MicroRNA-200a Promotes Anoikis Resistance and Metastasis by Targeting YAP1 in Human Breast Cancer

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Statement of translational relevance:

Distant metastasis remains a major obstacle to successful breast cancer treatment. Anoikis (detachment-induced apoptosis) has been suggested to act as a barrier to metastasis. Here, we report that miR-200a, functioning as an anoikis suppressor, promotes anoikis resistance by targeting YAP1 and subsequently leads to distant metastasis in breast cancer. Our study also provides evidence that overexpression of miR-200a might affect the prognosis of breast cancer patients. Therefore, understanding the mechanism and function of miR-200a as a pro-metastatic miRNA may provide new therapeutic opportunities for breast cancer treatment.
Abstract:
Purpose:
The process of metastases involves the dissociation of cells from the primary tumor, penetration into the basement membrane, invasion, and exiting from the vasculature to seed and colonize distant tissues. miR-200a is involved in this multistep metastatic cascade. This study aimed to test the hypothesis that miR-200a promotes metastasis through increase anoikis resistance in breast cancer.

Experimental design:
Breast cancer cells transfected with mimic or inhibitor for miR-200a were assayed for anoikis in vitro. miR-200a expression was assessed by quantitative real-time PCR (qRT-PCR). Luciferase assays, colony formation assays, and animal studies were performed to identify the targets of miR-200a and the mechanism by which it promotes anoikis resistance.

Results:
We found that overexpression of miR-200a promotes while inhibition of miR-200a suppresses anoikis resistance in breast cancer cells. We identified Yes-associated protein 1 (YAP1) as a novel target of miR-200a. Our data showed that targeting of YAP1 by miR-200a resulted in decreased expression of pro-apoptotic proteins, which leads to anoikis resistance. Overexpression of miR-200a protected tumor cells from anoikis and promoted metastases in vivo. Furthermore, knockdown of YAP1 phenocopied the effects of miR-200a overexpression, whereas restoration of YAP1 in miR-200a overexpressed breast cancer cells reversed the effects of miR-200a on anoikis and metastasis. Remarkably, we found that YAP1 expression was inversely correlated with miR-200a expression in breast cancer clinical specimens and miR-200a expression was associated with distant metastasis in breast cancer patients.

Conclusions:
Our data suggest that miR-200a function as anoikis suppressor, and contributes to metastasis in breast cancer.
Introduction:
Breast cancer is the second-leading cause of cancer-related death in women. Exciting progress has been made in the systemic therapy and earlier diagnosis of breast cancer (1). However, distant metastases remain a major obstacle in the successful treatment of breast cancer, and the mechanism of breast cancer metastasis is still not completely understood (2, 3).

Emerging evidence indicates that the expression of several microRNAs (miRNAs), including miR-10b, miR-335 and miR-31, is altered in the metastatic cascade (4-6). The miR-200 family has multiple functions in regulation of cancer stem cells, chemosensitivity and apoptosis (7-10). It was recently reported that the miR-200a suppress the epithelial-mesenchymal transition (EMT), the initiating step of metastasis (11-14). Interestingly, miR-200 family promotes breast cancer metastasis to lung in an isogenic model (15, 16). miRNAs are known to target the metastatic cascade and enhance both pro- and anti-metastatic signals, especially in the early and late steps of tumor metastasis (4, 6, 17).

Anoikis is an important barrier to distant metastasis. Resistance to anoikis enhances the survival of cancer cells during systemic circulation, thereby facilitating secondary tumor formation in distant organs (14, 15). miR-200a, a member of the miR-200 family, involves in multiple steps of metastasis, such as suppressing EMT and promoting the lung metastasis. However, the function of miR-200a on the regulation of anoikis remains unknown.

YAP1 (Yes-associated protein 1), a key node of the Hippo signaling pathway, has been implicated as a tumor suppressor in breast (18). Binding of Tyr-357 phosphorylated YAP1 with the PY motif allows p73 to escape Itch-mediated ubiquitination (19-21). Loss function of YAP1 was reported to protect cells from anoikis(18). In this study, we identified a new role of miR-200a in anoikis in breast cancer and confirmed that YAP1 is a novel target of miR-200a. miR-200a induced anoikis resistance was phenocopied by knockdown of YAP1. Furthermore, ectopic expression of miR-200a promoted both anoikis resistance and metastasis of human breast cancer cells in animal models. In summary, we combine experimental and clinical studies to establish a role for miR-200a in anoikis resistance. We show that, by targeting YAP1, miR-200a allows cells to evade anoikis and embark on a metastasis spread.

