A Regulatory Loop Involving miR-22, Sp1, and c-Myc Modulates CD147 Expression in Breast Cancer Invasion and Metastasis

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Abstract
Breast cancer is the most common cancer in women for which the metastatic process is still poorly understood. CD147 is upregulated in breast cancer and has been associated with tumor progression, but little is known about its regulatory mechanisms. In this study, we demonstrated that CD147 was overexpressed in breast cancer tissues and cell lines, and the high expression correlated with tumor invasion and metastasis. We also found that the transcription factors Sp1 and c-Myc could bind to the CD147 promoter and enhance its expression. The CD147 mRNA has a 748-bp 3′-untranslated region (UTR) with many miRNA target sites, suggesting possible regulation by miRNAs. We discovered that miR-22 repressed CD147 expression by directly targeting the CD147 3′UTR. We also determined that miR-22 could indirectly participate in CD147 modulation by downregulating Sp1 expression. miR-22 could form an autoregulatory loop with Sp1, which repressed miR-22 transcription by binding to the miR-22 promoter. Together with the c-Myc–mediated inhibition of miR-22 expression, our investigation identified a miR-22/Sp1/c-Myc network that regulates CD147 gene transcription. In addition, miR-22 overexpression suppressed breast cancer cell invasion, metastasis, and proliferation by targeting CD147 \textit{in vitro} and \textit{in vivo}. Furthermore, we found that miR-22 was significantly downregulated in breast cancer tissues and that its expression was inversely correlated with the tumor–node–metastasis stage and lymphatic metastasis in patients. Our study provides the first evidence that an miR-22/Sp1/c-Myc network regulates CD147 upregulation in breast cancer and that miR-22 represses breast cancer invasive and metastatic capacities. Cancer Res; 74(14); 3764–78. ©2014 AACR.

Introduction
Breast cancer is the most commonly occurring cancer in women and is a major cause of cancer-related morbidity and mortality. Globally, approximately 350,000 women die from breast cancer each year (1, 2). A challenging problem is the high mortality due to the spread of tumor cells to distant organs, particularly, liver, lungs, bone, or brain (3). Therefore, many studies have been conducted to investigate the genes and gene products that drive the metastatic process.

CD147, also known as extracellular matrix metalloproteinase inducer (EMMPRIN), basigin and HAb18G, is a 58-kDa transmembrane glycoprotein belonging to the immunoglobulin superfamily (4, 5). Several studies have clearly shown a key role for CD147 in tumor progression and metastasis. Indeed, high CD147 expression levels have been reported in many tumors, including breast cancer, lymphoma, oral squamous cell carcinoma, glioma, melanoma, lung, bladder, liver, and kidney carcinomas (5–7). Accordingly, CD147 expression has been associated with known risk factors for breast cancer and with poor prognosis in patients with breast cancer (8–10). Nonetheless, although CD147 plays a critical role in breast cancer progression and prognosis, the mechanisms that underlie the upregulation of this molecule in breast cancer are not well elucidated.

Recent studies have shown that miRNAs can act as activators or inhibitors of tumor metastasis by targeting multiple signaling pathways involved in metastasis (11–13). miR-22 is a 22-nt noncoding RNA and originally identified in HeLa cells as a tumor-suppressing miRNA. Subsequently, ubiquitous miR-22 expression has been identified in a variety of tissues (14). We have identified that miR-22 is downregulated in gastric cancer and its overexpression inhibits cell migration and invasion (15). Lately, several targets of miR-22 have been reported to mediate its tumor-suppressive
effect, such as tumor-suppressive PTEN, Max genes, p21 and oncogene c-Myc expression, etc (14–18). In breast cancer cells, miR-22 might act as a tumor suppressor to repress cancer metastasis and progression by either downregulating EVI-1 oncogene expression and the estrogen signaling pathway or inducing cellular senescence (19–21). However, the function of miR-22 in breast cancer progression, especially its roles in human patients with breast cancer and mouse models of breast cancer, and the molecular mechanisms by which miR-22 exerts its functions and modulates the malignant phenotypes of breast cancer cells, are not yet fully understood.

