell fate**. *Progress in neurobiology* 2011, **93**(2):182-203.
15. Dey JH, Bianchi F, Voshol J, Bonenfant D, Oakeley EJ, Hynes NE: **Targeting fibroblast growth factor receptors blocks PI3K/AKT signaling, induces apoptosis, and impairs mammary tumor outgrowth and metastasis**. *Cancer research* 2010, **70**(10):4151-4162.
16. Vanhaesebroeck B, Stephens L, Hawkins P: **PI3K signalling: the path to discovery and understanding**. *Nature reviews Molecular cell biology* 2012, **13**(3):195-203.
17. Stephens L, Anderson K, Stokoe D, Erdjument-Bromage H, Painter GF, Holmes AB, Gaffney PR, Reese CB, McCormick F, Tempst P *et al*: **Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B**. *Science* 1998, **279**(5351):710-714.
18. Moule SK, Welsh GI, Edgell NJ, Foulstone EJ, Proud CG, Denton RM: **Regulation of protein kinase B and glycogen synthase kinase-3 by insulin and beta-adrenergic agonists in rat epididymal fat cells. Activation of protein kinase B by wortmannin-sensitive and -insensitive mechanisms**. *The Journal of biological chemistry* 1997, **272**(12):7713-7719.
19. Inoki K, Li Y, Zhu T, Wu J, Guan KL: **TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling**. *Nature cell biology* 2002, **4**(9):648-657.
20. Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, Tissenbaum HA, Ruvkun G: **The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans**. *Nature* 1997, **389**(6654):994-999.
21. Luo J, Manning BD, Cantley LC: **Targeting the PI3K-Akt pathway in human cancer: rationale and promise**. *Cancer cell* 2003, **4**(4):257-262.
22. Xue G, Restuccia DF, Lan Q, Hynx D, Dirnhofer S, Hess D, Ruegg C, Hemmings BA: **Akt/PKB-mediated phosphorylation of Twist1 promotes tumor metastasis via mediating cross-talk between PI3K/Akt and TGF-beta signaling axes**. *Cancer discovery* 2012, **2**(3):248-259.
23. Wen W, Ding J, Sun W, Fu J, Chen Y, Wu K, Ning B, Han T, Huang L, Chen C *et al*: **Cyclin G1-mediated epithelial-mesenchymal transition via phosphoinositide 3-kinase/Akt signaling facilitates liver cancer progression**. *Hepatology* 2012, **55**(6):1787-1798.
24. Wang L, Yin J, Wang X, Shao M, Duan F, Wu W, Peng P, Jin J, Tang Y, Ruan Y *et al*: **C-Type Lectin-Like Receptor 2 Suppresses AKT Signaling and Invasive Activities of Gastric Cancer Cells by Blocking**

Expression of Phosphoinositide 3-Kinase Subunits. *Gastroenterology* 2016, **150**(5):1183-1195 e1116.

25. Bartel DP: **MicroRNAs: target recognition and regulatory functions**. *Cell* 2009, **136**(2):215-233.
26. Zhang X, Liu J, Zang D, Wu S, Liu A, Zhu J, Wu G, Li J, Jiang L: **Upregulation of miR-572 transcriptionally suppresses SOCS1 and p21 and contributes to human ovarian cancer progression**. *Oncotarget* 2015, **6**(17):15180-15193.
27. Jiang C, Yu M, Xie X, Huang G, Peng Y, Ren D, Lin M, Liu B, Liu M, Wang W *et al*: **miR-217 targeting DKK1 promotes cancer stem cell properties via activation of the Wnt signaling pathway in hepatocellular carcinoma**. *Oncology reports* 2017, **38**(4):2351-2359.
28. Zhang X, Ren D, Wu X, Lin X, Ye L, Lin C, Wu S, Zhu J, Peng X, Song L: **miR-1266 Contributes to Pancreatic Cancer Progression and Chemoresistance by the STAT3 and NF-kappaB Signaling Pathways**. *Molecular therapy Nucleic acids* 2018, **11**:142-158.
29. Khew-Goodall Y, Goodall GJ: **Myc-modulated miR-9 makes more metastases**. *Nature cell biology* 2010, **12**(3):209-211.
30. Ma L, Young J, Prabhala H, Pan E, Mestdagh P, Muth D, Teruya-Feldstein J, Reinhardt F, Onder TT, Valastyan S *et al*: **miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis**. *Nature cell biology* 2010, **12**(3):247-256.
31. Dai Y, Wu Z, Lang C, Zhang X, He S, Yang Q, Guo W, Lai Y, Du H, Peng X *et al*: **Copy number gain of ZEB1 mediates a double-negative feedback loop with miR-33a-5p that regulates EMT and bone metastasis of prostate cancer dependent on TGF-beta signaling**. *Theranostics* 2019, **9**(21):6063-6079.
32. Cazzoli R, Buttitta F, Di Nicola M, Malatesta S, Marchetti A, Rom WN, Pass HI: **microRNAs derived from circulating exosomes as noninvasive biomarkers for screening and diagnosing lung cancer**. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer* 2013, **8**(9):1156-1162.
33. Wang W, Peng B, Wang D, Ma X, Jiang D, Zhao J, Yu L: **Human tumor microRNA signatures derived from large-scale oligonucleotide microarray datasets**. *International journal of cancer Journal international du cancer* 2011, **129**(7):1624-1634.
34. Gururajan M, Josson S, Chu GC, Lu CL, Lu YT, Haga CL, Zhau HE, Liu C, Lichterman J, Duan P *et al*: **miR-154* and miR-379 in the DLK1-DIO3 microRNA mega-cluster regulate epithelial to mesenchymal transition and bone metastasis of prostate cancer**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2014, **20**(24):6559-6569.
35. Formosa A, Markert EK, Lena AM, Italiano D, Finazzi-Agro E, Levine AJ, Bernardini S, Garabadgiu AV, Melino G, Candi E: **MicroRNAs, miR-154, miR-299-5p, miR-376a, miR-376c, miR-377, miR-381, miR-487b, miR-485-3p, miR-495 and miR-654-3p, mapped to the 14q32.31 locus, regulate proliferation, apoptosis, migration and invasion in metastatic prostate cancer cells**. *Oncogene* 2014, **33**(44):5173-5182.
36. Wu N, Ren D, Li S, Ma W, Hu S, Jin Y, Xiao S: **RCC2 over-expression in tumor cells alters apoptosis and drug sensitivity by regulating Rac1 activation**. *BMC cancer* 2018, **18**(1):67.