Materials and Methods:
Cell lines and breast tumor specimens
Breast cancer cell lines obtained from ATCC were grown according to the ATCC-recommended culture conditions. Bcap37 cells were obtained from the Cell Bank of China and maintained in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics (Invitrogen, San Diego, CA, USA). All primary human breast cancers specimens were obtained from patients undergoing surgery for breast cancer during 2003-2005 at the Shanghai Cancer Center and stored in liquid nitrogen until analysis. All patients provided written informed consent. The study was approved by the Ethics Committee of the Cancer Hospital, Fudan
siRNA, miRNA, Plasmid Construction, Transfection, and Luciferase Assays

Specific siRNAs and negative controls (scrambled siRNA) were purchased from Genepharma, and miR-200a mimics, inhibitors, and negative controls were obtained from Ribobio. Cells cultured in 6-well plates were transfected with 100 pmol/well siRNA and miRNA mimic or with 400 pmol/well antagomiRNA using the Hilymax Transfection Agent (Dojindo) according to the manufacturer’s protocol. The negative controls (miR mimics and antagomiRNA) consisted of random sequences had no detectable effects in human cell lines or tissues. The sequences of the siRNAs, miRNAs, and negative controls are listed in Table S1.

The coding domain sequence of human YAP1 mRNA was cloned into NotI/BamHI sites of the pQCXIH vector (Clontech). The 3′-UTR of human YAP1 mRNA was cloned into the Xhol/NotI sites of the psiCHECK2 Luciferase vector (Promega). The In-Fusion® Advantage PCR Cloning Kit (Clontech) was used in the plasmid cloning. For luciferase assays, HEK293T cells were plated in 6-well plates and transfected with 4 µg of the psiCHECK-YAP1 3′-UTR reporter per well and 100 pmol of the miR-200a mimic or 400 pmol antagomiR-200a or a negative control. Site-specific mutants of the 3′-UTR reporters for YAP1 were also tested. The clone and site mutant primer sequences are listed in the supplementary material Table S1.

Protein Extraction and Western Blot Analysis

Cells were lysed in SDS cell lysis buffer supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics) for 30 minutes on ice. The homogenates were centrifuged at 13,000 rpm for 10 minutes at 4 °C. Supernatants were collected, and protein concentrations were determined by the Bradford assay (BioRad). The proteins were then resolved on SDS/polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Millipore). The membranes were probed with the following primary antibodies: p73, cleaved caspase-3, and Bim (1:1000, Epitomics); Noxa (1:500) (AbD Serotec); YAP1 (1:1000), Bax (1:1000), and GAPDH (1:2000, Proteintech). After further washes, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000, Proteintech), and blots were developed using ECL (Millipore). Quantity One software (BioRad) was used to quantify YAP1 and GAPDH expression levels.

RNA Extraction and Quantification of Mature miRNAs

The tissues were homogenized with Polytron PT100. Total RNA was extracted using the TRIzol reagent (Invitrogen) or the miRVana miRNA isolation kit (Ambion). The primers for miR-200a and U6 detection assays were purchased from Ribobio. Total RNAs, isolated from cell lines or tissues, were reverse-transcribed using a specific stem-loop RT primer (50 nM) and the ReverTra Ace®qPCR RT Kit (Toyobo). The RT conditions consisted of 15 minutes at 42°C followed by 5 minutes at 98°C. Levels of mature miRNAs were quantified by qRT-PCR using the SYBR® Green Real-time PCR Master Mix (Toyobo). Quantitative PCR was performed using an Applied Biosystems 7900HT real-time PCR system, and data were collected and analyzed using ABI SDS version 2.3. To calculate relative concentrations, miR-200a and U6 CT values were obtained for all samples. The mRNA level of GAPDH was used as an internal control for gene-specific mRNA
analysis. The normalized expression of each sample was designated as CT and obtained by dividing the CT value of miR-200a by the U6 CT of the same sample. The relative amount of miRNA or mRNA in each sample was calculated using the comparative CT method. The results are presented as fold change of expression in cells or cancer tissues.

