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MicroRNA-224 targets RKIP to control cell invasion and expression of metastasis genes in human breast cancer cells

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ABSTRACT

The Raf kinase inhibitor protein (RKIP) is a tumor suppressor that protects against metastasis and genomic instability. RKIP is downregulated in many types of tumors, although the mechanism for this remains unknown. MicroRNAs silence target genes via translational inhibition or target mRNA degradation, and are thus important regulators of gene expression. In the current study, we found that miR-224 expression is significantly upregulated in breast cancer cell lines, and especially in highly invasive MDA-MB-231 cells, compared to human normal breast epithelial cells. In addition, miR-224 inhibits *RKIP* gene expression by directly targeting its 3′-untranslated region (3′-UTR). Moreover, metastasis, as assayed by Transwell migration, 3D growth in Matrigel, and wound healing, was enhanced by ectopic expression of miR-224 and inhibited by miR-224 downregulation. Promotion of metastasis in response to miR-224 downregulation was associated with derepression of the stroma-associated RKIP target genes, *CXCR4*, *MMP1*, and *OPN*, which are involved in breast tumor metastasis to the bone. Taken together, our data indicate that miR-224 play an important role in metastasis of human breast cancer cells to the bone by directly suppressing the RKIP tumor suppressor.

1. Introduction

Breast cancer is the most common female malignancy, accounting for approximately 22% of all new cancer cases worldwide, with more than 1.05 million new cases every year. Globally, 0.45 million patients died from breast cancer annually, accounting for 13.7% of female cancer deaths [1,2]. In the developing world, including China, breast cancer incidence has been rising in recent years [3]. Breast cancer is prone to metastasis and secondary sites include the lung, liver, bone, and brain. Metastasis can occur many years after the removal of the primary tumor, reducing the survival rate from 85% for early detection to 23% for patients with lung or bone metastasis, and is therefore the main cause of death for breast cancer patients [4]. Currently, the mechanisms controlling metastasis are poorly understood and treatments for metastatic late-stage breast cancer are inefficient and mainly palliative [5]. Hence, it is of great clinical value to understand the molecular mechanisms involved in primary tumor cell invasion and spread to distant sites, such as bone, and thus identify molecular targets for new therapies.

Several regulatory pathways, such as MAPK (MAP kinase) and Akt/protein kinase B (PKB), are important regulators of cell invasion and metastasis in malignant tumors [6,7]. Thus, inhibition of one or more key components of these signaling pathways may provide new therapeutic breakthroughs in cancer treatment. One therapeutic strategy for metastatic tumors is to target metastasis suppressor genes. Raf kinase inhibitory protein (RKIP, also named PEBP1) a metastasis tumor suppressor that regulates MAPK and NFκB signaling pathways [8–10]. Accumulated evidence has demonstrated that RKIP-mediated tumor suppression specifically affects metastatic but not primary tumors in prostate and breast tumor xenografts [11,12]. Moreover, RKIP expression is significantly reduced or absent in a variety of cancer cells, including breast, esophageal, gastric, and prostate cancer [11–14]. Taken together, these studies highlight RKIP as a general suppressor of metastasis.

Metastasis is a complex process involving a series of steps: cellular epithelial–mesenchymal transition leading to basement

Abbreviations: NBEC, normal breast epithelial cells; RKIP, Raf kinase inhibitor protein; HCC, hepatocellular cancer.

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invasion, intravasation into blood or lymph vessels, extravasation from vessels, mesenchymal–epithelial transition, and metastatic colonization of distal tissues [15]. RKIP repression of genes implicated in the development of bone metastasis, Chemokine (C-X-C motif) receptor 4(CXCR4), the matrix metalloproteinase1 (MMP1), the integrin-binding glycoprotein osteopontin(OPN), is reported to be mediated by MAPK inhibition and enhanced transcription of the miRNA let-7 [12,16–19]. MMP1 has important functions in facilitating colonization in bone via expression of bone extracellular matrix degrading enzymes, OPN-mediated osteoclast activation and adhesion to the bone surface, and CXCR4 targets specific homing of tumor cells to the bone microenvironment [20]. In conclusion, CXCR4, MMP1, and OPN are important downstream targets of the RKIP pathway that inhibits bone metastasis, and their expression forms the bone metastasis signature. MicroRNAs (miRNAs) are a class of small, non-coding, single-stranded RNAs that inhibit gene expression at the post-transcriptional level, mainly by binding to a sequence in the 3′-untranslated region (UTR) of target mRNAs [21,22]. miRNAs are involved in regulating various processes, including the proliferation, invasion, metastasis, and prognosis of cancers such as breast, lung, and prostate cancer, and glioma [23–25]. Breast cancer progression requires altered expression of multiple oncogenes and tumor suppressors, and it is likely that miRNAs play a pivotal role in the regulation of these genes [26,27]. miRNAs are now known to be linked to breast cancer metastasis: some have been identified as metastasis promoters (e.g. let-7, miR-9, and miR-10b) and others as metastasis suppressors (e.g. miR-31, miR-126, and miR-145) [28–33]. However, few studies have focused on tissue-specific metastasis of breast cancer, especially to the bone, which is one of the most frequent site. Thus, specific miRNAs may have a potential clinical application in bone metastatic breast cancer, for example as biomarkers and therapeutic targets.