37. Edens BM, Vissers C, Su J, Arumugam S, Xu Z, Shi H, Miller N, Rojas Ringeling F, Ming GL, He C *et al*: **FMRP Modulates Neural Differentiation through m(6)A-Dependent mRNA Nuclear Export**. *Cell reports* 2019, **28**(4):845-854 e845.
38. Miller N, Feng Z, Edens BM, Yang B, Shi H, Sze CC, Hong BT, Su SC, Cantu JA, Topczewski J *et al*: **Non-aggregating tau phosphorylation by cyclin-dependent kinase 5 contributes to motor neuron degeneration in spinal muscular atrophy**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2015, **35**(15):6038-6050.
39. Li X, Liu F, Lin B, Luo H, Liu M, Wu J, Li C, Li R, Zhang X, Zhou K *et al*: **miR150 inhibits proliferation and tumorigenicity via retarding G1/S phase transition in nasopharyngeal carcinoma**. *International journal of oncology* 2017.
40. Shi H, Deng HX, Gius D, Schumacker PT, Surmeier DJ, Ma YC: **Sirt3 protects dopaminergic neurons from mitochondrial oxidative stress**. *Human molecular genetics* 2017, **26**(10):1915-1926.
41. Chen J, Liu A, Wang Z, Wang B, Chai X, Lu W, Cao T, Li R, Wu M, Lu Z *et al*: **LINC00173.v1 promotes angiogenesis and progression of lung squamous cell carcinoma by sponging miR-511-5p to regulate VEGFA expression**. *Molecular cancer* 2020, **19**(1):98.
42. Lin C, Liu A, Zhu J, Zhang X, Wu G, Ren P, Wu J, Li M, Li J, Song L: **miR-508 sustains phosphoinositide signalling and promotes aggressive phenotype of oesophageal squamous cell carcinoma**. *Nature communications* 2014, **5**:4620.
43. Liu YN, Yin JJ, Abou-Kheir W, Hynes PG, Casey OM, Fang L, Yi M, Stephens RM, Seng V, Sheppard-Tillman H *et al*: **MiR-1 and miR-200 inhibit EMT via Slug-dependent and tumorigenesis via Slug-independent mechanisms**. *Oncogene* 2013, **32**(3):296-306.
44. Lang C, Dai Y, Wu Z, Yang Q, He S, Zhang X, Guo W, Lai Y, Du H, Wang H *et al*: **SMAD3/SP1 complex-mediated constitutive active loop between lncRNA PCAT7 and TGF-beta signaling promotes prostate cancer bone metastasis**. *Molecular oncology* 2020.
45. Zhu C, Li J, Cheng G, Zhou H, Tao L, Cai H, Li P, Cao Q, Ju X, Meng X *et al*: **miR-154 inhibits EMT by targeting HMGA2 in prostate cancer cells**. *Molecular and cellular biochemistry* 2013, **379**(1-2):69-75.
46. Zheng Y, Zhu C, Ma L, Shao P, Qin C, Li P, Cao Q, Ju X, Cheng G, Zhu Q *et al*: **miRNA-154-5p Inhibits Proliferation, Migration and Invasion by Targeting E2F5 in Prostate Cancer Cell Lines**. *Urologia internationalis* 2017, **98**(1):102-110.
47. Tang Y, Pan J, Huang S, Peng X, Zou X, Luo Y, Ren D, Zhang X, Li R, He P *et al*: **Downregulation of miR-133a-3p promotes prostate cancer bone metastasis via activating PI3K/AKT signaling**. *Journal of experimental & clinical cancer research : CR* 2018, **37**(1):160.
48. Zhao X, Ji Z, Xie Y, Liu G, Li H: **MicroRNA-154 as a prognostic factor in bladder cancer inhibits cellular malignancy by targeting RSF1 and RUNX2**. *Oncology reports* 2017, **38**(5):2727-2734.
49. Song J, Guan Z, Li M, Sha S, Song C, Gao Z, Zhao Y: **MicroRNA-154 Inhibits the Growth and Invasion of Gastric Cancer Cells by Targeting DIXDC1/WNT Signaling**. *Oncology research* 2017.
50. Wong TS, Liu XB, Wong BY, Ng RW, Yuen AP, Wei WI: **Mature miR-184 as Potential Oncogenic microRNA of Squamous Cell Carcinoma of Tongue**. *Clinical cancer research : an official journal of*

the American Association for Cancer Research 2008, **14**(9):2588-2592.