Anoikis assay
Anoikis was induced by polyHEMA (Sigma, St. Louis, MI, USA) culture. A solution containing 20 mg/ml polyHEMA in 95% ethanol was made, and 0.95 ml/mm² of this solution was pipetted into 35-mm wells. The plates were allowed to sit, partially covered, until the ethanol evaporated and the poly-HEMA had solidified. Periodically, the plates were rocked by hand to help ensure even coating. Prior to use, the wells were washed twice with phosphate-buffered saline (PBS) and once with DMEM to remove residual ethanol. In all, 10⁵ cells of each line, suspended in 2 ml DMEM with 10% FBS, were incubated in the polyHEMA-coated wells for 48 hours in a humidified (37°C, 5% CO₂) incubator. After incubation, the cells were used for further analyses.

Apoptosis and colony formation assays of cells treated in suspension culture
Following induction of anoikis for 48 hours, caspase-3 activity was measured using the Caspase-Glo 3/7 assay kit (Promega) according to the manufacturer’s instructions. Apoptosis was detected by FITC-Annexin V/propidium iodide (PI) double staining (Invitrogen). After washing twice with PBS, cells were labeled with FITC-Annexin V and PI for 10 min in the dark at room temperature, and cellular fluorescence was measured using a FACScan flow cytometer (Becton Dickinson). Each experiment was performed in triplicate. After suspension culture, cells were seeded into normal plates at a density of 2000 cells/100-mm² dishes. For colony formation assays, plates were incubated for 2 weeks, and the resulting colonies were stained with crystal violet and counted.

Animals studies
MDA-MB-231 cells were transfected with synthetic hsa-mir-200a, YAP1 siRNA, or scrambled RNA and then labeled with 1,1’-Diocatadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI, Invitrogen). Labeled cells were resuspended in culture medium at a concentration of 10⁷ cell/ml and injected into the center of the yolk sac of transgenic fl:EGFP zebrafish (25 embryos/group, and 100 cells/embryo). One and three days after injection, DiI-labeled MDA-MB-231 cells were detected in green fluorescent protein (GFP)-labeled vessels using a fluorescence microscope, and surviving cells were counted in the tail vein (22). Cell-Injected zebrafish were examined with Olympus BX61/SZX12 microscopes and photographed with a DP70 digital camera. Images were processed using Adobe Photoshop software. The data were derived from three replicated experiments.

4- to 6-week-old athymic female BALB/c nu/nu mice were provided by the Shanghai Institute of Materia Medica at the Chinese Academy of Science. MDA-MB-231 cells were infected with pMR-miR-200a and pMR-miR-Ctrl (Genecopoeia). YAP1 reintroduced MDA-MD-231 cells were also used in the experiments. GFP labeled MCF-7 cells were transfected with antagomiR-200a and antagomiR-Ctrl. Those cells were resuspended in PBS at a concentration of 10⁷ cells/ml, and
animals were injected with 100 µl of each cell line. In all experiments, the cells were introduced via tail vein injection. The animals were sacrificed and autopsied 3 weeks after injection. Metastasis was assessed by macroscopic observation of the major organs for secondary tumors and confirmed by histology. All animal experiments were performed with approval from the Shanghai Medical Experimental Animal Care Commission.

**Statistical analysis**

Data were shown as mean ± SEM unless otherwise noted. Statistical significance was analyzed by the unpaired Student’s t test, and \( P < 0.05 \) was considered to be statistically significant.