Our previous studies reported that transcription factor Sp1 could regulate CD147 expression in lung and liver cancers (22, 23). Recently, Sp1 was identified as a direct miR-22 target involved in the cancer cell senescence program (20). The Myc transcription factor heterodimerizes with Max and binds to the E-box motif CACGTG, where it activates transcription by cooperating with multiple coactivator complexes (24). Myc serves a master regulator of various biologic functions and has been linked to cancer metastasis. An initial analysis of the CD147 promoter region revealed potential c-Myc–binding sites, suggesting that c-Myc might be involved in CD147 transcription regulation (25). Lately, it has become evident that, in addition to the upregulation of the protumorigenic miR17-92 cluster by Myc (26), the predominant effect of Myc on miRNA expression is widespread downregulation (27). Interestingly, miR-22 is repressed by c-Myc, and miR-22 inhibits Myc transcriptional partners, thus mediating a positive feedback loop to suppress Myc expression levels (14). However, the interplay between transcription factors (Sp1 and c-Myc) and miR-22 in the modulation of the CD147 gene regulatory networks involved in breast cancer cell invasion and metastasis remains largely unknown.

In this study, we demonstrated that CD147 expression was upregulated in metastatic breast cancer tissues and cell lines and that the transcription factors Sp1 and c-Myc could bind to the CD147 promoter and enhance its expression. miR-22 could repress CD147 expression through directly binding to its 3′-untranslated region (UTR) or indirectly inhibiting the transcription factor Sp1 and c-Myc partner expression to block the gene expression. In turn, Sp1 and c-Myc served as transcriptional repressors of miR-22 to downregulate miR-22 expression. Therefore, we have identified a microcircuitry mechanism in which Sp1 and c-Myc repress miR-22 transcription and this repression subsequently increases Sp1 expression level sufficient to participate with c-Myc in CD147 transcriptional upregulation. In addition, the overexpression of miR-22 suppressed the invasion, metastasis, and proliferation in breast cancer cell by targeting CD147 both in vitro and in vivo. Furthermore, we found that miR-22 was significantly downregulated in metastatic breast tissues compared with the expression in localized breast tissues and that miR-22 expression levels correlated with the tumor–node–metastasis (TNM) stage and histologic grade of breast cancer. Therefore, our findings provide valuable clues toward an understanding of the mechanisms of human breast cancer invasion and metastasis and present an opportunity to develop more effective clinical therapies in the future.

Materials and Methods

Tissue specimens and IHC analysis

Fifty paired tissue specimens of breast cancer (T) and matched adjacent normal tissues (ANT) were obtained at the time of surgical resection and were immediately frozen to −80°C until their use. Three fresh normal mammary gland tissues (NT) were also collected as normal controls. All samples were collected from the Department of Vascular Surgery, Xijing Hospital, which is affiliated with the Fourth Military Medical University (FMMU, Xi’an, China) from 2009 to 2010 and were histologically confirmed by staining with hematoxylin and eosin (H&E). All individuals provided written informed consent, and the study was approved by the hospital Ethics Committee. Immunohistochemistry was performed using a CD147 diagnostic kit (jiangsu Pacific Menuoke Biopharmaceutical Company) according to the manufacturer’s manual. Immunopositivity was evaluated as described in Supplementary Methods.

Cell culture

The human breast cancer cell lines MDA-MB-453, MDA-MB-468, MCF-7, BT-549, SK-BR-3, and MDA-MB-231 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The immortalized breast epithelial cell line MCF-10A was obtained from the ATCC. All cell lines were routinely cultured using standard protocols. Cell line authentication was assessed using short tandem repeat (STR) DNA profiling method every year in our laboratory and the latest verification was done in March 2013.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed as described previously (22). The expression of miR-22 was quantified by TaqMan microRNA assays from Applied Biosystems. Expression data were uniformly normalized to either U6 or GAPDH as an internal control, and the relative expression levels were evaluated using the ΔΔCt method (7, 28). Primers are listed in Supplementary Table S1.