51. Yang L, Yan Z, Wang Y, Ma W, Li C: **Down-expression of miR-154 suppresses tumourigenesis in CD133(+) glioblastoma stem cells**. *Cell biochemistry and function* 2016, **34**(6):404-413.
52. Mian C, Pennelli G, Fassan M, Balistreri M, Barollo S, Cavedon E, Galuppini F, Pizzi M, Vianello F, Pelizzo MR *et al*: **MicroRNA profiles in familial and sporadic medullary thyroid carcinoma: preliminary relationships with RET status and outcome**. *Thyroid : official journal of the American Thyroid Association* 2012, **22**(9):890-896.
53. Zhu C, Shao P, Bao M, Li P, Zhou H, Cai H, Cao Q, Tao L, Meng X, Ju X *et al*: **miR-154 inhibits prostate cancer cell proliferation by targeting CCND2**. *Urologic oncology* 2014, **32**(1):31 e39-16.
54. Turke AB, Zejnullahu K, Wu YL, Song Y, Dias-Santagata D, Lifshits E, Toschi L, Rogers A, Mok T, Sequist L *et al*: **Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC**. *Cancer cell* 2010, **17**(1):77-88.
55. Chen WW, Schoeberl B, Jasper PJ, Niepel M, Nielsen UB, Lauffenburger DA, Sorger PK: **Input-output behavior of ErbB signaling pathways as revealed by a mass action model trained against dynamic data**. *Molecular systems biology* 2009, **5**:239.
56. Ferretti C, Bruni L, Dangles-Marie V, Pecking AP, Bellet D: **Molecular circuits shared by placental and cancer cells, and their implications in the proliferative, invasive and migratory capacities of trophoblasts**. *Human reproduction update* 2007, **13**(2):121-141.
57. Engelman JA, Mukohara T, Zejnullahu K, Lifshits E, Borras AM, Gale CM, Naumov GN, Yeap BY, Jarrell E, Sun J *et al*: **Allelic dilution obscures detection of a biologically significant resistance mutation in EGFR-amplified lung cancer**. *The Journal of clinical investigation* 2006, **116**(10):2695-2706.
58. Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, Arora VK, Kaushik P, Cerami E, Reva B *et al*: **Integrative genomic profiling of human prostate cancer**. *Cancer cell* 2010, **18**(1):11-22.
59. Wang S, Gao J, Lei Q, Rozengurt N, Pritchard C, Jiao J, Thomas GV, Li G, Roy-Burman P, Nelson PS *et al*: **Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer**. *Cancer cell* 2003, **4**(3):209-221.
60. Baylin SB, Jones PA: **A decade of exploring the cancer epigenome - biological and translational implications**. *Nature reviews Cancer* 2011, **11**(10):726-734.
61. Al-Qatati A, Akrong C, Stevic I, Pantel K, Awe J, Saranchuk J, Drachenberg D, Mai S, Schwarzenbach H: **Plasma microRNA signature is associated with risk stratification in prostate cancer patients**. *International journal of cancer Journal international du cancer* 2017, **141**(6):1231-1239.
62. Chen KE, Bustamante K, Nguyen V, Walker AM: **Involvement of miR-106b in tumorigenic actions of both prolactin and estradiol**. *Oncotarget* 2017, **8**(22):36368-36382.
63. Nip H, Dar AA, Saini S, Colden M, Varahram S, Chowdhary H, Yamamura S, Mitsui Y, Tanaka Y, Kato T *et al*: **Oncogenic microRNA-4534 regulates PTEN pathway in prostate cancer**. *Oncotarget* 2016, **7**(42):68371-68384.
64. D'Angelo E, Fassan M, Maretto I, Pucciarelli S, Zanon C, Digito M, Rugge M, Nitti D, Agostini M: **Serum miR-125b is a non-invasive predictive biomarker of the pre-operative chemoradiotherapy**

responsiveness in patients with rectal adenocarcinoma. *Oncotarget* 2016, **7**(19):28647-28657.

65. Huang J, Wu J, Li Y, Li X, Yang T, Yang Q, Jiang Y: **Deregulation of serum microRNA expression is associated with cigarette smoking and lung cancer**. *BioMed research international* 2014, **2014**:364316.