In this study, publicly available algorithms (TargetScan, PicTar, and miRanda) indicated that miR-224, the only conserved miRNA, may directly target the 3′-untranslated region (3′-UTR) of RKIP. We now report that miR-224 is upregulated and inhibits RKIP expression in human breast cancer cells. We observed that miR-224 inhibits breast cancer cell invasion and motility by directly targeting the 3′-UTR of the RKIP transcript, resulting in upregulation of bone metastasis genes, including MMP1, OPN, and CXCR4. Our results suggest that miR-224 might have an important role in promoting the development and progression of breast cancer metastasis to bone.

2. Materials and methods

2.1. Cell culture

Normal breast epithelial cells (NBEC) were obtained from Clonetics-Biowhittaker (Walkersville, MD, USA) and cultured in KSFM medium (Clonetics-Biowhittaker). Breast cancer cell lines MCF-10A, MDA-MB-468, T47D, MCF-7, MDA-MB-453, MDA-MB-231were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in D-Medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Plasmids and transfection

The *RKIP* 3′-UTR was PCR amplified from NBEC RNA and cloned into the *Sac*I/*Xma*I sites of the pGL3-control luciferase reporter (Promega, Madison, WI, USA) and pGFP-C3 (Clontech, Mountain View, CA, USA) plasmids. The primers used were: *RKIP*-3′UTR-wt-up, 5′-GCCCCGCGGCTGTCCTGGAGGCCCCAAGCCATG-3′; *RKIP*-3′UTR-wt-dn, 5′-GCCCTGCAG GAGGTTAGCCTCAATGCCAG-3′; *RKIP*-3′UTR-mu-up, 5′-GCCCCGCGGGATGGTAGTTGAGGGTGACAAT -3′; and *RKIP*-3′UTR-mu-dn, 5′- GCCCTGCAG GAGGTTAGCCTCAATGC-CAG -3′. The miR-224 mimics, negative control, and anti-miR-224 inhibitor were purchased from RiboBio (Guangzhou, Guangdong, China).

2.3. Western blotting

Western blot analysis was performed according to standard methods using anti-RKIP, anti-MMP1, anti-OPN, anti-CXCR4, and anti-green fluorescent protein (GFP) antibodies (Cell Signaling, Danvers, MA, USA). Membranes were stripped and reprobed with an anti-α-tubulin monoclonal antibody (Sigma, St. Louis, MO, USA) to control for loading.

2.4. RNA extraction and real-time quantitative PCR

Total miRNA was purified from cultured cells and using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions and cDNA was synthesized from 5 ng of total RNA using the TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). miR-224 expression was quantified using a miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems). miRNA expression levels were defined based on the threshold cycle (C_t), and relative expression levels were calculated as $2^{-[(Ct\ of\ miR\text{-}224)-(Ct\ of\ U6)]}$ and normalized to U6 small nuclear RNA using the formula $2^{-[(Ct\ of\ miR\text{-}224)-(Ct\ of\ U6)]}$.

Real-time reverse transcription PCR (RT-PCR) was performed using the Applied Biosystems 7500 Sequence Detection system using the following primers: *MMP1*, (forward, 5′-TGTGGACC ATGCCATTGAGAA-3′; reverse, 5′-TCTGCTTGACCCTCAGAGACC-3′); OPN,(forward,5′-ACGGAGACCATGCAGAGAGC-3′;reverse,5′-TTGTG TGCTGGCAGTGAAGG-3′); and CXCR4 (forward, 5′-CAG CAG GTAG CA AAG TGA CG-3′; reverse, 5′-CAG GGT TCC TTC ATG GAG TC-3′). Expression data were normalized to the geometric mean expression of the housekeeping gene, *GAPDH* (forward, 5′-GACTCATGACCA CAGTCCATGC-3′; reverse, 3′-AGAGGCAGGGATG ATGTTCTG-5′) and calculated using $2^{-[(Ct\ of\ MMP1,\ OPN,\ or\ CXCR4)-(Ct\ of\ GAPDH)]}$, where C_t represents the threshold cycle for each transcript.

2.5. Three-dimensional spheroid invasion assay

Cells (1×10^4) were trypsinized and seeded in 24-well plates coated with Matrigel (2%; BD Biosciences), and medium was changed every second day. Microscope images were taken at 2-days intervals for 8 days.

2.6. Transwell matrix penetration assay

Cells (1×10^4) were plated into the upper chamber of the BioCoat Invasion Chambers (BD, Bedford, MA) containing a polycarbonate Transwell filter coated with Matrigel and incubated at 37 °C for 22 h, followed by removal of cells remaining inside the upper chamber using cotton swabs. Cells that had invaded the lower membrane surface were fixed in 1% paraformaldehyde, stained with hematoxylin, and counted (ten random fields per well at 100× magnification). Cell counts are expressed as the mean number of cells per field of view. Three independent experiments were performed and the data are presented as mean ± standard deviation (SD).

2.7. Statistical analysis

A two-tailed Student's *t*-test was used to evaluate the significance of the differences between two groups; *p* values <0.05 were considered significant.

3. Results

3.1. MiR-224 is upregulated in highly invasive MDA-MB-231breast cancer cells

Real-time RT-PCR analysis revealed that miR-224 expression was markedly increased in highly invasive MDA-MB-231 cell lines, and increased to a lesser extent in other breast cancer cells, compared to NBEC (Fig. 1A). This data indicates that miR-224 is strongly upregulated in highly invasive breast cancer cells.