Vector construction, siRNA, miRNA, and luciferase reporter assay

All vectors were constructed as described in Supplementary Methods. siRNAs targeting Sp1, c-Myc and CD147 and negative control siRNA were purchased from Ambion. The miRNA mimics, antisense miR-22 (As-miR-22), and negative control were synthesized by GenePharma. Cell transfection and dual luciferase reporter assay were performed as described previously (22, 23).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed using the EZ ChIP Assay Kit (Millipore) following the supplied protocol. The antibodies against Sp1 or c-Myc and
control IgG were from Santa Cruz. The primers specific for CD147 or miR-22 promoter are listed in Supplementary Table S1.

**Western blot analysis**

Western blotting was performed as described previously (22, 23). Anti-CD147 mAb was prepared by our laboratory (7). Primary antibodies against Sp1, c-Myc, or tubulin were from Santa Cruz. Anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase were from Pierce.

**Immunofluorescence**

Immunofluorescence was performed as described previously (7) using Nikon A1 confocal laser microscope system.

**In vitro invasion assay and migration assay**

In vitro invasion assay was performed as described previously (7) with MilliCell chambers (Millipore). The migration assay was the same as the invasion assay, except that no Matrigel was used and the cell permeating time was 12 hours.

**Wound-healing assay**

The wound-healing assay was performed as described previously (15).

**Cell proliferation assay**

Cell proliferation assay was assessed using MTT method as described previously (7).

**In vivo metastasis assay**

Female BALB/c nude mice at 4 to 6 weeks of age were provided by the Laboratory Animal Research Center of FMMU, and the animal study was reviewed and approved by the Animal Care and Use Committee of FMMU. The MDA-MB-231 cells (2 × 10^6) stably expressing GFP were resuspended in 100 μL Matrigel and orthotopically injected into the mammary fat pad on day 0. The miR-22 mimics and control miRNA with the mammary fat pad on day 0. The miR-22 mimics and control miRNA with in vivo-jetPEI (Polyplus Transfection) complex at the ratio of 1:1 in a volume of 100 μL (20 μg/site) were injected intratumorally every other day from day 13 to 31 after inoculation. The animals were imaged for 35 days using Carestream MS FX Pro in vivo imaging system (Carestream Health). For in vivo fluorescence imaging, mice were anesthetized with isoflurane, and a whole-body image was acquired for 20 seconds with an excitation filter at 480 nm and an emission filter at 535 nm. Data were analyzed using Carestream MI image analysis software. Fluorescence signals were normalized to photons per second per millimeter squared (p/s/mm²).

After the final imaging, the mice were sacrificed, and the organs (liver, lung, stomach, spleen, kidney, and intestines) were examined for metastases. Tumor volume was determined using direct measurement and calculated using the formula length × width²/2. Tumor tissues were then fixed, embedded in paraffin, and serially sectioned at a thickness of 4 μm. H&E staining was performed, and sections were examined by a pathologist to verify the tumor.

**Statistical analysis**

All statistical analyses were performed using the SPSS statistical software package (version 16.0). Each in vitro quantitative test was independently replicated, and all data are presented as means ± SEM. One-way ANOVA or the Student t test was used to compare the expression levels, luciferase activities, transfilter cell numbers, fluorescence signals, tumor volumes, and grayscale values of IHC staining among the different groups. Two-way repeated measures ANOVA analyses and Bonferroni tests were used to measure the proliferation curves in vitro and in vivo. The Spearman rank correlation coefficient was used as a statistical measure of association. All the statistical tests were 2-sided, and P < 0.05 was considered with statistical significance.

**Results**

**CD147 is overexpressed in metastatic breast cancer tissues and cell lines**

To investigate the role of CD147 in the progression of human breast cancer, we compared the CD147 expression levels between clinical breast cancers and paired adjacent normal tissues from 50 cases of patients with breast cancer by IHC. Our results showed that CD147 was predominantly located in the cytoplasm and membrane of breast cancer cells (Fig. 1A). CD147-positive expression found in breast cancer tissues was 78% (39 of 50), which was significantly higher than the 12% (6 of 50) in their adjacent tissues (P < 0.001, Fig. 1B). We also confirmed that CD147 expression was significantly increased in metastasis tumors (P = 0.004) and advanced TNM stages (P = 0.0093, Fig. 1B).

