c-MYC regulated miR-23a~24-2~27a cluster promotes mammary carcinoma cell invasion and hepatic metastasis by targeting Sprouty2

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Running title: miR-23a~24-2~27a cluster promotes mammary carcinoma cell invasion

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Disclosure statement: The authors declare no conflict of interest.

Keywords: miR-23a~24-2~27a cluster, c-MYC, mammary carcinoma, EGF, metastasis mechanism

Capsule:

Background: The regulation of miR-23a~24-2~27a cluster is largely unknown.

Results: EGF induced c-MYC expression to promoted the expression of miR-23a~24-2~27a cluster, resulting decreased expression of Sprouty2 and increased activation of p44/42 MAPK to stimulate mammary carcinoma cell invasion and subsequent hepatic metastases.

Conclusion: EGF promoted mammary carcinoma cell invasion and hepatic metastasis.

Significance: miR-23a~24-2~27a cluster might be used as biomarker for breast cancer metastasis.
Abstract
Emerging evidence indicates that the miR-23a~24-2~27a cluster may possess a causal role in mammary tumorigenesis and function as a novel class of oncogenes. However, the regulatory mechanism of the miR-23a~24-2~27a cluster in mammary carcinoma cell invasion and migration is still largely unknown. We observed that the expression levels of miR-23a, miR-24-2 and miR-27a were significantly higher in breast cancer with lymph node metastasis, compared with that from patients without lymph node metastasis or normal tissue. Forced expression of the miR-23a~24-2~27a cluster promoted mammary carcinoma cell migration, invasion and hepatic metastasis, through targeting Sprouty2 (SPRY2), and consequent activation of p44/42 MAPK. Epidermal growth factor induced the expression of the transcription factor c-MYC, which promoted the expression of mature miR-23a, miR-24-2 and miR-27a, and subsequently decreased expression of SPRY2 and activated p44/42 MAPK to promote mammary carcinoma cell migration and invasion. We therefore suggest a novel link between epidermal growth factor and the miR-23a~24-2~27a cluster via the regulation of c-MYC, providing the potential for the miR-23a~24-2~27a cluster to be used as biomarker in the diagnosis and/or treatment of breast cancer.

INTRODUCTION
microRNAs (miRNAs) are small noncoding RNAs that regulate the translation of protein coding genes by repressing translation of protein coding mRNA or enhancing mRNA degradation (1-15). They are predicted to modulate the expression levels of at least one-third of all human protein coding genes (16,17). Current target-prediction computer programs (18,19) predict that one specific miRNA may target tens to hundreds of genes. Thus, expect that miRNAs play important roles in coordinating many cellular processes (8,20-23). Deregulation of miRNAs has been reported to modulate normal cell growth and differentiation, potentially leading to a variety of disorders including cancer (24-27). Thus, the identification of miRNAs that are associated with pathology provides new approaches for understanding disease processes (28,29).

The miR-23a~24-2~27a cluster is a miRNA cluster, existing intergenically in the vertebrate genome (30). Members of the cluster are involved in cell cycle control and differentiation, in various cell types (31). The cluster has also been suggested to play a role in promoting apoptosis by both caspase-dependent and caspase-independent pathways (32). However, the mechanisms of miR-23a~24-2~27a cluster regulation in cancer progression remains poorly understood. Only a few target genes for the miR-23a~24-2~27a cluster have thus far been identified.

Sprouty2 (SPRY2), an inhibitor of the Ras/MAPK pathway, is one of four highly conserved family members of Sprouty signal modulatory proteins (33,34). SPRY2 is recognized to be deregulated in various types of cancers, such as breast, liver and prostate cancer, amongst others (35,36). SPRY2 might be an important modulator of pathways central to cancer progression, including cell growth, migration and invasion (37-39).

In this report, we observed that the expression levels of members of the miR-23a~24-2~27a cluster were significantly higher in mammary carcinoma with lymph node metastasis compared with that from patients without lymph node metastasis or normal tissue. We further described the mechanism by which the miR-23a~24-2~27a cluster contributed to mammary carcinoma cell migration and invasion. We demonstrated epidermal growth factor (EGF) induced the
expression of c-MYC, which increased the expression of mature miR-23a, miR-24-2 and miR-27a, subsequently decreased the expression of the target gene, SPRY2, and promoted cell migration and invasion through activation of p44/42 MAPK.

EXPERIMENTAL PROCEDURES
Cell culture—All human breast cancer cell lines used in this study were obtained from the American Type Culture Collection (Rockville, MD) and cultured in conditions as recommended. All cells were maintained in a humidified incubator at 37°C and 5% CO2.

Reagents—Media, sera and antibiotics for cell culture were from Life Technologies, Inc. (Grand Island, NY, USA). Protein electrophoresis reagents were from Bio-Rad Laboratories (Richmond, VA, USA). Iressa (Gefitinib) were purchased from Tocris Bioscience (Ellisville, MO, USA). U0126 were purchased from Calbiochem (San Diego, CA, USA). All other chemicals were from Sigma (St Louis, MO, USA).