3.2. MiR-224 expression levels correlate with invasiveness in breast cancer cells

To investigate the function of miR-224 in breast cancer, we transfected the hsa-miR-224 mimic into MDA-MB-231 and MCF-7 breast cancer cells and measured the effect on cell invasion. MCF-7 and MDA-MB-231 are breast cancer cell lines: MCF-7 cells were isolated from an in situ breast adenocarcinoma and MDA-MB-231 cells were isolated from a distant metastatic site. MiR-224 overexpression in MDA-MB-231 and MCF-7 cells following transfection was shown by real-time RT-PCR (Fig. 1B). The invasive capacity of transfected cells was then examined using the Transwell–Matrigel penetration assay, which showed that transfection with miR-224 in MDA-MB-231 and MCF-7 cells increased the number of cells that penetrated the gel–membrane barrier (Fig. 1C; $p < 0.01$). Furthermore, in a three-dimensional (3D) spheroid invasion assay, miR-224-transfected cells had more outward projections compared with control cells (Fig. 1D), thus displaying an altered morphology typical of highly invasive cells. Moreover, cell migration measured using the wound healing assay was increased in MDA-MB-231 and MCF-7 cells transfected with miR-224 mimic than in negative controls (Fig. 1E). These results demonstrate that miR-224 upregulation promotes invasion in breast cancer cells.

We next transfected a miR-224 inhibitor into highly invasive MDA-MB-231 breast cancer cells, which led to significant repression of miR-224 (Fig. 2A; $p < 0.01$). Consistent with our previous findings, the Transwell–Matrigel penetration assay indicated that fewer MDA-MB-231 cells transfected with miR-224 inhibitor than transfected negative controls could pass through the gel–membrane barrier (Fig. 2B, $p < 0.01$). Furthermore, the 3D culture in Matrigel and the wound healing assays showed that the invasive ability of MDA-MB-231cells was significantly decreased following transfection with the miR-224 inhibitor (Fig. 2C and D). Taken together, our data suggest that miR-224 controls the invasive ability of MDA-MB-231 breast cancer cells.

3.3. MiR-224 directly targets the RKIP 3′-UTR in breast cancer cells

A previous study revealed that RKIP transcriptionally represses a series of genes relevant to bone metastasis, including *CXCR4*, *MMP1*, and *OPN* [12,16–19]. Interestingly, our bioinformatic

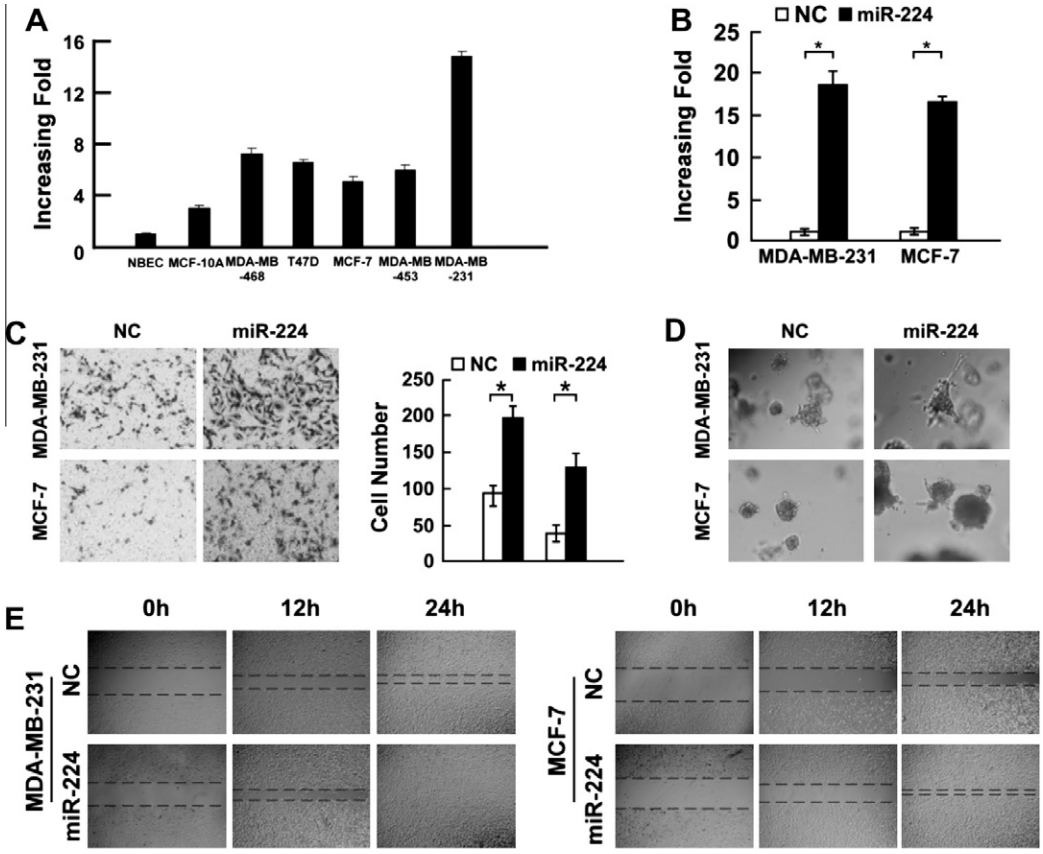

Fig. 1. MiR-224 upregulation promotes breast cancer cell metastasis. (A) Real-time RT-PCR analysis of miR-224 expression in the highly invasive breast cancer cell line MDA-MB-231 and other breast cell lines, including NBEC, MCF-10A, MCF-7, MDA-MB453, T47D, MDA-MB-468. (B) Real-time RT-PCR analysis of miR-224 expression in indicated cells. (C) Transwell assays indicate that invasiveness of miR-224-transfected cells is increased compared to negative control (NC)-transfected cells. Cells that penetrated the gel–membrane barrier are counted. (D) 3D growth capacity is increased in miR-224-overexpressing MDA-MB-231 and MCF-7 cells. (E) Wound healing is increased in MDA-MB-231 and MCF-7cells transfected with miR-224 mimic compared to negative controls (NC). Bars represent the mean ± SD of three independent experiments; $^{*}p < 0.05$.