**Results:**

**miR-200a is a determinant of anoikis in breast cancer**

miR-200a expression was evaluated in seven breast cancer cell lines by qRT-PCR. Three cell lines (MCF-7, MDA-MB-468, and HBL100) had high levels of miR-200a, whereas the remaining cell lines showed significantly lower expression (Fig. 1A) consistent with the previous report(23). To examine the role of miR-200a in anoikis, MDA-MB-231 (i.e., low miR-200a expression) and MCF-7 (i.e., high miR-200a expression) cells were transfected with a miR-200a mimic and antagoniR-200a, respectively. The transfected cells were transferred into polyHEMA-coated plates for 48 hours incubation, which prevents cell attachment and induces anoikis (Fig. 1B, C and supplementary Fig1). First, Annexin V/PI staining showed that MCF-7 antagoniR-200a cells were sensitive to suspension-induced apoptosis (Fig. 1B, C, and \( P = 0.003 \)). In contrast, the MDA-MB-231 miR-200a mimic cells were resistant to suspension-induced apoptosis (Fig. 1B, C, and \( P < 0.01 \)). Second, Caspase-3 cleavage was observed in MCF-7-antagomiR-200a cells when they were brought into suspension. In contrast, cleavage of Caspase-3 was suppressed in MDA-MB-231 cells transfected with miR-200a mimic (Fig. 1D). Third, following 48 hours of suspension culture, the cells were replated and cultured in normal conditions, and the colonies were scored after an additional two weeks of culture. Transfection of miR-200a mimic increased colony formation in MDA-MB-231 cells (Fig. 1E, \( P < 0.01 \)). However, silencing miR-200a with antagoniR-200a reduced colony formation in MCF-7 cells (Fig. 1E, \( P < 0.01 \)). Together, these results demonstrate a critical role for miR-200a in the suppression of anoikis in breast cancer cells.

**YAP1 is negatively regulated by miR-200a**

To identify the miR-200a targets we performed an in silico search using Targetscan, MiRanda, and Diana (24-27). Cytoscape was used to find an intersection between genes that regulate the apoptotic processes (GO: 0042981) and predicted target genes of miR-200a (Fig. 2A) (28, 29). Among the potential targets which were listed in Supplementary Table S2, 5 candidate genes (TGFB2, TIAM1, FOXA1, YAP1, and ITSN1) were selected. We carried out colony formation assay to screen the genes. YAP1 attracted our attention because it mostly promoted colony formation after suspension culture. (Supplementary Fig. S3). YAP1 expression was also reported lost in human breast tumors (18).

To confirm YAP1 is a target of miR-200a, 3′-UTR of the YAP1 gene was cloned into the
Luciferase construct psiCHECK2 (Fig. 2B). Luciferase assay revealed that miR-200a significantly reduced the activity of the luciferase reporter gene fused to the YAP1 3' UTR by 52.5% ± 4.9% compared to miR-Ctrl in HEK293T cells. Intriguingly, miR-200a did not decrease the luciferase activity of a mutant construct that contained substitutions at three nucleotides within the miR-200a binding site (Fig. 2C). In contrast, when the wild-type reporter was co-transfected with the antagomiR-200a, the relative luciferase activity of the reporter was significantly enhanced (P < 0.01) (Fig. 2D). These results suggested that YAP1 was a direct target of miR-200a. Furthermore, we found that transfection with the miR-200a mimic caused a 60% reduction of YAP1 protein expression in MDA-MB-231 cells (Fig. 2C). In contrast, suppression of miR-200a with antagomiR-200a increased YAP1 protein expression in MCF-7 cells (Fig. 2D). These data indicate that miR-200a directly targets the 3' UTR of YAP1 to repress the expression of YAP1 protein.

**miR-200a suppresses anoikis by targeting YAP1**

We next investigated the correlation between miR-200a and YAP1 expression in breast cancer cell lines. Four among seven cell lines expressed high levels of miR-200a presented undetectable or low-levels of YAP1. Two cell lines (MDA-MB-231 and ZR-75-30) that expressed low levels of miR-200a expressed high levels of YAP1 (Fig. 3A). To further explore the effect of YAP1 on suspension-induced apoptosis, MDA-MB-231 and ZR-75-30 cells were transfected with YAP1 siRNA followed treated with 48 hours of suspension culture, then replated, and cultured under normal conditions. Both cell lines exhibited increased colony formation (Supplementary Fig. S1A, B).

Because YAP1 is a key modulator of p73, which regulates pro-apoptotic genes, we examined caspase 3 cleavages and caspase 3/7 activity in YAP1 siRNA transfected cells. The data showed knockdown of YAP1 inhibited caspase-3 cleavage in both MDA-MB-231 and ZR-75-30 cells (Fig. 3B) and the caspase 3/7 activity also repressed by YAP1 siRNA in those cells. Furthermore, we examined whether the expression of noxa, bax, and Bim, three pro-apoptotic proteins, was affected by miR-200a in breast cancer cell lines (19, 30-32). Western blotting analysis revealed that basal levels of p73, noxa, bax, and Bim were reduced following transfection with miR-200a and YAP1 siRNA in MDA-MB-231 and ZR-75-30 cells treated in suspension culture (Fig. 3D). These data suggested that miR-200a targeting of YAP1 suppresses the pro-apoptotic protein expression and allows the cells to evade anoikis.