Patients and specimens—The female patient population consisted of 14 consecutive non-invasive mammary ductal carcinoma patients, 14 consecutive invasive mammary ductal carcinoma patients and 13 consecutive normal mammary (benign) tissue patients who underwent surgery at the First Affiliated Hospital of Anhui Medical University (Hefei, Anhui, China) between 2009 and 2010. The pathohistological diagnosis of the specimens was consistent with breast neoplasm in accordance with WHO guidelines (40). Histology grade was based on the Scarff–Bloom–Richardson system (41). The protocol for the use of tissue samples from patients and follow-up study was approved by the Institutional Review Board and patients signed informed consent. Our study procedures were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Transfections (siRNA and miRNA)—Cells were transfected with double-stranded hsa-miRNA mimics 23a, hsa-miR-24-2 or hsa-miR-27a or 2′-O methylated single-stranded hsa-miR-23a, hsa-miR-24-2 or hsa-miR-27a ASO or SPRY2 siRNA or their respective negative controls (GenePharma, Shanghai) using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Cells were harvested in Trizol (Invitrogen) for RNA extraction and in RIPA lysis buffer for protein extraction. SPRY2 specific siRNA#1 (sense: GCCCUUACAGAAAGCCUUUATT and antisense: UAAAGGCUUUCUGUAAGGCTT), siRNA#2 (sense: GCAGGUACAGUUCUGUCUTT and antisense: AGACAAGACAGUUGCUGCTT), were all purchased from GenePharma.

Transwell migration and invasion assay—Assays were performed in BioCoat Matrigel invasion chambers (Corning Costar, Acton, MA) as described previously (42). Values for cell migration or invasion were expressed as the average number of cells per microscopic field.

RNA analysis, Western Blot analysis and Luciferase reporter assay—These procedures were carried out as described previously (44). Membranes were blocked with 5% milk powder in PBS and then incubated with: anti-SPRY2 (Millipore, 1:2000), anti-p44/42 MAPK (Cell Signaling, 1:1000), anti-phospho-p44/42 MAPK (Cell Signaling, 1:1000), anti-beta-tubulin (Sigma-Aldrich, 1:1000), anti-beta-ACTIN (Calbiochem, 1:10,000).

ChIP assay—Chromatin immunoprecipitation was performed using EZ ChIP kit (Upstate, Lake Placid, NY) as described previously (45). Immunoprecipitations were performed using 2 ug each of Anti-c-MYC (Santa Cruz Biotechnology,
Plasmid constructs and Mutagenesis—The miR-23a~24-2~27a gene promoter expression plasmids were kind gift from Dr. V. Narry Kim (Seoul National University). miR-23a, -24-2, -27a precursors including up- and downstream 80-100nt sequences were amplified from human genomic DNA. The miRNA expression vector was created by inserting the PCR products downstream of the U6 promoter of the pLL3.7 plasmid followed by ST. To construct the luciferase reporter vector, the psiCHECK-2 plasmid (Promega) was modified by introducing a new Eco72I site at the multiple cloning regions downstream of XhoI site. The 3’UTR sequence of SPRY2 was amplified with primers Fwd-5′-CCGCTCGAGGAAGTTGGGATAGTC TTTGCTGT-3′, Rev-5′-TATGCATCTGTAACCCCTCATTTG-3′, and it was cloned into the XhoI and Eco72I sites downstream of the Renilla luciferase (Rluc) report gene of the modified psiCHECK-2 plasmid. Site-directed mutagenesis was performed by using the QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). For 3’UTR of SPRY2, the seed sequence of miR-23a was replaced from AATGTGA to TTACACT, miR-24-2 TGTGCCG was substituted by ACGCGGT, and miR-27a was changed from ACTGTGAA to TGACACTT.

Generation of lentivirus expressing microRNA—Generation of lentivirus vectors was performed by cotransfecting pLL3.7 carrying the miRNA-expression cassette with helper plasmid pCMV-VSV-G (envelope) and pHR’ 8.9△VPR (core protein) in 293T cells using Effectene Transfection Reagent (QIAGEN). The viral supernatant was collected 48 hr after transfection. 

Transduction with lentivirus vectors—MCF-7 cells were infected with 2 ml of lentiviral supernatant with miR-23a, miR-24-2 or miR-27a, respectively, in RPMI-1640 medium containing 10% FBS and 8 μg/ml polybrene for 2 hr at 1,200 × g on 2 consecutive days (multiplicity of infection [MOI], 1 to 5), followed by incubation for 2 hr at 37°C and cultivation with fresh medium.

Tumor xenograft studies—All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23, revised 1985). An Institutional Animal Care and Use Committee (IACUC) approved all animal protocols. The procedure was carried out as described previously (44). The 5 weeks old female BALB/C nude mice (Hunan SJA Laboratory Animal Co., Ltd.) were used for studies. The 5 weeks old female BALB/C nude mice (Hunan SJA Laboratory Animal Co., Ltd.) were injected with 4.5x10⁶ cells via tail vein. When animals were sacrificed, lungs and livers were harvested and fixed overnight in 4% paraformaldehyde, embedded in paraffin and cut into 10μm-thick sections for histological studies and real-time PCR analysis for hHPRT to evaluate metastasis (46).

Statistical analysis—All experiments were performed at least three times. All statistical analyses were performed using SPSS software system for Windows (version 13.0; SPSS, Chicago, IL). The chi-squared (χ²) test was used to analyze the difference in miRNA expression levels among BC and normal samples. Numerical data are expressed as mean ±S.E.M. (standard error of the mean) from a representative experiment performed in triplicate. The statistical significance of each correlation was determined using the χ² test, P<0.05 was considered statistically significant.