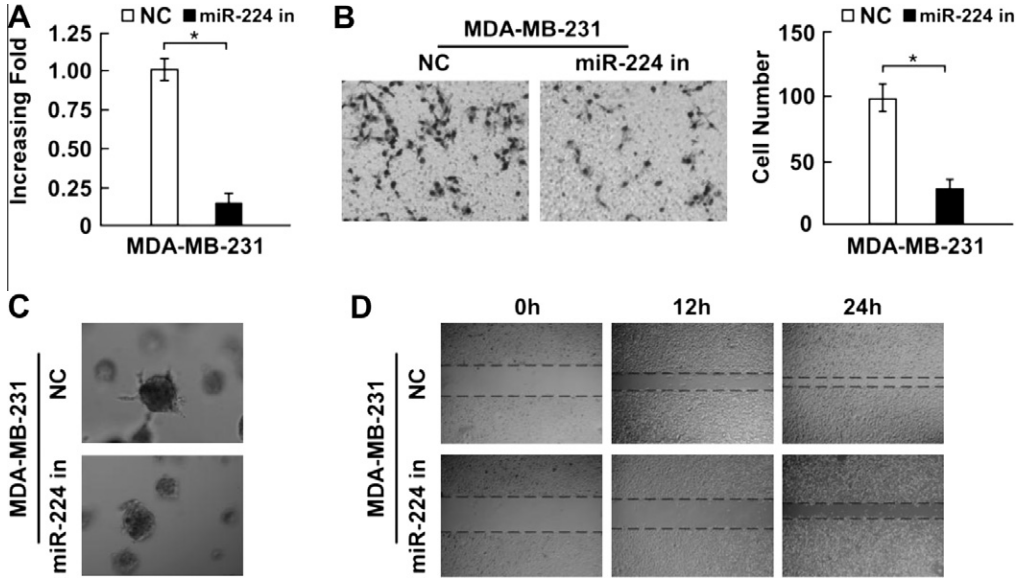

Fig. 2. Inhibition of miR-224 represses MDA-MB-231 cell invasion. (A) Real-time RT-PCR analysis of miR-224 expression in MDA-MB-231 cells transfected with the miR-224 inhibitor. (B) Transwell assays indicate that membrane penetration is reduced in MDA-MB-231 cells transfected with the miR-224 inhibitor. (C) 3D growth capacity is reduced in MDA-MB-231 cells transfected with the miR-224 inhibitor. (D) Wound healing is reduced in MDA-MB-231 cells transfected with the miR-224 inhibitor. Bars represent the mean ± SD of three independent experiments; $^{*}p < 0.05$.

analysis indicated that miR-224 may directly target *RKIP* 3′-UTR (Fig. 3A). RKIP is downregulated in the highly invasive breast cancer cell line MDA-MB-231 (data not shown), in which miR-224 is upregulated (Fig. 1A). We observed that ectopic miR-224 expression decreased RKIP protein levels in MDA-MB-231 cells (Fig. 3B and C), supporting our identification of *RKIP* as a potential miR-224 target gene. In addition, miR-224-dependent *RKIP* downregulation was associated with significant increases in MMP1, OPN, and CXCR4 expression (Fig. 3B).

To confirm that miR-224 inhibition of *RKIP* is mediated by binding to the 3′-UTR, we cloned the *RKIP* 3′-UTR into both GFP (pEGFP-C3) and luciferase (pGL3) reporter plasmids, and investigated the effect of miR-224 and miR-224 inhibitor on reporter activity. GFP fluorescence was dramatically reduced by ectopic expression of miR-224 in MDA-MB-231 cells transfected with the GFP reporter, compared to those transfected with a control plasmid, suggesting that miR-224 specifically targets the *RKIP* 3′-UTR (Fig. 3C). Furthermore, miR-224 transfection reduced the luciferase activity of the *RKIP* 3′-UTR luciferase reporter plasmid in MDA-MB-231 breast cancer cells in a dose-dependent manner (Fig. 3D). MiR-224 repression of the *RKIP* 3′-UTR was abrogated by point mutations in the miR-224 seed region of the *RKIP* 3′-UTR (Fig. 3D). Moreover,