**Transfection of miR-200a promoted in vivo breast cancer cell metastasis in zebrafish and mouse models**

To analyze the in vivo effect of miR-200a on tumor cell metastasis, transgenic fli:EGFP zebrafish embryos with GFP-labeled blood vessels were prepared as an animal model. The transparent nature of zebrafish embryos allows visualization tumor cell dissemination in living fish. Immune privilege of zebrafish embryos facilitates implantation of mammalian tumor cells (22, 33). MDA-MB-231 cells were transfected with miR-200a mimic or YAP1 siRNA, and MCF-7 cells were transfected with antagomiR-200a or antagomiR-Ctrl. After 48 hours, the transfected cells were labeled with DiI, and injected into the abdomen of zebrafish embryos. After injection, cells that seeded in the blood vessels of the zebrafish were detected, and zebrafish embryos containing metastatic cells were counted at 24 hour, and 72 hour separately. (Fig. 4A and Supplementary Figure S1A, B).
Table S3). Cells transfected with the YAP1 siRNA or miR-200a mimic were seeding in nearly 75% injected zebrafish embryos, respectively. However, control MDA-MB-231 cells were observed in 40%. The metastatic cells in the tail vessels were also counted (Fig. 4B). Approximately 40% of miR-200a mimic or YAP1 siRNA transfected cells were able to seed in the tail vein, but only 20% of the control transfected cells could do so (supplementary Table S4). In addition, MCF-7 antagomiR-200a transfected MCF-7 cells showed an obvious decrease of survival cells in zebrafish tail vein compared with antagomiR-Ctrl transfected cells (Fig. 4A, B).

To further examine the effects of miR-200a on metastasis in a mouse model, the metastatic potential of stably miR-200a and scramble transfected MDA-MB-231 cells were injected into tail vein in BALB/c nude mice. Intravenous injection of pMR-miR-200a-transfected MDA-MB-231 cells labeled with GFP induced marked lung colony formation in vivo, whereas MDA-MB-231 cells transfected with the control vector induced fewer lung metastases (Fig. 4C, Supplementary Fig. S1C). Statistic results showed a significant difference between the number of lung colonies derived from miR-200a-overexpressing MDA-MB-231 cells and control cells (Fig. 4D). In addition, we introduced a construct expressing YAP1 into miR-200a stably expressing cell line of MDA-MB-231. The restoration of YAP1 expression in the stable cell line was confirmed by western blot (supplementary Fig.S4C). The tail vein injection study showed that restoration of YAP1 could significantly reverse the metastasis promotion imposed by miR-200a (Fig 4C, D). Collectively, the data suggest that overexpression of miR-200a or silencing of YAP1 may promote breast cancer cells evasion of anoikis in the circulation, thereby facilitating cancer metastasis.

**Inverse correlation between YAP1 and miR-200a expression in breast cancer patients**

To address whether the expression of YAP1 is associated with miR-200a in breast cancer patients, miR-200a and YAP1 expression were examined in specimens from 16 breast cancer patients by qRT-PCR and western blotting, respectively. Our investigation showed that the expression of miR-200a was inversely correlated with that of YAP1 in 16 patients, such that YAP1 was expressed at a lower level in specimens overexpressing miR-200a (Fig. 5A, B). Moreover, analysis of 40 human breast cancer specimens by qRT-PCR showed a significant negative correlation between YAP1 and miR-200a expression levels by Pearson's correlation analysis ($R = -0.562, P = 0.000159, n = 40$) (Fig. 5C). This data provides further evidence of a functional link between miR-200a and YAP1 in breast cancer.