Figures

Figure 1

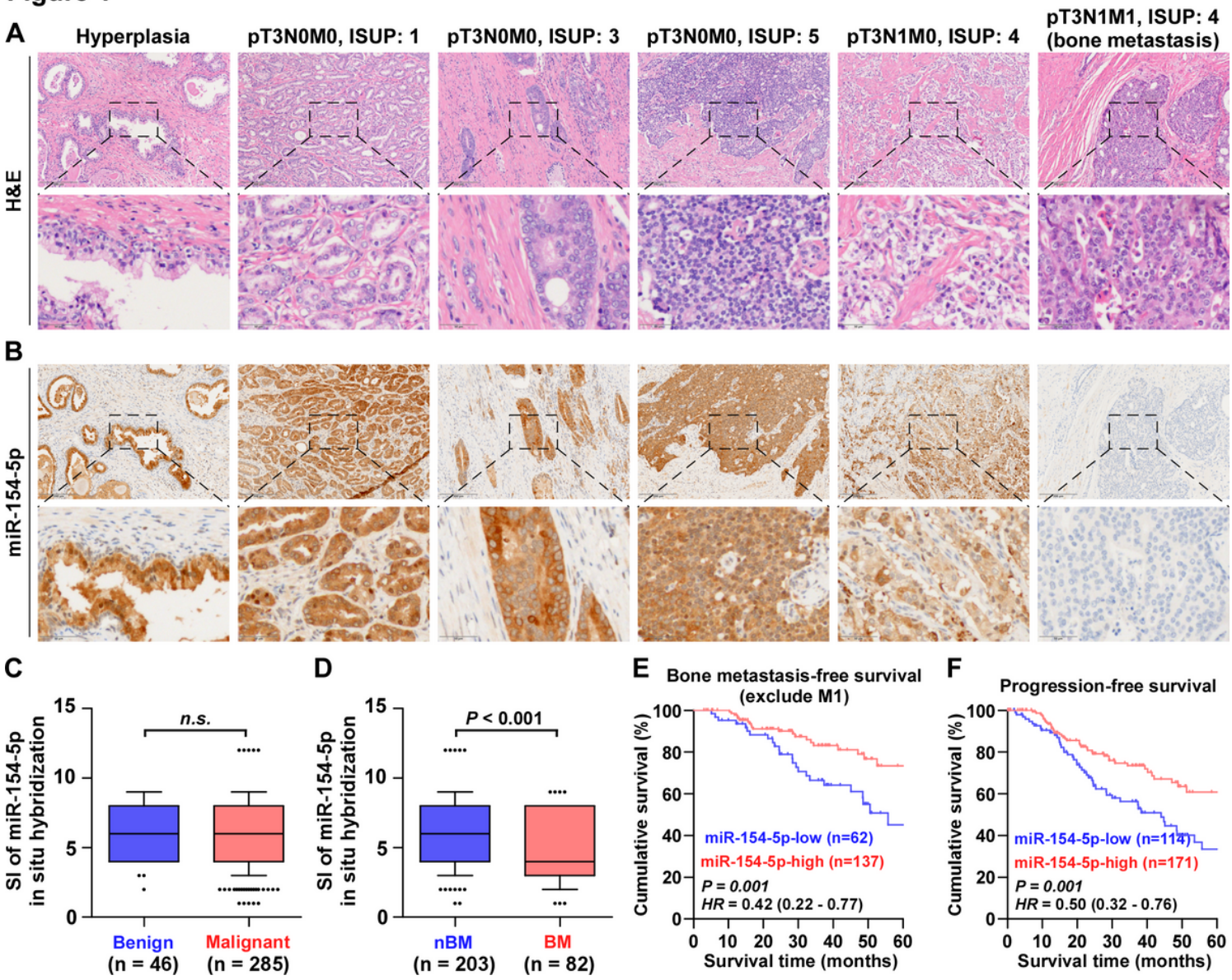

Figure 1

miR-154-5pexpression is downregulatedin bone metastatic PCa tissues.(A)Representative images of low-power (100×, top) and high-power (400×, bottom) fields of H&E staining inbenign prostate hyperplasia tissues (BPH),T3N0M0 PCa tissues with ISUP grade I, T3N0M0PCa tissues with ISUP grade III, T3N0M0PCa tissues with ISUPgrade V, T3N1M0PCa tissues with ISUP grade IV without bone metastasis and T3N1M1PCa tissues with ISUP grade IV with bone metastasis. (B) Representative images of low-

power (100×, top) and high-power (400×, bottom) fields of miR-154-5p expression by in situ hybridization (ISH) in the aforementioned BPH and PCa tissues. Scale bars, 200 mm for 100× magnification and 50 mm for 400× magnification.(C) Staining index of miR-154-5p by ISHin 46 BPH and 285PCa tissues.n.s, means no significance.(D) Staining index of miR-154-5p by ISH in 203 PCa tissues without bone metastasis (nBM) and 82 PCa tissues with bone metastasis (BM).(E)Kaplan–Meier analysis of bone metastasis-free survival curves of PCapatients with high miR-154-5p expression (n = 137) versus low miR-154-5p expression (n = 62).(F)Kaplan–Meier analysis of progression-free survival curves of PCapatients with high miR-154-5p expression (n = 171) versus low miR-154-5p expression (n = 114).