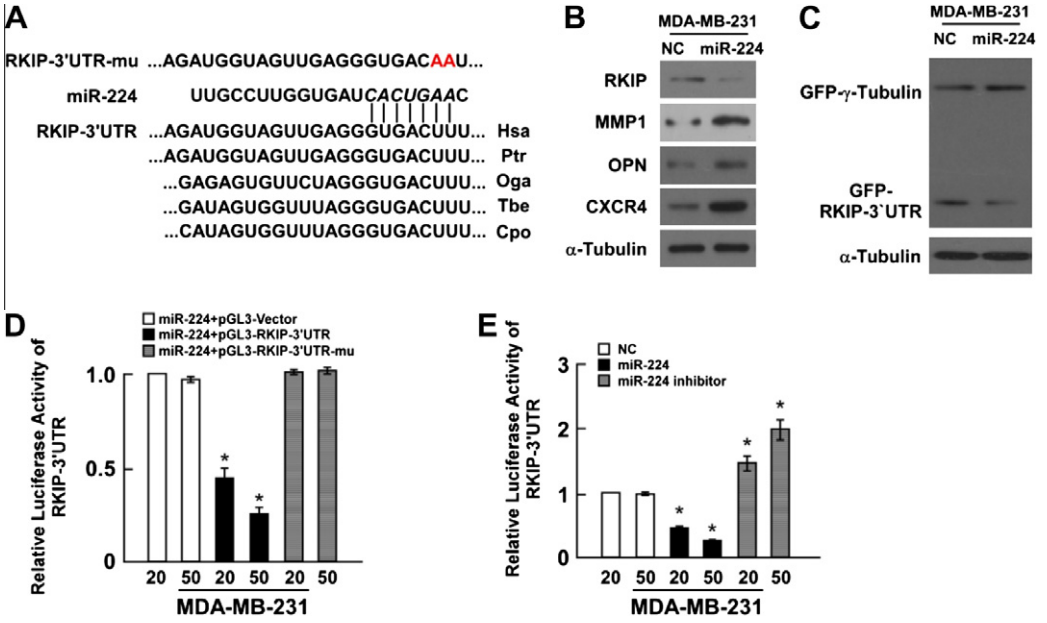

Fig. 3. MiR-224 downregulates RKIP by directly targeting the RKIP 3′-UTR. (A) Sequence of the *RKIP* 3′-UTR showing the miR-224 binding seed region and mutation of the *RKIP* 3′-UTR seed region to create RKIP-mu. Conservation of the miR-224 seed region in indicated species. (B) RKIP, CXCR4, MMP1, and OPN protein expression in MDA-MB-231 cells transfected with miR-224 or negative control (NC)-transfected cells. (C) GFP reporter gene expression in MDA-MB-231 cells transfected with miR-224 or NC-transfected cells. (D) Relative luciferase activity of MDA-MB-231 cells cotransfected with increasing amounts of miR-224 mimic oligonucleotides (20, 50 nM), and the pGL3 control reporter, pGL3-RKIP-3′UTR reporter, or pGL3-RKIP-3′UTR-mu reporter. (E) Relative luciferase activity of MDA-MB-231 cells co-transfected with increasing amounts of miR-224 mimic oligonucleotides (20, 50 nM) or miR-224 inhibitor oligonucleotides (20, 50 nM). Bars represent the mean ± SD of three independent experiments; $^{*}p < 0.05$.