RESULTS

miR-23a~24-2~27a cluster expression is increased in mammary carcinoma—The biological roles of
the miRNAs encoded by the miR-23a~24-2~27a cluster in cancer progression are yet to be fully delineated. To define the potential role of the miR-23a~24-2~27a cluster in the progression of mammary carcinoma, we performed quantitative real-time PCR analysis for expression of the members of the miR-23a~24-2~27a cluster on a panel of frozen mammary carcinoma samples. We observed that the expression levels of miR-23a, miR-24-2 and miR-27a were significantly higher in breast cancer with lymph node metastasis, compared with that from patients without lymph node metastasis or normal tissue (Fig. 1A-1C). Additionally, we observed a high correlation coefficient for expression of the individual members of the miR-23a~24-2~27a cluster (Fig. 1D), indicative of cluster co-expression in breast cancer.

miR-23a~24-2~27a cluster promotes mammary carcinoma cell migration and invasion in vitro—Given the positive correlation between the level of each individual miRNA in the cluster and metastasis in patients with mammary carcinoma, we next assessed the potential impact of miR-23a~24-2~27a cluster on mammary carcinoma cell migration and invasion, through manipulation of the expression level of members of the miR-23a~24-2~27a cluster by transfection of either antisense oligonucleotide (ASO) or synthetic miRNA mimic.

As shown in Figure 2, forced expression of miR-23a, miR-24-2, or miR-27a, respectively, resulted in significantly increased MCF-7 cell migration (P < 0.01, Fig. 2A) and penetration through the matrigel-coated membrane (invasion) compared with negative control (P < 0.01, Fig. 2B). In contrast, depletion of miR-23a, miR-24-2 or miR-27a with ASO, respectively, resulted in a significant reduction in cell migration (P < 0.01, Fig. 2C) and invasion (P < 0.01, Fig. 2D). Cotransfection of mimics of the members of the miR-23a~24-2~27a cluster did not result in supererogatory enhancement of cell migration (Fig. 2E) nor invasion (Fig. 2F). All individual members of the miR-23a~24-2~27a cluster failed to influence MCF-7 cell proliferation (Fig. 3A and 3B), suggestive of a specific role in regulating migration and invasion. Furthermore, combined cotransfection of mimics of the miR-23a~24-2~27a cluster did not alter MCF-7 cell proliferation either (Fig. 3C). Hence, no member of the miR-23a~24-2~27a cluster, individually or combined, altered MCF-7 cell proliferation.

miR-23a~24-2~27a cluster initiates distant metastasis in vivo—We further determined whether expression of the members of the miR-23a~24-2~27a cluster would promote metastasis of MCF-7 cells in vivo. To this end, MCF-7 cells were infected with lentivirus expressing miR-23a, miR-24-2 or miR-27a, respectively, or the control pll3.7 plasmid (Fig. 2G), and injected via the tail vein of female BALB/c nude mice. Metastases were only detected in the livers of experimental mice (3/7) injected with MCF-7 cells with lentivirus expressing miR-27a by H&E staining (data not shown) 8 weeks post tail vein injection, whereas no metastases were detected in the livers of control mice. Furthermore, the burden of micrometastases was determined by quantitative real-time PCR analysis for the expression of human HPRT (hHPRT). We observed that the expression levels of hHPRT were significantly higher in the livers of all mice injected with MCF-7 cells with lentivirus expressing miR-23a, miR-24-2 or miR-27a, compared with the livers of control mice (Fig. 2H). In comparison, there is no significantly higher expression of hHPRT in the lungs of mice injected with MCF-7 cells with forced expression of miR-23a, miR-24-2 or miR-27a, compared with the levels in the lungs of the control mice (Fig. 2I),
indicative that the miR-23a~24-2~27a cluster could specifically promote breast cancer metastasis to liver.

SPRY2 is directly targeted by the miR-23a~24-2~27a cluster via its 3’UTR—To determine the mechanism by which these miRs promote migration and invasion, we sought to determine specific targets for this cluster of miRs. Four bioinformatics software programs PicTar, TargetScan, Rna22 and miRanda (1, 2), revealed that all three miRs have putative binding sites in the 3’UTR of SPRY2 that are evolutionarily conserved among vertebrate species (47). To determine whether the miR-23a~24-2~27a cluster directly regulates SPRY2, we cloned the 3’ untranslated region (UTR) of SPRY2, which contains miR-23a, miR-24-2, and miR-27a target sequences, respectively, downstream of a luciferase reporter gene. Reporter assays using SPRY2 3’UTR-psi-CHECK2 for each miRNA mimic showed that forced expression of each individual miRNA (miR-23a, miR-24-2, or miR-27a) significantly repressed SPRY2 3’UTR luciferase activity (Fig. 4A). Concordantly, antisense oligonucleotide (ASO) to each miRNA increased luciferase reporter activity (Fig. 4B), indicating that the miR-23a~24-2~27a cluster directly targets SPRY2.

The predominant mechanism of miRNA action is thought to be degradation of mRNA or inhibition of translation (1). We therefore investigated the effect of a control oligonucleotide and members of the miR-23a~24-2~27a cluster on repression of endogenous SPRY2 mRNA and protein. We observed that both SPRY2 mRNA (Fig. 4C and 4G) and protein (Fig. 4E, 4F, 4I and 4J) were selectively decreased in the presence of miR-23a, miR-24-2, or miR-27a. In contrast, both SPRY2 mRNA (Fig. 4D and 4H) and protein (Fig. 4E, 4F, 4I and 4J) were increased after transfection of ASO for miR-23a, miR-24-2, or miR-27a, respectively, indicating SPRY2 is a bona fide target of miR-23a~24-2~27a cluster. Thus, SPRY2 expression is directly controlled through 3’UTR regulation by each miRNA of the miR-23a~24-2~27a cluster.