Figure 2

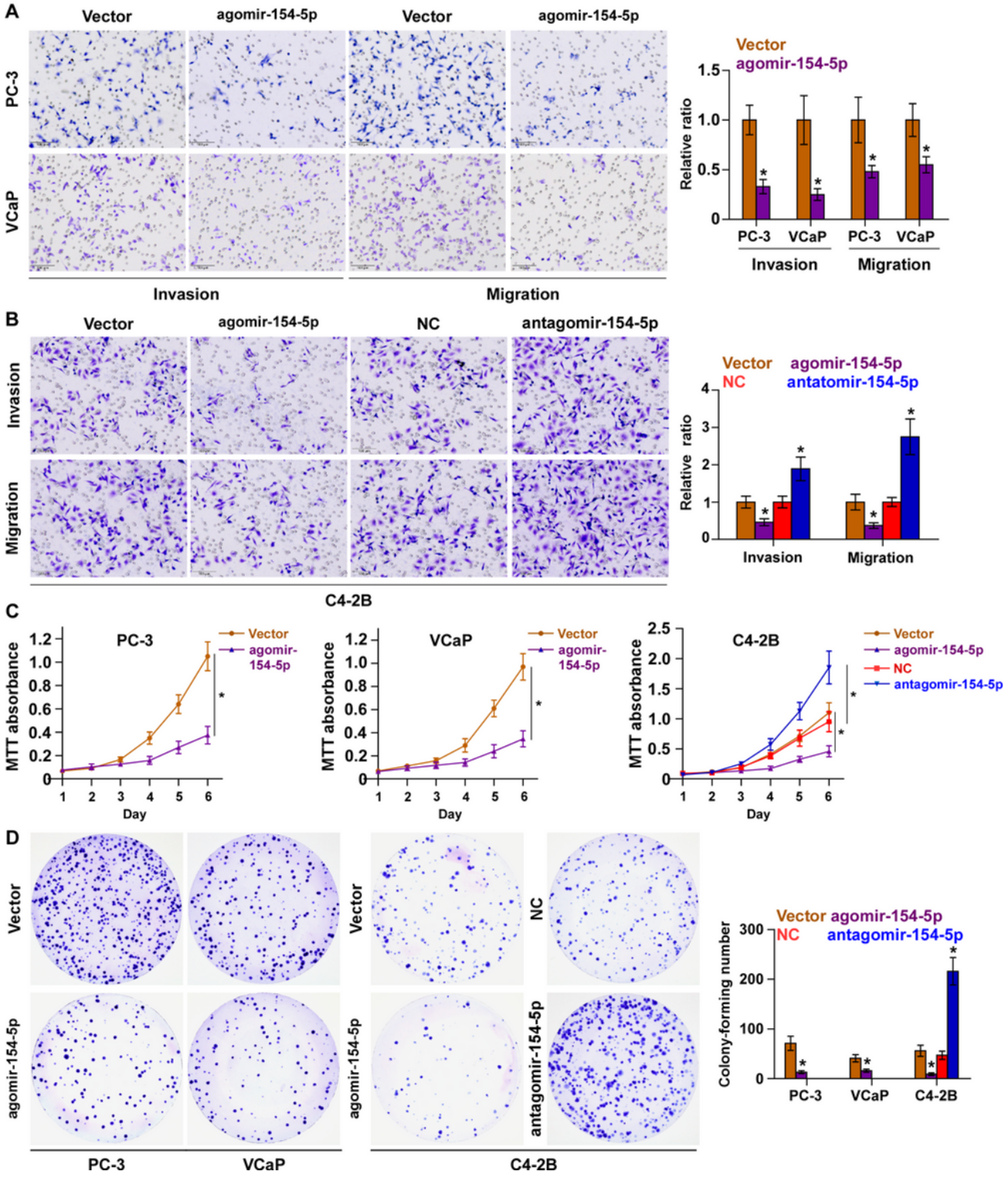

Figure 2

agomir-154-5pinhibits invasion, migrationand proliferation abilities ofPCa cellsin vitro.(A)The effect of agomir-154-5p or antagomir-154-5p on invasionand migration abilities in PC-3 and VCaP cells. Error bars represent the mean ± S.D. of three independent experiments. *P < 0.05.(B)The effect of agomir-154-5p or antagomir-154-5p on invasionand migration abilityin C4-2B cells. Error bars represent the mean ± S.D. of three independent experiments. *P < 0.05.(C) The effect of agomir-154-5p or antagomir-154-5p on the

proliferation of PCa cells assessed by MTT assay.*P< 0.05. (D)The effect of agomir-154-5p or antagomir-154-5p on thecolony number in PCa cells according to the colony formation assay.Error bars represent the mean ± S.D. of three independent experiments. *P < 0.05.