We further evaluated the expression levels of CD147 in breast cancer cell lines. The results showed that the expression levels of CD147 were significantly increased in all tumorigenic breast cancer cell lines than that in nontumorigenic cell lines and normal tissues (Fig. IC and D). In addition, the metastatic cancer cells showed the highest expression level of CD147 (Fig. IC and D). Notably, the expression level of CD147 was 6-fold higher in the metastatic MDA-MB-231 cell line than in the nonmetastatic MDA-MB-468. We also detected the expression of Sp1 and c-Myc in the breast cancer cell lines. The result showed that Sp1 and c-Myc were highly expressed in the metastatic breast cancer cells (Fig. 1D). The CD147 protein expression was correlated with Sp1 and c-Myc, respectively (R = 0.7526, P = 0.0011 and R = 0.6475, P = 0.005; Fig. 1E). This correlation indicates that CD147 might be regulated by Sp1 and c-Myc and has a causal role in breast cancer metastasis.

**Sp1/c-Myc upregulate CD147 expression in breast cancer cells**

To unravel the regulatory mechanisms of CD147 expression in breast cancer, we examined the CD147 core promoter region (23) for transcription factor–binding sites and identified binding sites for both Sp1 and c-Myc in this 217-bp region. To determine the roles of Sp1 and c-Myc in CD147 transcription, we cloned the human CD147 promoter fragment (nucleotides –217 to +1) into pGL3 vector for a luciferase activity assay. CD147 transcriptional activity was induced by Sp1 and c-Myc
Figure 1. CD147 is overexpressed in breast cancer tissues and cell lines. A, IHC analysis of CD147 protein expression in breast cancer and paired adjacent normal tissues. Pictures of representative areas are presented at different staining intensities (negative, weak, and strong) in ANT and tumor tissues. Scale bars, 50 μm. B, analysis of the expression pattern of CD147 in breast tissues detected by IHC. Stages I-IV, TNM stages. Statistical analyses were performed using a Student paired t test and one-way ANOVA. C, real-time RT-PCR analyzed the expression of CD147 in a series of human breast cancer cell lines and normal tissues. D, expression of CD147, Sp1, and c-Myc were detected by Western blotting. E, analysis for correlation of CD147 and Sp1/c-Myc protein expression level in human breast cancer cell lines and normal tissues by the Spearman rank correlation coefficient.
overexpression, respectively (Fig. 2A). These results suggested that Sp1 and c-Myc participate in CD147 transcription regulation.

To dissect the promoter region required for CD147 transcription by Sp1 and c-Myc, we generated 2 fragments from the CD147 promoter. The transcriptional activities of the 2 fragments containing the c-Myc (fragment B) or 4 Sp1-binding motifs (fragment C) were induced by the c-Myc or Sp1 overexpression, which suggested that Sp1 and c-Myc could affect CD147 transcription by recognizing their own binding sites, respectively (Fig. 2B). To validate this notion, we mutated these binding sites individually and used them in a reporter assay. The results showed that the mutations in either c-Myc–binding sites or the E3 Sp1–binding motif significantly impaired the effect of c-Myc or Sp1 on CD147 transcription activation. However, mutations in other Sp1–binding E-boxes did not obviously affect the ability of Sp1 to induce CD147 transcription activation (Fig. 2C), suggesting that c-Myc and Sp1 could bind to their special binding motifs to regulate CD147 transcription.

To corroborate this notion, we performed in vivo ChIP assays to address whether c-Myc and Sp1 bind to the CD147 promoter region. The ChIP assay revealed that endogenous c-Myc and Sp1 bound to the CD147 promoter (Fig. 2D, top). To further assess the biologic roles of Sp1 and c-Myc in CD147 expression, we applied loss- and gain-of-function approaches. We showed that the ectopic expression (Fig. 2D, middle) or siRNA knockdown (Fig. 2D, bottom), respectively, increased or reduced Sp1 and c-Myc enrichment on the CD147 promoter, and resulted, respectively, in CD147 upregulation or downregulation protein expression in breast cancer cells (Fig. 2E). We also showed the combined effects of Sp1 and c-Myc on CD147 expression (Supplementary Fig. S1). Our results showed that the CD147 expression could be significantly affected by Sp1 and c-Myc expression alteration. The roles of Sp1 and c-Myc in CD147 gene transcription were further elucidated by immunofluorescence. As shown in Fig. 2F, the c-Myc was cytoplasmic staining (green) and cell nuclei were dyed with DAPI (blue). The Sp1 protein was detected nuclear localization (red). The expression of CD147 was changed with the Sp1 and c-Myc expression alteration and the increased CD147 was mostly localized on the breast cancer cell membrane (green), which were consistent with the Western blot results. Hence, these results suggest that Sp1 and c-Myc serve as the transcriptional factors to activate CD147 transcription and upregulate its expression.