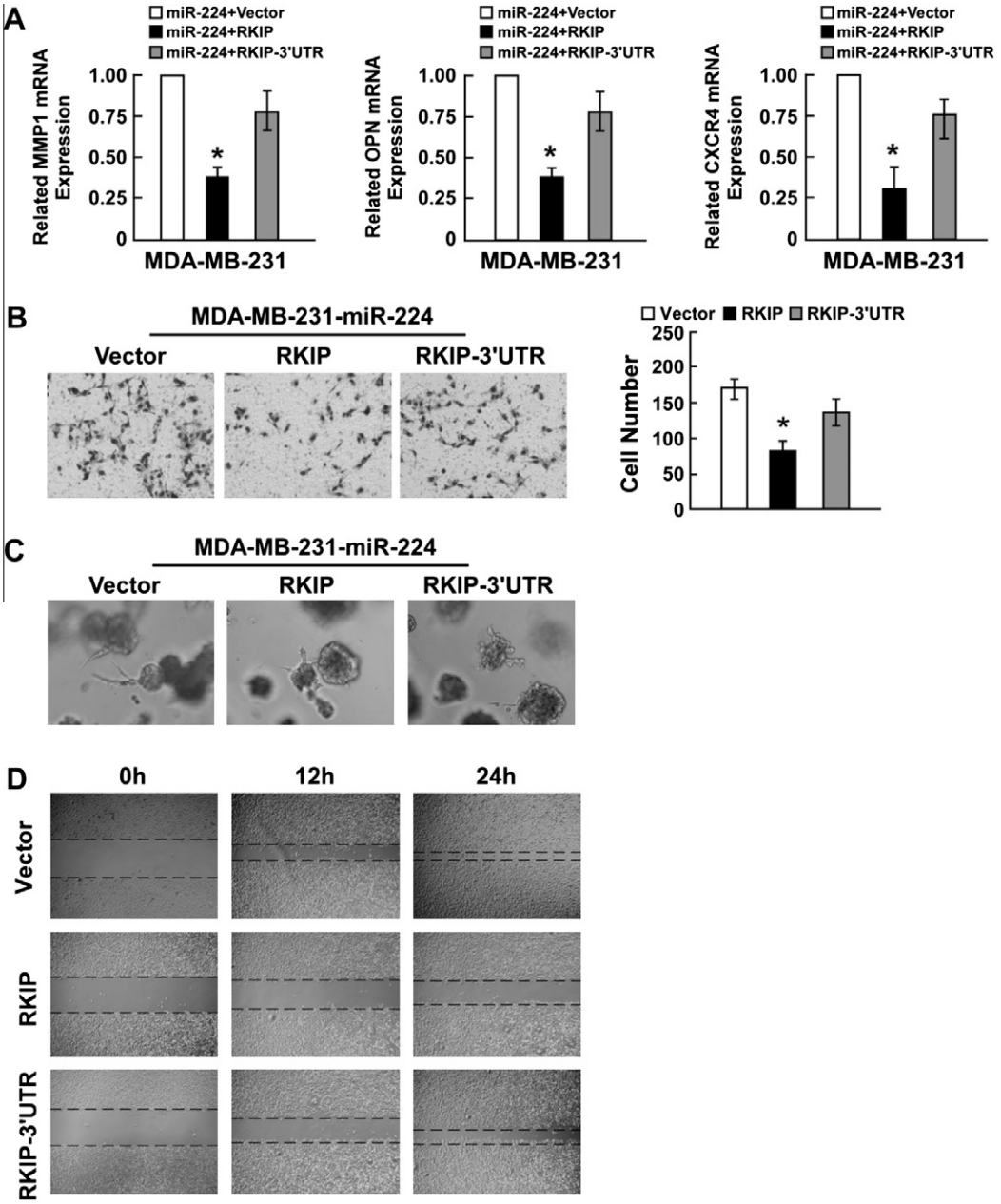

Fig. 4. MiR-224-induced breast cancer cell metastasis is mediated by RKIP. (A) Real-time RT-PCR analysis of *CXCR4*, *MMP1*, and *OPN* mRNA expression in transfected or control MDA-MB-231 cells. Expression was normalized to *GAPDH*. (B) Transwell migration assay of MDA-MB-231 overexpressing miR-224 cells transfected with RKIP or RKIP-3′-UTR expression vectors or empty control. Cells that penetrated the gel–membrane barrier are counted. (C) 3D culture assay shows the invasive capacity of MDA-MB-231 cells transfected with RKIP or RKIP-3′-UTR expression vectors or empty control. (D) Wound healing assay showing the migration rate of MDA-MB-231 cells overexpressing miR-224 cells cotransfected with RKIP or RKIP-3′-UTR expression vectors or empty control. Bars represent the mean ± SD values of three independent experiments; $^{*}p < 0.05$.

transfection of a miR-224 inhibitor increased basal activity of the pGL3-RKIP-3′UTR reporter in MDA-MB-231 cancer cells in a dose-dependent manner (Fig. 3E). These results confirm *RKIP* to be a miR-224 target.

Importantly, RKIP overexpression and miR-224 inhibition could both reduce mRNA levels of the RKIP targets *CXCR4*, *MMP1*, and *OPN* (Supplemental Fig. S1A and B)

3.4. MiR-224-induced breast cancer cell metastasis is modulated by RKIP

To investigate the role of RKIP in miR-224-induced cell invasion, *RKIP* cDNA (lacking the 3′-UTR), *RKIP*-3′-UTR (including the 3′-UTR) expression plasmids, and control vector were transfected into MDA-MB-231 cells. Expression of *MMP1*, *OPN*, and *CXCR4* mRNA was reduced more greatly in cells cotransfected with miR-224 and RKIP, compared to cells transfected with miR-224 and RKIP-3′-UTR (Fig. 4A). Furthermore, MDA-MB-231 cell invasion, assayed by Transwell migration, 3D Matrigel culture, and wound healing, was significantly inhibited following cotransfection of miR-224 and RKIP compared to cells cotransfected with miR-224 and RKIP-3′-UTR or with miR-224 and vector (Fig. 4B–D). In conclusion, our data suggest that miR-224-induced breast cancer cell invasion is directly mediated by *RKIP* suppression.

4. Discussion

In the past decade, the importance of tumor–stroma interactions for the progression of primary tumors to an aggressive and

invasive phenotype has been recognized. Through an interdisciplinary approach, RKIP was identified as an inhibitor of invasion and metastasis through the regulation of stroma-associated genes [13]. RKIP binds to Raf-1 and inhibits Raf-1-mediated MEK phosphorylation [34–36]and can regulate various cellular processes, in particular metastasis [36]. In breast cancer, RKIP suppresses metastasis to the bone by regulating CXCR4, MMP1, and OPN [13]. In the current study, we identified the *RKIP* tumor suppressor gene as a putative miR-224 target using bioinformatic analysis, and confirmed that miR-224 can target the RKIP 3′-UTR in breast cancer cells.MiRNAs are important regulators of many biological processes [37,38]and miR-224 expression is frequently upregulated in various human tumor types, including colorectal cancer, hepatocellular cancer (HCC), and renal cancer [39–41]. In addition, miR-224 is significantly overexpressed in highly invasive and metastatic pancreatic ductal adenocarcinoma (PDAC) and CD40 protein levels are significantly reduced in these tumors when miR-224 is upregulated, suggesting that *CD40* is a target of miR-224 [15]. Moreover, miR-224 expression in clear cell renal cell carcinoma is increased more than fourfold compared to control samples [40]. Furthermore, miR-224 is one of the most highly differentially expressed miRNAs in methotrexate-resistant cells, and its overexpression induces the resistant phenotype in HT29 colon cancer cells [41]. Taken together, these studies suggest that miR-224 is an onco-miR. In agreement, our findings link miR-224 upregulation to breast cancer invasion and also indicate that miR-224 may function as an onco-miRNA.

However, the mechanism of miR-224 upregulation in breast cancer remains unclear. Increased miR-224 expression in HCC tumors correlates with overexpression of the EP300 transcriptional coactivator and increased EP300 binding to the Xq28 locus, where the miR-224 gene is located [39]. These findings suggest that miR-224 is upregulated through an epigenetic mechanism. An alternative explanation is that gene amplification of the Xq28 locus upregulates miR-224. However, further research is required to determine whether these or other mechanisms regulate miR-224 expression in breast cancer.