**miR-200a is associated with breast cancer metastasis**

To further understand the relationship between miR-200a and breast cancer metastasis, we performed miR-200a expression analyses on a series of breast tumor samples ($n = 110$, Cancer Hospital Fudan University collection). The relationship between the miR-200a expression levels and clinicopathologic parameters of breast cancer was summarized in Table 1. The results showed that no significant correlation between the miR200a expression and age, histological type, size, stage, ER, PR, and Her2. However, we found that miR-200a expression was upregulated by more than 7-fold in lymph node positive specimens compared with lymph node negative specimens. Moreover, in metastasis positive patients the miR-200a expression was 10-fold compared with metastasis negative patients (Table 1). Thus, these findings suggested that high miR-200a levels were associated with metastatic behavior in breast cancer.
Discussion

In this study, we demonstrate that miR-200a promotes breast cancer metastasis by targeting YAP1. We also proved that upregulation of miR-200a is associated with anoikis resistance. Our data show that miR-200a could reduce the expression of YAP1 by directly binding to the 3'-UTR of YAP1. As a result, caspase-3, p73 and its downstream pro-apoptotic proteins were indirectly inhibited by miR-200a. Silencing YAP1 largely phenocopied miR-200a induced anoikis resistance. In addition, an inverse correlation between miR-200a and YAP1 expression was observed in breast cancer cell lines and tumor specimens.

The miR-200 family is known to suppress EMT, the initiating step of metastasis, by targeting Zeb1 and Zeb2 (13). To verify if miR-200a influence EMT of breast cancer cells, we examined the expression of four EMT marker (E-cadherin, N-cadherin, vimentin and Fibronectin) by qRT-PCR in MDA-MB-231 and MCF-7 cell lines. With miR-200a mimic transfection, MDA-MB-231 cells showed lower vimentin expression and higher E-cadherin expression compared with control group. In contrast, we observed elevated vimentin and decreased E-cadherin in antagomiR-200a transfected MCF-7 cells (Supplementary Fig.S2). These results, together with those previous studies, indicate that overexpression miR-200a may suppress metastasis in breast cancer (12, 13, 23, 34). However, some evidence showed overexpression of miR-200 in mouse breast cancer cells promoted lung metastasis (15, 16). Previous identified miR-200a targets were inadequate to explain miR-200a pro-metastatic phenotypes. It is implied miR-200a may be involved in multiple steps of metastasis. A recent paper reported that miR-200s promote 4TO7 mouse carcinoma cells metastatic colonization by directly targeting Sec23a, which mediates secretion of metastasis-suppressive proteins (16, 35). However, relatively little is known about the role of miR-200a in the translocation of tumor cells. The function of miR-200a in anoikis is still need to be elucidated. Herein we identify a novel role for miR-200a in imbuing human breast cancer cells with anoikis resistance. In vivo mouse model showed antagomiR-200a transfected MCF-7 cells failed to form lung metastasis. Considering tumor metastasis include several steps, and several regulators involve in the phenotype of metastatic potential (14, 36). The impact of miR-200a expression on the metastasis of breast cancer seems to vary according to histopathologic character, and stage of cancer. Our results provide an explanation for how miR-200a inhibits EMT but promotes lung metastasis. At the initial stage, low levels of miR-200a promote invasion and intravasation. However, once tumor cells enter into the circulation, high miR-200a expression is required for efficient survival and further colonization of secondary organs. Thus, miR-200a may exert its pro-metastatic effects at multiple steps in the metastatic cascade.

The proper execution of apoptosis is a fundamental safeguard against tumorigenesis and metastasis. YAP1 is a well-defined pro-apoptotic transcriptional factor (19). The inhibition of YAP1 expression greatly reduces detachment-induced apoptosis. Nuclear translocation of YAP1 was inhibited by phosphorylation at the Ser-127 site (37). Co-transcriptional function of YAP1 was related to this posttranslational modification (38). Our study has shown that miR-200a directly targets the 3'-UTR of YAP1 to repress the expression of YAP1. These findings suggested that miR-200a negatively regulates YAP1 not only provides an underlying mechanism for the aberrant expression of YAP1 in breast cancer but also demonstrates the importance of
post-transcriptional regulation of YAP1.

Resistance to anoikis, which is a critical step in the metastatic cascade, allows cells to survive within the circulatory and lymphatic systems (35, 39). Our findings demonstrate that miR-200a promotes tumor cell survival in the circulatory system through confer anoikis resistance, indicating that miR-200a may play roles in the prognosis of cancer. In ovarian cancer, overexpression of the miR-200a correlated with a poor prognosis(40). Patients with high expression of miR-200a had a shorter median overall survival time compared to those lacking significant miR-200a expression (27.5 months Vs. 61.0 months, respectively)(41). The present results indicated that miR-200a expression correlates with lymph node metastasis and distant metastasis, which are significant prognosis factors in breast cancer patients. However, the correlation was not observed between miR-200a expression and traditional pathologic markers, such as histological type, hormone receptor status, and TNM stage. One of the reason may be a relatively limited number of patients was used. The other could be the complex function of miR-200a in cancer progression.