It has previously been shown that not only Sp1 but also Sp3 and Sp4 are highly expressed in breast cancer cells (29), and Sp3 and particularly Sp4 regulate many Sp1-dependent genes in cancer cells. Therefore, we detected the roles of Sp3 and Sp4 in the regulation of CD147. The coding sequences of Sp3 and Sp4 were amplified and cloned into pcDNA3.1. We co-transfected the CD147 promoter luciferase vector with Sp3 or Sp4 overexpression vectors, respectively. The results showed that CD147 transcriptional activity was induced by Sp3 overexpression. However, this upregulation induced by Sp3 was significantly lower than that of Sp1 function on CD147 transcription regulation. Furthermore, there was no significant upregulation of CD147 by Sp4 overexpression (Supplementary Fig. S2). Western blotting also proved the above results (Supplementary Fig. S3). These results suggested that Sp3 rather than Sp4 could participate in the CD147 transcription regulation. Among the Sp family members, Sp1 plays the major effect on CD147 transcription regulation.

miR-22 targets the 3′UTR of the CD147 mRNA and downregulates its expression

To associate miRNAs with the regulation of CD147 expression, a bioinformatics search was performed for potential miRNAs targeting mRNA of CD147 by using 4 common databases such as microRNA.org, Target-Scan, Pictar, and miRanda. These analyses had identified 7 miRNAs as the potential miRNA to target CD147 (Fig. 3A). To find which miRNA directly targets the 3′UTR region of CD147, we subcloned a 748-bp fragment of the full-length 3′UTR region of CD147 mRNA and inserted it into pmirGLO vector named as pmirGLO-CD147′UTR. MDA-MB-231 cells were transiently transfected with pmirGLO-CD147′UTR and miRNAs mimics or negative control. miR-22 mimics rather than other mimics significantly suppressed the luciferase activity, which indicated that miR-22 participates in the CD147 posttranscriptional regulation (Fig. 3A). Moreover, we constructed the miR-22–binding sites mutant vector containing three mutated bases on the predicted sites and the miR-22 overexpression vector. As shown in Fig. 3B, overexpression of miR-22 notably inhibited the luciferase activity and the inhibition was rescued when the binding sites were mutated. The result was also confirmed in BT-549 cells.

To further prove that CD147 is a target gene of miR-22, MDA-MB-231 and BT-549 cells were transiently transfected with miR-22 expression plasmid. Meanwhile, MDA-MB–468 and MCF-7 cells were transiently transfected with antisense miR-22 (As-miR-22). Notably, the expression of CD147 substantially decreased after miR-22 transfection, and in contrast, obviously increased expression of CD147 was observed after As-miR-22 transfection (Fig. 3C). Real-time quantitative PCR analysis showed that miR-22 overexpression or inhibition could affect the CD147 mRNA level, which indicates the
miR-22-induced degradation of the CD147 mRNA, accounting for some of the repression (Fig. 3D). However, we could also conclude the possibility that translational repression contributes to the overall repression of CD147 expression. Immunofluorescence also validated the above point (Fig. 3E). Collectively, these results make it evident that CD147 is a direct target of miR-22, and miR-22 affects CD147 expression by directly binding to the 3' UTR region of CD147.

miR-22 modulates CD147 expression by targeting Sp1 through an autoregulatory loop