To further identify a single miR-23a~24-2~27a cluster cognate binding site, genomic location and sequence analysis of the miR-23a~24-2~27a cluster in the SPRY2 gene were analyzed and is shown in Fig. 4K. We performed a bioinformatics analysis with RNA-hybrid (3), finding several potential binding sites for members of the miR-23a~24-2~27a cluster. To confirm whether members of the miR-23a~24-2~27a cluster target these potential binding sites, we cloned full-length fragments of SPRY2 3’UTR (either wild-type or mutant) into the luciferase reporter plasmid psi-CHECK2. We then transfected miR-23a, miR-24-2, or miR-27a mimic, respectively, with SPRY2 3’UTR constructs into MCF-7 cells and observed that miR-23a, miR-24-2, or miR-27a, markedly repressed the relative luciferase activities of the wild-type SPRY2 3’UTR respectively (Fig. 4L, 4M and 4N). Consistently, further mutations of the partially complementary miR-23a, miR-24-2, or miR-27a site(s) in SPRY2 3’UTR abrogated responsiveness to miR-23a, miR-24-2, or miR-27a, respectively (Fig. 4L, 4M and 4N). Additionally, cotransfection of mimics of the members of the miR-23a~24-2~27a cluster showed that similar repression levels of SPRY2 3’UTR luciferase activity (Fig. 4O) compared with each individual miR in the cluster. Thus, the SPRY2 gene is a direct and specific downstream target of the miR-23a~24-2~27a cluster.

The miR-23a~24-2~27a cluster promotes migration and invasion via SPRY2—To further determine whether SPRY2 was required for miR-23a~24-2~27a cluster mediated mammary carcinoma cell migration and invasion, we used
SPRY2 siRNA for depletion of SPRY2 expression. SPRY2 siRNA efficiently depleted SPRY2 as observed by qPCR analysis (Fig. 4P). Cotransfection of SPRY2 siRNA and ASO to each of the miR-23a~24-2~27a cluster ASO demonstrated that depletion of SPRY2 expression significantly abrogated the repression of MCF-7 cell migration (Fig. 4Q) and invasion (Fig. 4R) modulated by the miR-23a~24-2~27a cluster ASO. Thus, SPRY2 is a critical mediator for the enhancement of mammary carcinoma cell migration and invasion by the miR-23a~24-2~27a cluster.

miR-23a~24-2~27a cluster activates MAP kinase signaling—We further investigated the downstream signaling pathway modulated by SPRY2 to mediate the effects of the miR-23a~24-2~27a cluster on cell migration and invasion. SPRY2 is recognized as an inhibitor of the p44/42 MAPK signaling pathway (9,33,34). We and others have also previously reported that p44/42 MAPK enhanced breast cancer cell (MCF-7) migration and invasion (48,49).

To determine if the miR-23a~24-2~27a cluster modulated the p44/42 MAPK signaling pathway, we assessed p44/42 MAPK activation by determination of phospho-p44/42 MAPK levels. Phospho-p44/42 MAPK was significantly increased by the forced expression of miR-23a, miR-24-2 or miR-27a, respectively (Fig. 4S). In contrast, phospho-p44/42 MAPK was decreased after transfection of miR-23a ASO, miR-24-2 ASO, or miR-27a ASO, respectively. Additionally, we quantified the effects of miR-23a, miR-24-2, and miR-27a, respectively, on the levels of phospho-p44/42 MAPK (Fig. 4T). Together, our findings indicate that the miR-23a~24-2~27a cluster increased p44/42 MAPK activation.

To further determine whether MAPK activity was required for miR-23a~24-2~27a cluster mediated mammary carcinoma cell migration and invasion, MCF-7 cells with forced expression of miR-23a, miR-24-2, and miR-27a, respectively, were treated with the MEK1/2 specific inhibitor U0126. Inhibition of MEK1/2 activity was demonstrated to diminish cell migration and invasion while forced expression of miR-23a, miR-24-2, and miR-27a, respectively, promoted cell migration and invasion. U0126 decreased cell migration and invasion stimulated by forced expression of miR-23a, miR-24-2, and miR-27a, respectively (Fig. 4U and 4V). It is therefore apparent that p44/42 MAPK signaling was utilized to enhance cell migration and invasion consequent to forced expression of miR-23a~24-2~27a cluster.

c-MYC transcriptionally up-regulates the miR-23a~24-2~27a cluster—We next sought to delineate regulation of the expression of the miR-23a~24-2~27a cluster. Bioinformatics analysis revealed one putative c-MYC regulatory element is present in the promoter of the miR-23a~24-2~27a cluster. Hence, we investigated whether c-MYC drove the expression of the miR-23a~24-2~27a cluster. To determine this possibility, we used an adenovirus to force the expression of c-MYC in MCF-7 cells. Real-time PCR analysis showed that the mature miR-23a, -24-2, -27a and pri-miR cluster transcripts were significantly increased above control levels respectively (Fig. 5A). Together, these findings demonstrate that c-MYC increased the expression of the miR-23a~24-2~27a cluster in mammary carcinoma cells.

c-MYC acts on an E-Box to drive miR-23a~24-2~27a cluster expression—Having demonstrated that c-MYC increased the expression of the miR-23a~24-2~27a cluster, we next searched for response elements (the recognition site of c-MYC) in the miRNA cluster promoter and observed that a region containing an E-Box (a promoter element known to be bound by c-MYC) was present. This region was isolated from
genomic DNA by PCR and cloned upstream of a luciferase reporter gene. Reporter assays demonstrated that luciferase activity was increased by c-MYC in cells transfected with the E-Box-luciferase construct (PL842; PL639; PL439); however, no increase in luciferase activity was observed in cells transfected with the pGL3-Basic plasmid lacking the E-box (Fig. 5B).