In summary, the key finding of our current study is that miR-224 upregulation increases MDA-MB-231 breast cancer cell invasion by inhibiting RKIP. We have therefore shown that miR-224 plays an essential role in regulating breast cancer cell invasion and metastasis and may function as an onco-miRNA. Understanding the precise role of miR-224 in breast cancer progression will not only advance our knowledge of breast cancer biology but may also verify miR-224 as a novel therapeutic target for the treatment of breast cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.025.

References

[1] D.M. Parkin, Global cancer statistics in the year 2000, Lancet Oncol. 2 (2001) 533–543.
[2] A. Jemal, F. Bray, M.M. Center, J. Ferlay, E. Ward, D. Forman, Global cancer statistics, CA Cancer J. Clin. 61 (2011) 69–90.
[3] P. Porter, "Westernizing" women's risks? Breast cancer in lower-income countries, N. Engl. J. Med. 358 (2008) 213–216.
[4] G. Lorusso, C. Ruegg, New insights into the mechanisms of organ-specific breast cancer metastasis, Semin Cancer Biol 22 (2012) 226–233.
[5] J.H. Lee, A. Nan, Combination drug delivery approaches in metastatic breast cancer, J. Drug. Deliv. 2012 915375.
[6] D.J. Mulholland, N. Kobayashi, M. Ruscetti, A. Zhi, L.M. Tran, J. Huang, M. Gleave, H. Wu, Pten loss and RAS/MAPK activation cooperate to promote EMT and metastasis initiated from prostate cancer stem/progenitor cells, Cancer Res 72 (2012) 1878–1889.
[7] A. Carnero, The PKB/AKT pathway in cancer, Curr. Pharm. Des. 16 (2010) 34–44.
[8] G. Antoun, P.B. Cannon, H.Y. Cheng, Regulation of MAPK/ERK signaling and photic entrainment of the suprachiasmatic nucleus circadian clock by Raf kinase inhibitor protein, J. Neurosci. 32 (2012) 4867–4877.
[9] K. Deiss, C. Kisker, M.J. Lohse, K. Lorenz, Raf kinase inhibitor protein (RKIP) dimer formation controls its target switch from Raf1 to G protein coupled receptor kinase (GRK) 2, J. Biol. Chem. 287 (2012) 23407–23417.
[10] B. Bonavida, S. Baritaki, The novel role of Yin Yang 1 in the regulation of epithelial to mesenchymal transition in cancer via the dysregulated NF-kappaB/Snail/YY1/RKIP/PTEN Circuitry, Crit. Rev. Oncog. 16 (2011) 211–226.
[11] G. Ren, S. Baritaki, H. Marathe, J. Feng, S. Park, S. Beach, P.S. Bazeley, A.B. Beshir, G. Fenteany, R. Mehra, S. Daignault, F. Al Mulla, E. Keller, B. Bonavida, I. de la Serna, K.C. Yeung, Polycomb protein EZH2 regulates tumor invasion via the transcriptional repression of the metastasis suppressor RKIP in breast and prostate cancer, Cancer Res. 72 (2012) 3091–3104.
[12] E. Bevilacqua, C.A. Frankenberger, M.R. Rosner, RKIP Suppresses Breast Cancer Metastasis to the Bone by Regulating Stroma-Associated Genes, Int. J. Breast Cancer 2012 124704.
[13] Y. Fujimori, M. Inokuchi, Y. Takagi, K. Kato, K. Kojima, K. Sugihara, Prognostic value of RKIP and p-ERK in gastric cancer, J Exp Clin Cancer Res. 31 (2012) 30.
[14] P. Birner, B. Jesch, A. Schultheis, S.F. Schoppmann, RAF-kinase inhibitor protein (RKIP) downregulation in esophageal cancer and its metastases, Clin. Exp. Metastasis. 29 (2012) 551–559.
[15] D.X. Nguyen, J. Massague, Genetic determinants of cancer metastasis, Nat. Rev. Genet. 8 (2007) 341–352.
[16] S. Dangi-Garimella, J. Yun, E.M. Eves, M. Newman, S.J. Erkeland, S.M. Hammond, A.J. Minn, M.R. Rosner, Raf kinase inhibitory protein suppresses a metastasis signalling cascade involving LIN28 and let-7, EMBO J. 28 (2009) 347–358.
[17] J. Yun, C.A. Frankenberger, W.L. Kuo, M.C. Boelens, E.M. Eves, N. Cheng, H. Liang, W.H. Li, H. Ishwaran, A.J. Minn, M.R. Rosner, Signalling pathway for RKIP and Let-7 regulates and predicts metastatic breast cancer, Embo. J. 30 (2011) 4500–4514.
[18] A. Boire, L. Covic, A. Agarwal, S. Jacques, S. Sherifi, A. Kuliopulos, PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells, Cell 120 (2005) 303–313.
[19] Z. Zhou, Z.W. Chen, X.H. Yang, L. Shen, X.H. Ai, S. Lu, Q.Q. Luo, Establishment of a biomarker model for predicting bone metastasis in resected stage III non-small cell lung cancer, J Exp Clin Cancer Res. 31 (2012) 34.
[20] P.H. Anborgh, J.C. Mutrie, A.B. Tuck, A.F. Chambers, Role of the metastasis-promoting protein osteopontin in the tumour microenvironment, J Cell. Mol. Med. 14 (2010) 2037–2044.
[21] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[22] M.V. Iorio, C.M. Croce, MicroRNAs in cancer: small molecules with a huge impact, J. Clin. Oncol. 27 (2009) 5848–5856.
[23] X.B. Shi, L. Xue, J. Yang, A.H. Ma, J. Zhao, M. Xu, C.G. Tepper, C.P. Evans, H.J. Kung, R.W. deVere White, An androgen-regulated miRNA suppresses Bak1 expression and induces androgen-independent growth of prostate cancer cells, Proc Natl Acad Sci U S A 104 (2007) 19983-19988.
[24] A. Musiyenko, V. Bitko, S. Barik, Ectopic expression of miR-126*, an intronic product of the vascular endothelial EGF-like 7 gene, regulates prostein translation and invasiveness of prostate cancer LNCaP cells, J. Mol. Med. (Berlin) 86 (2008) 313–322.
[25] Z. Lu, M. Liu, V. Stribinskis, C.M. Klinge, K.S. Ramos, N.H. Colburn, Y. Li, MicroRNA-21 promotes cell transformation by targeting the programmed cell death 4 gene, Oncogene 27 (2008) 4373–4379.
[26] S. Pollari, S.K. Leivonen, M. Perala, V. Fey, S.M. Kakonen, O. Kallioniemi, Identification of MicroRNAs Inhibiting TGF-beta-Induced IL-11 Production in Bone Metastatic Breast Cancer Cells, PLoS One 7 (2012) e37361.
[27] K. Tjensvoll, K.N. Svendsen, J.M. Reuben, S. Oltedal, B. Gilje, R. Smaaland, O. Nordgard, MiRNA expression profiling for identification of potential breast cancer biomarkers, Biomarkers 17 (2012) 463–470.
[28] Y. Khew-Goodall, G.J. Goodall, Myc-modulated miR-9 makes more metastases, Nat Cell Biol 12 (2010) 209–211.
[29] F. Yu, H. Yao, P. Zhu, X. Zhang, Q. Pan, C. Gong, Y. Huang, X. Hu, F. Su, J. Lieberman, E. Song, Let-7 regulates self renewal and tumorigenicity of breast cancer cells, Cell 131 (2007) 1109–1123.
[30] L. Ma, F. Reinhardt, E. Pan, J. Soutschek, B. Bhat, E.G. Marcusson, J. Teruya-Feldstein, G.W. Bell, R.A. Weinberg, Therapeutic silencing of miR-10b inhibits metastasis in a mouse mammary tumor model, Nat. Biotechnol. 28 (2010) 341–347.
[31] D.M. Pedrioli, T. Karpanen, V. Dabouras, G. Jurisic, G. van de Hoek, J.W. Shin, D. Marino, R.E. Kalin, S. Leidel, P. Cinelli, S. Schulte-Merker, A.W. Brandli, M.