Figure 3

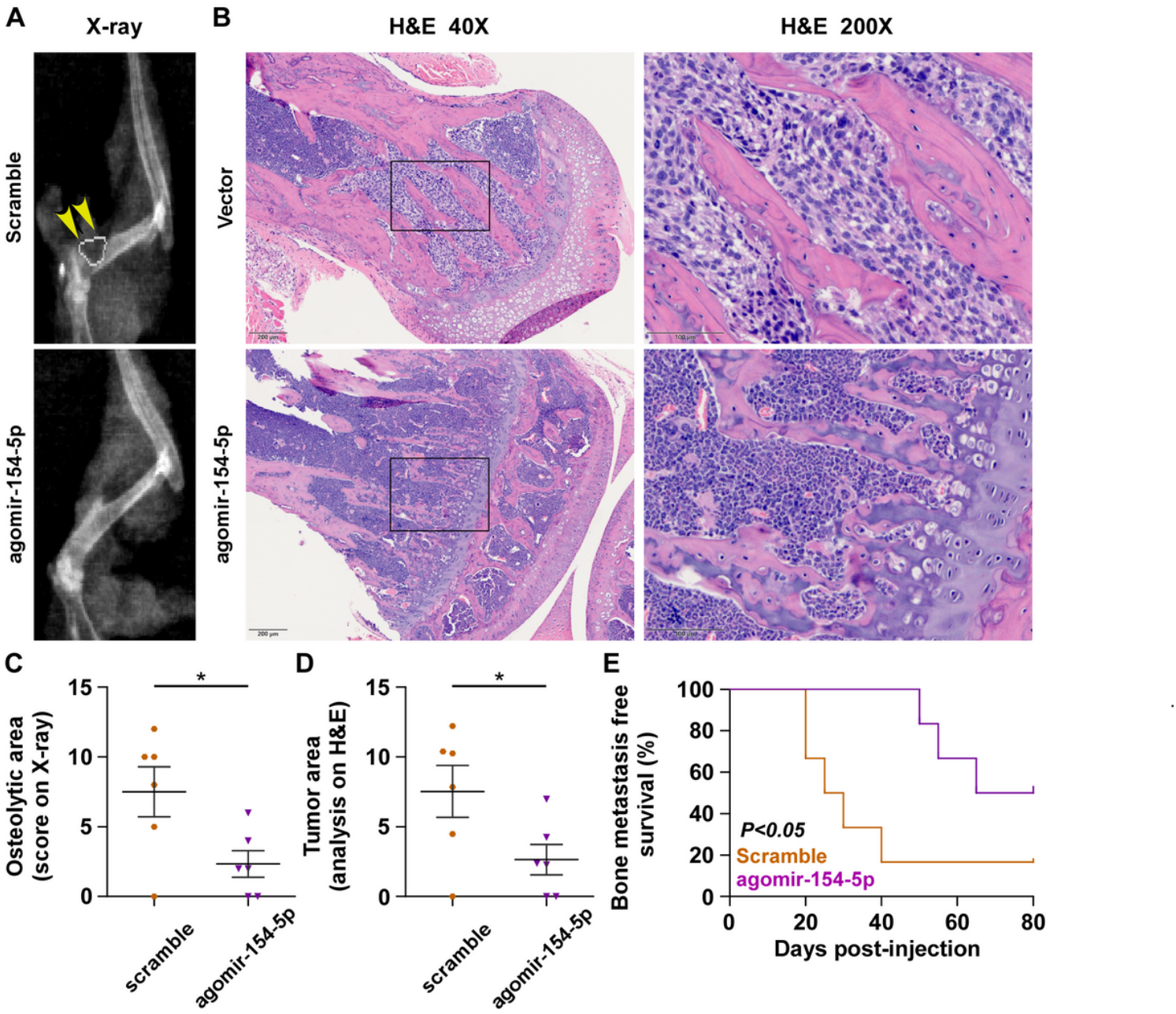

Figure 3

agomir-154-5prepresses bone metastasis of PC-3 cells in vivo. (A) Representative radiographic images of bone metastases in the indicated mice (yellow arrows indicate osteolytic lesions). (B) Representative H&E-stained sections of tibias from the indicated mouse. (C)The sum of bone metastasis scores for each mouse in scramble(n = 6) or agomir-154-5p (n = 6) group of mice. (D)Histomorphometric analysis of bone osteolytic areas in the tibia of the indicated groups.Mean ± SEM; *, P < 0.05. (E) Kaplan-Meyer analysis of mouse bone metastasis-free survivalin the scramble or agomir-154-5p group.