In summary, we combined experimental and clinical studies to establish a role for miR-200a in anoikis resistance. We showed that miR-200a promotes anoikis resistance and metastasis via targeting YAP1.

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References


Table 1. The relationship between miR-200a expression and clinicopathologic parameters in breast cancer

<table>
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<th>Clinicopathologic parameters</th>
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<th>Median expression of miR-200a (normalized CT)*</th>
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<td>10.06 ± 3.096</td>
<td></td>
</tr>
<tr>
<td>Distant metastasis**</td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Absent</td>
<td>76</td>
<td>10.95 ± 3.351</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>34</td>
<td>7.574 ± 2.418</td>
<td></td>
</tr>
<tr>
<td>All cases</td>
<td>110</td>
<td>11.12 ± 3.770</td>
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</table>

* normalized \( C_T = C_T \text{miR-200a} - C_T \text{U6} \)

** In this assay metastasis defined as distant metastasis after surgery.
Figure legends
Fig. 1
miR-200a induces anoikis resistance in breast cancer cells.
A, miR-200a and U6 expression in 7 breast cancer cell lines was determined by qRT-PCR. Relative expression was calculated by obtaining normalized CT values, such that the CT value of miR-200a was normalized to the U6 CT value from the same sample. The resulting value was then transformed by applying $2^{-\Delta CT}$. Mean ± SD of normalized CT from three independent experiments.
B, Apoptosis was evaluated after culturing MDA-MB-231 and MCF-7 cells in adherent and suspension, and staining with Annexin-V at 48 h. The flow cytometry profile depicts Annexin-V-FITC staining on the x-axis and PI staining on the y-axis.
C, The number represents the percentage of early apoptotic cells in each condition. Mean ± SEM of apoptotic cells from three independent experiments.
D, After suspension culture, the transfected cells were lysed for western blotting. The protein levels of cleaved caspase-3 were normalized to GAPDH.
E, Forty-eight hours after the induction of anoikis, transfected MDA-MB-231 and MCF-7 cells were used for the colony formation assay. Colonies were stained with crystal violet after 2 weeks in culture (left panel). Mean ± SEM of colonies from three independent experiments was shown in the right panel.

**$P < 0.01$**

Fig. 2
miR-200a targets YAP1 through an interaction with the YAP1 3’-UTR.
A, The intersection between genes that regulate the apoptotic processes (GO: 0042981) and Targetscan, MiRanda, and Diana predicted target genes is defined as the overlapping fraction of all four database.
B, Schematic diagrams of human YAP1 3’-UTR luciferase constructs with wild-type and mutant (YAP1-3’-UTR) miR-200a target sequences.
C, HEK293T cells were transfected with the luciferase constructs shown in B and 100 pmol miR-200a and harvested 48 hours later. Firefly luciferase activity is normalized to Renilla luciferase activity. Values are expressed as the mean ± SD of three replicate, independent experiments. MDA-MB-231 cells were transfected with a miR-200a mimic or control oligonucleotide and subjected to western blotting (top panel).
D, HEK293T cells were transfected with the luciferase constructs shown in B and 400 pmol antagomiR-200a and harvested 48 hours later. Firefly luciferase activity is normalized to Renilla luciferase activity. Values are expressed as the mean ± SD of three replicate, independent
experiments. MCF-7 cells were transfected with the antagomiR-200a and control oligonucleotide for 48 hours and then subjected to western blotting (top panel). The protein levels of YAP1 were normalized to GAPDH.