Detmar, MiR-31 functions as a negative regulator of lymphatic vascular lineage-specific differentiation in vitro and vascular development in vivo, Mol Cell Biol. 30 (2010) 3620–3634.

[32] M. Sachdeva, Y.Y. Mo, MicroRNA-145 suppresses cell invasion and metastasis by directly targeting mucin 1, Cancer Res. 70 (2010) 378–387.

[33] R. Feng, X. Chen, Y. Yu, L. Su, B. Yu, J. Li, Q. Cai, M. Yan, B. Liu, Z. Zhu, MiR-126 functions as a tumour suppressor in human gastric cancer, Cancer Lett. 298 (2010) 50–63.

[34] K. Yeung, T. Seitz, S. Li, P. Janosch, B. McFerran, C. Kaiser, F. Fee, K.D. Katsanakis, D.W. Rose, H. Mischak, J.M. Sedivy, W. Kolch, Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP, Nature 401 (1999) 173–177.

[35] R.I. Gregory, R. Shiekhattar, MicroRNA biogenesis and cancer, Cancer Res. 65 (2005) 3509–3512.

[36] G.A. Calin, C.M. Croce, MicroRNA signatures in human cancers, Nat. Rev. Cancer 6 (2006) 857–866.

[37] Y. Wang, C.G. Lee, Role of miR-224 in hepatocellular carcinoma: a tool for possible therapeutic intervention?, Epigenomics 3 (2011) 235–243

[38] J. Boguslawska, A. Wojcicka, A. Piekielko-Witkowska, A. Master, A. Nauman, MiR-224 targets the 3'UTR of type 1 5'-iodothyronine deiodinase possibly contributing to tissue hypothyroidism in renal cancer, PLoS One 6 (2011) e24541.

[39] N. Mencia, E. Selga, V. Noe, C.J. Ciudad, Underexpression of miR-224 in methotrexate resistant human colon cancer cells, Biochem. Pharmacol. 82 (2011) 1572–1582.

[40] S.T. Mees, W.A. Mardin, S. Sielker, E. Willscher, N. Senninger, C. Schleicher, M. Colombo-Benkmann, J. Haier, Involvement of CD40 targeting miR-224 and miR-486 on the progression of pancreatic ductal adenocarcinomas, Ann. Surg. Oncol. 16 (2009) 2339–2350.

[41] Y. Kang, P.M. Siegel, W. Shu, M. Drobnjak, S.M. Kakonen, C. Cordon-Cardo, T.A. Guise, J. Massague, A multigenic program mediating breast cancer metastasis to bone, Cancer Cell 3 (2003) 537–549.