To demonstrate whether c-MYC occupied the promoter of the miR-23a-24-2-27a cluster, we performed chromat immunoprecipitation assays for c-MYC binding sites. We observed a significant enrichment of the miR-23a-24-2-27a cluster promoter amplicon in c-MYC ChIP samples as compared with ChIP samples generated with control IgG (Fig. 5C).

A previous report (50) has demonstrated that c-MYC suppresses invasion and metastasis of mammary carcinoma cells including MCF-7 cells. We further determined the possible role of c-MYC in breast cancer cell migration and invasion. Surprisingly, forced expression of c-MYC resulted in significantly increased migration and invasion in a number of breast cancer cell lines including MCF-7 cells compared with the control (Fig. 5D and 5E, data not shown). In contrast, depletion of c-MYC expression by siRNA resulted in a significant reduction in cell migration (Fig. 5F) and invasion (Fig. 5G). It is therefore apparent that forced expression of c-MYC enhances MCF-7 mammary carcinoma cell migration and invasion.

EGF regulates the expression of the miR-23a-24-2-27a cluster in vitro—We further sought to determine whether pro-metastatic growth factors, such as EGF (51,52), should contribute to promotion of cell migration and invasion through regulation of the miR-23a-24-2-27a cluster expression. We first demonstrated EGF-stimulated cell migration (Fig. 6A) and invasion (Fig. 6B) were significantly inhibited by transfection of miR-23a, -24-2, or -27a ASO, respectively. Next, treatment of MCF-7 cells with EGF resulted in increased phosphorylation of p44/42 MAPK as expected (Fig. 6C). Transfection of miR-23a, -24-2, or -27a ASO, consistently, decreased EGF induced phosphorylation of p44/42 MAPK, indicating that EGF stimulated p44/42MAPK phosphorylation is modulated by the combinatorial functionality of miR-23a-24-2-27a cluster (Fig. 6C and 6D).

To further determine whether EGF modulated the expression of the miR-23a-24-2-27a cluster, we stimulated MCF-7 cells with EGF at different time intervals and assessed changes in miRNA abundance. qPCR results demonstrated that EGF induced a significant increase in mature miR-23a, -24-2, and -27a expression in MCF-7 cells, respectively (Fig. 6E). Furthermore, we treated MCF-7 cells with the EGFR antagonist Iressa (53). qPCR results demonstrated that Iressa markedly decreased the expression of mature miR-23a, -24-2, and -27a in MCF-7 cells, respectively (Fig. 6F-6H). Further analysis demonstrated that the expression of the miR-23a-24-2-27a cluster precursor (Pri-miRNA Cluster) was also increased (Fig. 6I). Additionally, reporter assays demonstrated that luciferase activity from the miR-23a-24-2-27a cluster promoter was significantly increased by EGF; however, no EGF dependent increase in luciferase activity was observed in cells transfected with the pGL3-Basic plasmid lacking the promoter sequences (Fig. 6J). Thus, the miR-23a-24-2-27a cluster is modulated by EGF in vitro.

EGF regulates the expression of miR-23a-24-2-27a cluster partly via c-MYC—EGF has previously been demonstrated to regulate c-MYC expression (54,55). We demonstrated above that c-MYC enhanced miR-23a-24-2-27a cluster expression by directly binding to the promoter region of the miR-23a-24-2-27a cluster. We next examined
whether the increased miR-23a-24-2-27a cluster expression stimulated by EGF is modulated via c-MYC. We used c-MYC siRNA for depletion of c-MYC expression as described. c-MYC siRNA efficiently depleted c-MYC as evidenced by qPCR analysis (Fig. 6K). Transfection of c-MYC siRNA substantially impaired the stimulatory effect of EGF on miR-23a (Fig. 6L), miR-24-2 (Fig. 6M), miR-27a (Fig. 6N), and the miR-23a-24-2-27a cluster precursor (Fig. 6O), respectively. Thus, EGF-mediated regulation of the miR-23a-24-2-27a cluster may be mediated in part through increased expression of c-MYC.

**DISCUSSION**

There is a paucity of reports on the mechanisms of miR-23a-24-2-27a cluster regulation in cancer progression. In this study, the expression of miR-23a-24-2-27a cluster members was examined in clinical specimens. Strikingly, we observed that the expression levels of the members of the miR-23a-24-2-27a cluster were significantly higher in breast cancer with lymph node metastasis, compared with that from patients without lymph node metastasis or normal tissue, suggesting that the miR-23a-24-2-27a cluster may be linked to metastasis in mammary carcinoma. We further demonstrated a role of the miR-23a-24-2-27a cluster members in promoting mammary carcinoma cell migration and invasion. The mechanistic insight of the clinical correlation was gained further by in vitro and in vivo studies. The finding herein therefore assist in understanding the functional role of the miR-23a-24-2-27a cluster in breast cancer progression and may provide new understanding of the mechanism of neoplastic progression. The miR-23a-24-2-27a cluster is expected to possess other functional roles in addition to our finding here. For example, functional activity of the miR-23a-24-2-27a cluster during osteoblast differentiation is indicative of a central role for the miR-23a-24-2-27a cluster in both progression and maintenance of the osteocyte phenotype (56). Also, miR-23a-24-2-27a cluster members are enriched in endothelial cells and highly vascularized tissues, suggesting that increased miR-23a-24-2-27a cluster expression may possess important function in neovascular age-related macular degeneration or tumor-related angiogenesis (30).