Figure 4

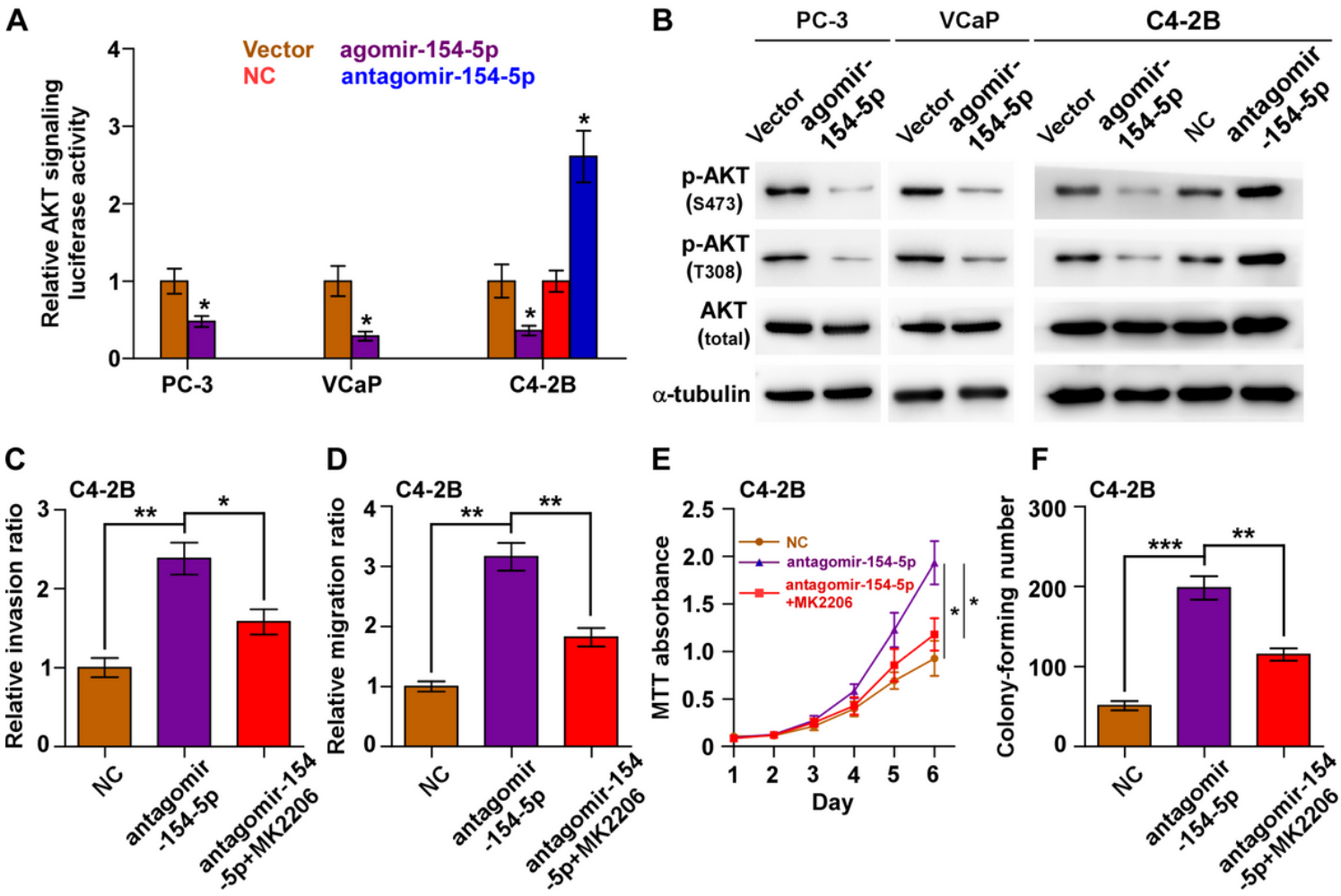

Figure 4

miR-154-5prepresses activity of AKT signalingin PCa cells. (A) Luciferase reporter analysis of AKT signaling activityin the indicated cells. *P < 0.05. (B)Western blotting of p-AKT expressionat S473 and T308 in the indicated cells. α-Tubulin served as the loading control.(C and D) AKT inhibitors MK2206(1 μM) attenuated invasion (C) and migration(D)abilities in antagomir-154-5p-treatedC4-2B cells.*P < 0.05. (E)AKT inhibitors MK2206(1 μM) attenuated proliferation ability in antagomir-154-5p-treatedC4-2B cells.*P < 0.05.(F)AKT inhibitors MK2206(1 μM) attenuated colony formation ability in antagomir-154-5p-treatedC4-2B cells.*P < 0.05.

Figure 5

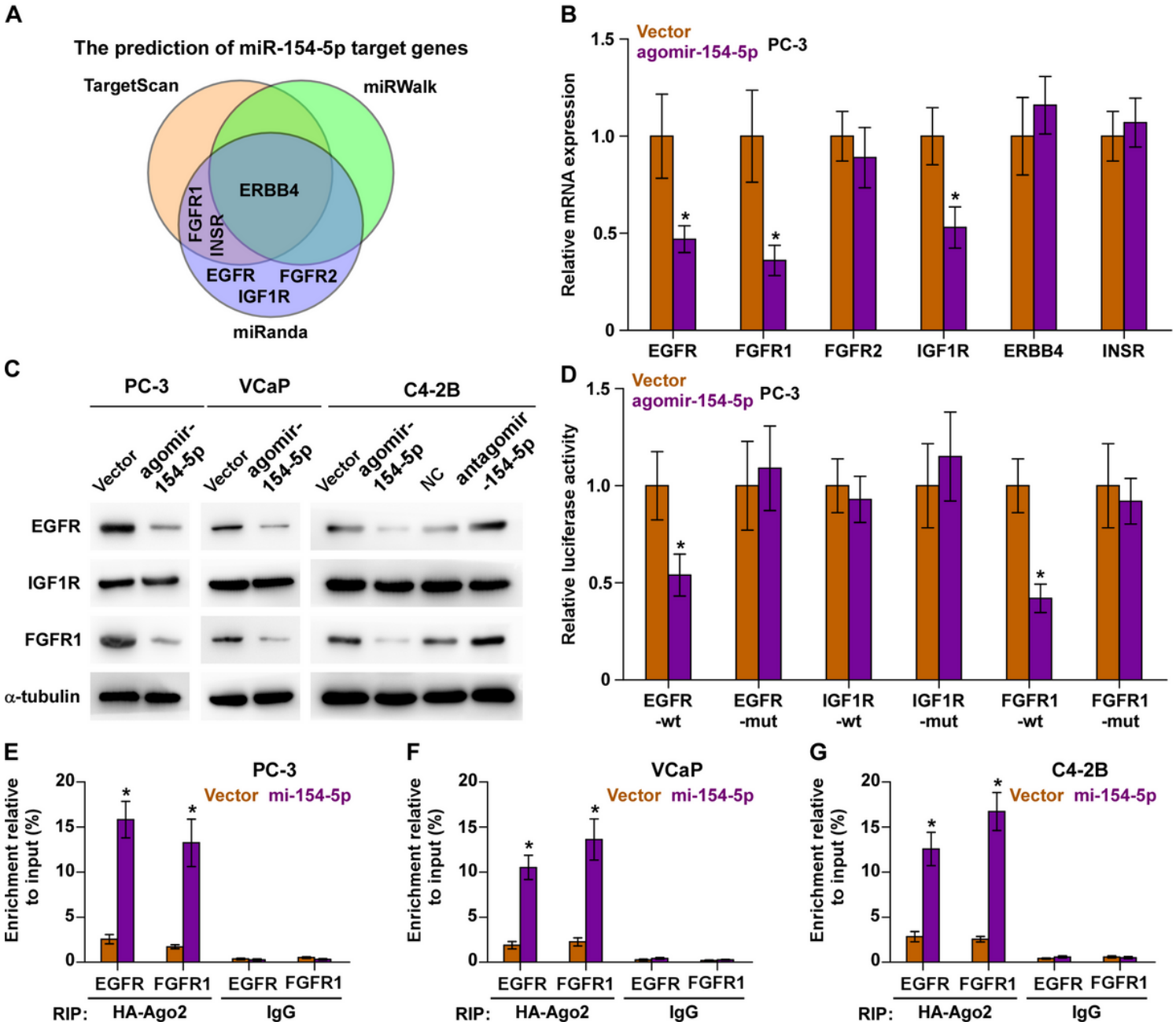

Figure 5

miR-154-5p targets EGFR and FGFR1.(A)Predicted target of miR-154-5p in TargetScan, miRwalk and miRanda. (B) Real-time PCR analysis of EGFR,FGFR1, FGFR2,IGF1R, ERBB4 and INSR expression in the indicated PC-3cells. Transcript levels were normalized by GAPDH expression. Error bars represent the mean ± s.d. of three independent experiments. *P < 0.05.(C) Western blotting of EGFR, IGF1R and FGFR1expressionin the indicated PCacells. α-Tubulin served as the loading control.(D)Luciferase assay of cells transfected with pmirGLO-3'UTRreporter of EGFR, IGF1R and FGFR1in the indicated PC-3 cells, respectively. *P < 0.05.(E-G)MiRNP IP assay showing the association between miR-154-5p and EGFR and FGFR1transcripts in PCa cells. Pulldown of IgGantibody served as the negative control.*P < 0.05.