**P < 0.01

Fig. 3
miR-200a attenuates anoikis induced apoptosis in breast cancer cells.
A, Relative expression levels of YAP1 and miR-200a in human breast cancer cell lines and Western blot analysis of YAP1 protein expression. Pearson’s correlation of the YAP1 and miR-200a expression levels in cell lines (n = 7, R = -0.803, P = 0.03).
B, After adherent or suspension culture, the transfected cells were lysed for western blotting. The protein levels of cleaved caspase-3 were normalized to GAPDH. The data were derived from three replicated experiments.
C, Forty-eight hours after the induction of anoikis, lysates were collected from MDA-MB-231 and ZR-75-30 cells transfected with YAP1 siRNA to analyze the activation of Caspases-3 and -7 using the Caspase GLO assay system. Values are expressed as the mean ± SD of three replicate, independent experiments.
D, Cellular lysates of the miR-200a mimic, YAP1 siRNA, and control oligonucleotide transfected MDA-MB-231 and ZR-75-30 cells were used for western blotting analyses to detect YAP1, p73, Noxa, Bim, BAX, and GAPDH expression. The data were derived from three replicated experiments.

**P < 0.01

Fig. 4
Detection of metastatic breast cancer cells in in vivo animal models.
A, MDA-MB-231 cells were transfected with miR-200a mimic, miR-Ctrl, YAP1 siRNA, or scramble RNA. MCF-7 cells were transfected with antagomiR-200a and antagomiR-Ctrl. All transfected cells were labeled with Dil, and injected into the center of the yolk sac of transgenic fltl:EGFP zebrafish embryos. 24 hours and 72 hours after injection, Dil-labeled MDA-MB-231 and MCF-7 cells were detected in GFP-labeled vessels using a fluorescence microscope. The data were derived from three replicated experiments.
B, The number of metastatic cells in zebrafish tail vessels at 72 hours are expressed as the mean ± SD of three replicate, independent experiments.
C, Representative GFP and H&E-stained lung sections from mice intravenously injected with MDA-MB-231 cells. The arrowhead points to the tumour focus formed in the lung. The number of metastases in the lung were counted and analysed.
D, Lung colony numbers were macroscopically counted using dissecting microscopes.

*P < 0.05, **P < 0.01

Fig. 5
miR-200a expression is inversely correlated with YAP1 expression in breast cancer tissue.
A, Western analyses of YAP1 expression in 16 breast cell lines (top two panels).
B, Ranks of YAP1 and miR-200a expression.
C, Pearson’s correlation of the YAP1 and miR-200a expression levels in breast cancer patients (n
\[ R = -0.5621, P = 0.00158. \]
Regulation of apoptotic process

Hsa-miR-200a
YAP1 3’ UTR BS
YAP1 3’ UTR Mutant BS

SV40 pro → Renilla luciferase → 3’ UTR → polyA → TK pro → Firefly luciferase → SV40 polyA

3’ UGUAGCAUGGUCUGUCACAUA
5’ AGAAUUCUAACCAUAUCAGUGUUG
5’ AGAAUUCUAACCAUAAGAGUUG

**Fig 2**

A

Diana
miRanda
497
3420
8
657
1238

GO: 0042981
Regulation of apoptotic process

B

C

miR-Ctrl
miR-200a mimic

YAP1

GAPDH

D

AntagomiR-Ctrl
AntagomiR-200a

YAP1

GAPDH

**Luciferase activity (FLU/RLU)**

**Luciferase activity (FLU/RLU)**

YAP1 3’ UTR
YAP1 MT3’ UTR

YAP1 3’ UTR
YAP1 MT3’ UTR

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Fig4

A

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<th>0 hour</th>
<th>0 hour</th>
<th>24 hour</th>
<th>72 hour</th>
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<tr>
<td>miR-200a</td>
<td>miR-Ctrl</td>
<td>YAP1 siRNA</td>
<td>Scramble</td>
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<tr>
<td>antagomiR-200a</td>
<td>antagomiR-Ctrl</td>
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<td></td>
</tr>
</tbody>
</table>

B

![Graph showing cell numbers in tail vein for different conditions.]

C

<table>
<thead>
<tr>
<th>pMR-miR-200a</th>
<th>pMR-miR-Ctrl</th>
<th>pMR-miR-200a+pQCXIH</th>
<th>pMR-miR-200a+pQCXIH-YAP1</th>
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<tbody>
<tr>
<td>GFP</td>
<td>H&amp;E</td>
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D

![Graph showing number of micrometastases per section for different conditions.]

**Research.**
on October 24, 2017. © 2013 American Association for Cancer Research.
Clinical Cancer Research

MicroRNA-200a Promotes Anoikis Resistance and Metastasis by Targeting YAP1 in Human Breast Cancer

San-Jian Yu, Jing-ying Hu, Xia-Ying Kuang, et al.

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