A prior report has indicated that SPRY2 was down-regulated in hepatocellular carcinoma (35). Interestingly, we also observed that the miR-23a-24-2-27a cluster members promoted micrometastases to murine liver but not to lung (Fig. 2H and 2I), indicative that this cluster of miRNAs might be specifically modulated in hepatic specific metastases.

In this study, we demonstrated that EGF, a major pro-metastatic growth factor in breast cancer (57-60), induced the expression of c-MYC (61-65) that transcriptionally up-regulated the miR-23a-24-2-27a cluster, subsequently decreasing the expression of SPRY2. Decreased SPRY2 in turn increased the activity of p44/42 MAPK, promoting mammary carcinoma cell migration and invasion. However, it has previously been reported that c-MYC decreased the transcription of several miRNAs, including all members of the miR-23a-24-2-27a cluster, in the human p493 B cell line, bearing a tetracycline-repressible MYC construct, wherein tetracycline withdrawal results in rapid induction of c-MYC and mitochondrial biogenesis (66,67). We have however been able to repeat these results with the same p493 cell lines herein (data not shown). Interestingly, a recent report has demonstrated that c-MYC suppresses invasion and metastasis of mammary carcinoma cells (50). We however could not repeat these results with the strains of MDA-MB-231 and BT549 cells in our possession (data not shown). Our data suggest that
c-MYC exerts an alternate functional role in a highly context-dependent manner in the cell lines of different origin. We propose that EGF stimulates the expression of c-MYC to promote gene transcription of \textit{miR-23a-24-2-27a} cluster in mammary carcinoma cells and further triggers the activation of p44/42 MAPK via repressed expression of SPRY2 to promote mammary carcinoma cell invasion and metastasis (Fig. 7).

Prior studies reported \textit{miR-23a} and/or \textit{miR-27a} can target SPRY2 in pancreatic cancer cells (68) or endothelial cells (30) without an overall examination of the functionality of \textit{miR-23a-24-2-27a} cluster. In this study, we have defined the mechanism of regulation of the whole \textit{miR-23a-24-2-27a} cluster, affecting both migration and invasion by specifically targeting different sites of \textit{SRPY2} 3'UTR in a mammary carcinoma cell. Furthermore, we have now also defined a specific and coordinated disease association (metastatic mammary carcinoma) for this miRNA cluster.

In this study, we demonstrated that the \textit{miR-23a-24-2-27a} cluster enhanced MCF-7 cell migration and invasion but not proliferation. This might be due to distinct functions of the MAPK pathway during tumor progression (69). From another angle, multiple target proteins of this cluster of miRs, which could be either growth promoting or growth suppressive, might simultaneously affect cell growth depending on the particular microenvironment to exert differential cellular events (70,71). Furthermore, the proliferation and migration/invasion may be functionally uncoupled, even mutually exclusive in certain cellular models as suggested (72,73).

It should be noted that the \textit{miR-23a-24-2-27a} cluster could be expected to possess a wide range of functionalities due to its pleiotrophic regulation of additional genes in the progression of breast cancer. The cooperative expression and functionality of the members of the \textit{miR-23a-24-2-27a} cluster is yet to be fully defined (74). Further studies are therefore required to fully understand these functional roles and interactions of this miRNA cluster in breast cancer.
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Acknowledgments--We would like to thank Prof. V Narry Kim for offering the pri-miR-23a~24-2~27a promoter plasmids.

FOOTNOTES

This work is supported by the National Key Scientific Program of China (2012CB934002, 2010CB912804), National Natural Science Foundation of China (30971492, 81272925), Cancer Science Institute of Singapore and the Senior Foreign Expert Plan (6DW2012340015J), and the S&T Plan of Guangzhou (2012J4100082).

FIGURE LEGENDS

FIGURE 1. The miR-23a~24-2~27a cluster expression is correlated with mammary carcinoma metastasis. (A, B, C) Expression of the miR-23a~24-2~27a cluster in mammary carcinoma. Real-time PCR analysis of the expression of miR-23a (A), -24-2 (B) and -27a (C), respectively. Fresh tissue samples from patients with normal tissue (benign) and from patients with mammary carcinoma with or without lymph node metastasis (metastasis) were obtained. Total RNA was extracted from the tissues and subjected to real-time PCR analysis. U6 snRNA was used as loading control. *P<0.001. (D) Correlation of expression between the individual members of the miR-23a~24-2~27a cluster. Pearson correlation coefficients (R) and P-values (p) are indicated. All Sig.(2-tailed) were .000, N is the clinical sample number.

FIGURE 2. The miR-23a~24-2~27a cluster promotes mammary carcinoma cell migration/invasion and hepatic metastasis. (A, B) MCF-7 cells were grown and transiently transfected with the individual miR-23a~24-2~27a mimics or scrambled sequence oligonucleotides as negative control for 2 days and subjected to migration (A) and invasion (B) assays. (C, D) MCF-7 cells were grown and transiently transfected with the individual miR-23a~24-2~27a antisense oligonucleotides or scrambled sequence oligonucleotide as negative control for 2 days and subjected
to migration (C) and invasion (D) assays. (E, F) MCF-7 cells were grown and transiently transfected with mimics mixture of the miR-23a-24-2-27a cluster members or scrambled sequence oligonucleotide as negative control for 2 days and subjected to migration (E) and invasion (F) assays. (G) qPCR analysis of the expression level of miR-23a, -24-2 and -27a, respectively, in MCF-7 cells infected with lentivirus expressing miR-23a, miR-24-2 or miR-27a, respectively, or control vector. (H) qPCR analysis of the expression level of hHPRT in livers of mice that received the tail vein injection of MCF-7 cells infected with lentivirus expressing miR-23a, miR-24-2 or miR-27a, respectively, or control vector at week 8. (I) qPCR analysis of the expression level of hHPRT in lungs of mice that received tail vein injection of MCF-7 cells infected with lentivirus expressing miR-23a, miR-24-2 or miR-27a, respectively, or control vector at week 8. *P<0.01.