Figure 6

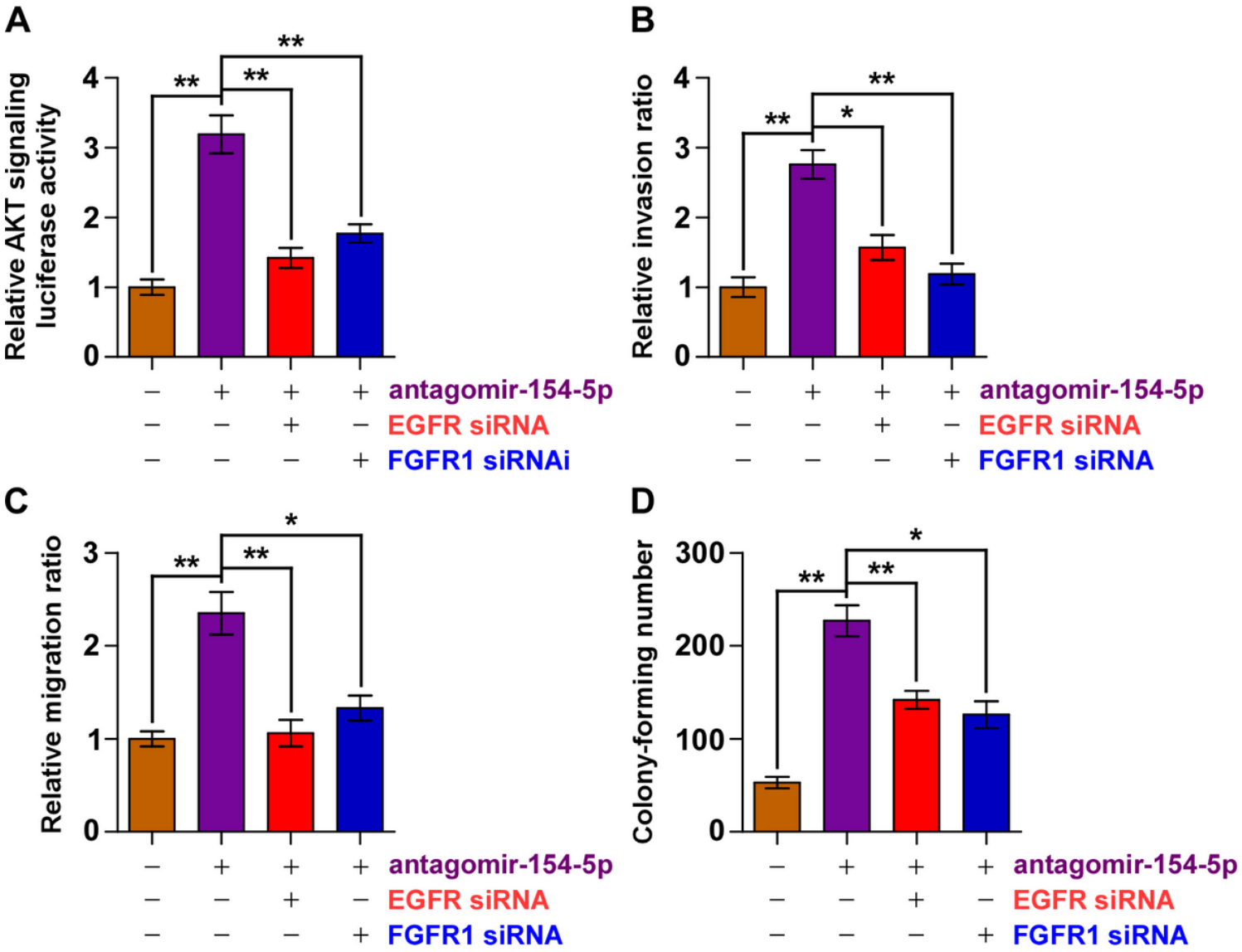

Figure 6

miR-154-5p inhibits invasion, migration and proliferation of PCa cells by targeting EGFR and FGFR1.(A) Individual downregulation of EGFR or FGFR1reversed the activity of AKT signaling increased by antagomir-154-5pin PCa cells.*P< 0.05 and **P< 0.01. (B and C) Individual downregulation of EGFR or FGFR1reversed the invasion (B) and migration(C)abilities enhanced by antagomir-154-5pin PCa cells.*P< 0.05 and **P< 0.01.(D) Individual downregulation of EGFR or FGFR1reversed the colony formation ability increased by antagomir-154-5pin PCa cells.*P< 0.05 and **P< 0.01.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile10FigureS6.pdf

- Additionalfile11FigureS7.pdf
- Additionalfile12FigureS8.pdf
- Additionalfile1TableS1.pdf
- Additionalfile13FigureS9.pdf
- Additionalfile14FigureS10.pdf
- Additionalfile2TableS2.pdf
- Additionalfile3TableS3.pdf
- Additionalfile4TableS4.pdf
- Additionalfile5FigureS1.pdf
- Additionalfile6FigureS2.pdf
- Additionalfile7FigureS3.pdf
- Additionalfile8FigureS4.pdf
- Additionalfile9FigureS5.pdf
- Supplementalfigureslegends.docx