FIGURE 3. The miR-23a-24-2-27a cluster does not modulate mammary carcinoma cell proliferation in vitro. (A, B, C) MTT Assay. MCF-7 cells were grown and transiently transfected with individual miR-23a-24-2-27a cluster mimics (A), miR-23a-24-2-27a cluster ASO (B) and mimics mixture of the miR-23a-24-2-27a cluster members (C) for 2 days and subjected to MTT (3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) Assay. The cells were cultured in 96-well plate for 1-5 days and cell viability defined by MTT (Invitrogen) according to the manufacturer’s instructions. *P<0.01.

FIGURE 4. Identification of SPRY2 and p44/42 MAPK as mediators of the miR-23a-24-2-27a cluster promoted mammary carcinoma cell migration and invasion. (A, B) Luciferase reporter assay. Cells were transfected with a reporter vector psiCHECK2-SPRY2 3'UTR or psiCHECK2 plus either the individual member of miR-23a-24-2-27a cluster mimic (A)/miR-23a-24-2-27a cluster antisense oligonucleotides (ASO) (B) or scrambled sequence oligonucleotides as negative control. (C, D, G, H) Quantitative real-time polymerase chain reaction (PCR) analysis of SPRY2 expression. MCF-7 breast cancer cells (C, D) / T47D breast cancer cells (G, H) were grown and transiently transfected with individual miR-23a-24-2-27a cluster mimics (C, G) or miR-23a-24-2-27a cluster antisense oligonucleotides (ASO) (D, H), respectively, and then subjected to RNA extraction and quantitative real-time PCR. (E, I) Western blot. MCF-7 cells (E)/T47D cells (I) were grown and transfected with the individual miR-23a-24-2-27a cluster mimics or miR-23a-24-2-27a cluster antisense oligonucleotides (ASO) or scrambled sequence oligonucleotides as negative control. Total cellular protein was isolated and subjected to Western blot analysis for SPRY2 expression. ß-TUBULIN (E)/ß-ACTIN (I) was used as loading control. (F, J) The quantitative result of western blot is shown. (K) Genomic location and sequence analysis of the miR-23a-24-2-27a cluster interactive sites with SPRY2. The cluster is present on Chromosome 19. (L, M, N) Luciferase reporter assays. Relative luciferase activity was analyzed after luciferase reporter plasmids with SPRY2 3'UTR constructs (either wild-type or mutant) or control reporter plasmid were cotransfected in MCF-7 cells with miR-23a (L), miR-24-2 (M), miR-27a (N) mimic or control mimic, respectively. (O) Luciferase reporter assay. Cells were transfected with a reporter vector psiCHECK2-SPRY2 3'UTR or psiCHECK2 plus either mimics of the members of the miR-23a-24-2-27a cluster members or scrambled sequence oligonucleotides as negative control. Luciferase reporter assays were performed and Renilla luciferase activity was normalized according to the internally controlled Firefly luciferase activity. (P) Verification of the efficacy of SPRY2 siRNA by qPCR. (Q, R) Transwell assays. MCF-7 cells were grown and transfected with SPRY2 small interfering RNA (siRNA), individual
miR-23a–24-2–27a cluster ASO, or individual miR-23a–24-2–27a cluster ASO plus SPRY2 siRNA, or control scrambled siRNA duplex. These cells were subsequently subjected to migration (Q) and invasion (R) assays. (S) Western blot. MCF-7 cells were grown and transfected with the individual miR-23a–24-2–27a cluster mimics/miR-23a–24-2–27a cluster antisense oligonucleotides (ASO) or scrambled sequence oligonucleotides as negative control. Total cellular protein was isolated and subjected to Western blot analysis for phospho-p44/42 MAPK. Total MAPK was used as an internal control. (T) The quantitative result of western blot is shown. (U, V) MCF-7 cells were grown and transiently transfected with the individual miR-23a–24-2–27a mimics or scrambled sequence oligonucleotide as negative control for 2 days and treated with 100ng/ml UO126 or Vehicle for 45 min. Cells were subsequently subjected to migration (U) and invasion (V) assays. *P<0.01.

FIGURE 5. c-MYC promotes miR-23a–24-2–27a cluster expression in MCF-7 cells. (A) Real-time PCR validated the expression of the miR-23a, -24-2, -27a transcript and the cluster precursors in the MCF-7 cell line, respectively. (B) c-MYC regulates the promoter of the miR-23a–24-2–27a cluster. Luciferase reporter assay. Cells were co-transfected with Vec/c-MYC plasmids and the luciferase reporter plasmid carrying various length promoters constructs (i.e. empty vector, 842bp, 639bp, 239bp) as indicated. Luciferase reporter gene activity was normalized by Renilla luciferase activity. Basal luciferase activity (unstimulated sample with empty vector) of the reporter construct was defined as 100%. The data are presented as means ±S.E.M. from three independent experiments each performed in triplicate. (C) Diagram representing the c-MYC binding sites in the miR-23a–24-2–27a cluster promoter region and ChIP assay. MCF-7 cells were transfected with pGL3–cMYC and vector plasmid, and ChIP was performed by IP with either anti-cMYC antibody or control IgG. (D, E) Transwell assays. MCF-7 cells were grown and transiently transfected with pGL3–cMYC or vector plasmid for 2 days and subjected to migration (D) and invasion (E) assays. (F, G) MCF-7 cells were grown and transiently transfected with c-MYC siRNA or control scrambled siRNA duplex for 2 days and subjected to migration (F) and invasion (G) assays. *P<0.01.

FIGURE 6. EGF regulates the expression of miR-23a–24-2–27a cluster partly via c-MYC in vitro. (A) Migration assay and (B) invasion assay. MCF-7 cells were grown and transiently transfected with individual miR-23a–24-2–27a cluster ASO or scrambled sequence oligonucleotide as negative control for 2 days. Serum deprived cells were subsequently treated with 20ng/ml EGF or Vehicle for 4 hours and subjected to migration and invasion assays. (C) Western blot. MCF-7 cells were grown and transiently transfected with the individual miR-23a–24-2–27a cluster antisense oligonucleotides (ASO) or scrambled sequence oligonucleotides as negative control for 2 days. Serum deprived cells were subsequently treated with 20ng/ml EGF or Vehicle for 4 hours. Total cellular protein was isolated and subjected to Western blot analysis for phospho-p44/42 MAPK expression. Total MAPK was used as an internal control. (D) The quantitative result of western blot is shown. (E) Real-time PCR validated the expression of the miR-23a, miR-24-2, miR-27a transcripts, respectively. (F, G, H) Iressa repressed the expression of miR-23a–24-2–27a cluster. Serum deprived MCF-7 cells were treated with Iressa for 30 min, and the expression of the miR-23a (F), miR-24-2 (G), miR-27a (H) transcript was analyzed by qPCR. U6 snRNA was used as loading control. (I) Real-time PCR validated that the expression of the miR-23a–24-2–27a cluster precursors was increased at all time intervals after treatment with EGF. (J) EGF regulated the promoter activity of the miR-23a–24-2–27a cluster. Luciferase reporter assay. MCF-7 cells were transfected with the luciferase reporter plasmid carrying various length promoter
constructs (i.e. empty vector, 2046bp, 1050bp, 639bp, 239bp) as indicated. Luciferase reporter gene activity normalized by Renilla/luciferase activity was analyzed 24 h after treatment with EGF. Basal luciferase activity (unstimulated sample with empty vector) of the reporter construct was defined as 100%. The data are presented as means±S.E.M. from three independent experiments each performed in triplicate. (K) Verification of the efficacy of c-MYC siRNA by qPCR. (L,M,N,O) Real-time PCR demonstrated the expression of the miR-23a, miR-24-2, miR-27a transcript and pri-miR Cluster, respectively. MCF-7 cells were transfected with c-MYC siRNA for 24 hours and subsequently treated with EGF for 4 hours. *P<0.01.

FIGURE 7. Proposed model to summarize the function of the miR-23a-24-2-27a cluster in mammary carcinoma cell migration and invasion involving EGF, c-MYC and SPRY2. EGF induced miR-23a-24-2-27a cluster expression partly via c-MYC to decrease SPRY2 expression, which further increased p44/42 MAPK activity to promote mammary carcinoma cell invasion and metastasis.
Figure 1

| miR-23a | miR-24-2 | miR-27a |
|---------|---------|---------|
| Correlation Coefficient | 1.000 | 0.783 | 0.866 |
| Significance (2-tailed) | - | <0.0001 | <0.0001 |
| N | 35 | 26 | 29 |

| miR-24-2 | miR-23a | miR-27a |
|---------|---------|---------|
| Correlation Coefficient | 0.783 | 1.000 | 0.744 |
| Significance (2-tailed) | <0.0001 | - | <0.0001 |
| N | 26 | 26 | 22 |

| miR-27a | miR-23a | miR-24-2 |
|---------|---------|---------|
| Correlation Coefficient | 0.866 | 0.744 | 1.000 |
| Significance (2-tailed) | <0.0001 | <0.0001 | - |
| N | 29 | 22 | 33 |
Figure 2
Figure 3

A

B

C

N.C.
- miR-23a mimic
- miR-24-2 mimic
- miR-27a mimic

N.C.
- miR-23a ASO
- miR-24-2 ASO
- miR-27a ASO

N.C.
- MIX
Figure 4

A. Relative Renilla/Firefly Luciferase Activity

B. Relative Renilla/Firefly Luciferase Activity

C. Relative Expression of SPRY2/GAPDH

D. Relative Expression of SPRY2/GAPDH

E. Mimics

F. Relative Expression of SPRY2/β-TUBULIN

G. Relative Expression of SPRY2/β-TUBULIN

H. Relative Expression of SPRY2/β-TUBULIN

I. Mimics

J. Relative Expression of SPRY2/β-ACTIN

K. chr 13 position 80,911,110

MRE

NC_000013.10

80,911,891
Figure 6

A. Relative expression of miRs/U6

B. Fold invasion

C. Phospho MAPK

D. Relative Expression of Phospho-p44/42MAPK

E. Relative expression of miRs/U6

F. Relative expression of miR-23a/U6

G. Relative expression of miR-24-2/U6

H. Relative expression of miR-27a/U6

I. Relative expression of Pri-miR Cluster/GAPDH

J. Relative Renilla/firefly Luciferase Activity
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Xiaoni Li, Xin Liu, Wei Yi Xu, Peng Zhou, Ping Gao, Songshan Jiang, Peter E. Lobie and Tao Zhu

J. Biol. Chem. published online May 6, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M113.478560

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