Reportedly, the Sp1 mRNA 3' UTR contains putative binding sites for miR-22, and these sites are broadly conserved among mammals (20). Therefore, we constructed pmirGLO constructs that contained full-length and mutated Sp1 3' UTR. As shown in Fig. 4A, in MDA-MB-231 and BT-549 cells, miR-22 significantly reduced the luciferase activities of pmirGLO-Sp13'UTR, compared with the activity of the control reporter. In contrast, the mutant reporters were not repressed by miR-22, indicating that these target sites directly mediate repression. Western blotting showed that miR-22 overexpression markedly downregulated Sp1 expression in MDA-MB-231 and BT-549 cells. In the MDA-MB-468 and MCF-7 cells, Sp1 expression was upregulated in response to miR-22 inhibitor transfection (Fig. 4B). These results provide experimental evidence that miR-22 can directly repress Sp1 expression by binding to its 3' UTR. We also determined whether miR-22 can regulate Sp3 and Sp4. However, our results indicated that miR-22 may not participate in the Sp3 and Sp4 posttranscriptional regulations (Supplementary Results and Supplementary Fig. S4).
miR-22 Loop Regulating CD147 in Breast Cancer

Given that miRNAs are frequently involved in feedback loops in which they target the same factors that regulate their expression (30) and that Sp1 participates in CD147 transactivation, we then hypothesized a microcircuitry mechanism in which Sp1 represses miR-22 transcription, which in turn increases Sp1 to levels sufficient to participate with c-Myc in CD147 transcriptional upregulation. Consistent with our hypothesis, exposure of MDA-MB-231 cells to mitomycin A, which interferes with Sp1 activities, resulted in increased miR-22 expression (Fig. 4C). Next, we focused on dissecting the underlying mechanisms of miR-22 transcriptional regulation. We identified three Sp1-binding sites within a 1-kb span of DNA upstream from the 5’end of the primary transcript of miR-22 on human chromosome 17 (using the software package available at www.generegulation.com; Fig. 4D). The miR-22 promoter was subcloned into a pGL3-basic vector, and a dual-luciferase reporter assay was performed to study the functionality of the interaction between Sp1 and miR-22. Transient Sp1 expression effectively inhibited miR-22 transcription (Fig. 4D). Next, a binding-site–directed mutant assay was used to determine which binding site was important for Sp1 recruitment. We found that Sp1 could bind to the −607 to −614 region in the miR-22 promoter and repress transcription (Fig. 4E). These data were confirmed in a ChIP assay that showed Sp1 enrichment on the miR-22 promoter (Fig. 4F). Gain- and loss-of-function assays also showed that forced Sp1 expression reduced miR-22 expression along with increased Sp1 enrichment on the miR-22 promoter. Conversely, siRNA-mediated Sp1 knockdown resulted in miR-22 upregulation, along with reduced Sp1 enrichment on the miR-22 promoter (Fig. 4F). We also found that the Sp3 and Sp4 could not participate in the miR-22 transcriptional regulation (Supplementary Results and Supplementary Fig. S5).

Collectively, these data suggest that miR-22 participates in Sp1 downregulation by directly binding to its 3’UTR and, in turn, that Sp1 represses miR-22 transcription to inhibit its expression. Therefore, we conclude that miR-22 indirectly participates in the modulation of CD147 expression by regulating Sp1 expression, whereas miR-22 forms an autoregulatory loop with Sp1 to regulate its own expression.

miR-22/Sp1/c-Myc network regulates CD147 expression

It has been reported that the c-Myc could inhibit the miR-22 expression (27), so we validated the idea using the gain- and loss-of-function approaches. As shown, c-Myc overexpression resulted in miR-22 downregulation (Fig. 5A, left) and upregulation of the miR-22 target gene Sp1 (Fig. 5A, middle), thereby resulting in higher levels of CD147 expression (Fig. 5A, right). In turn, reduced c-Myc expression yielded the opposite results (Fig. 5B). Altogether, these results support the idea that c-Myc–induced miR-22 downregulation, which occurs upon Sp1 protein activation, leads to CD147 gene overexpression via the miR-22/Sp1/c-Myc network. A summary diagram that outlines the above-described regulatory network is shown in Fig. 5C.

miR-22 decreases the invasive, metastatic, and proliferative capacities of breast cancer cells in vitro via CD147 downregulation

To determine whether miR-22 regulates human breast cancer cell migration and invasion, we selected MDA-MB-231 cells, which show strong migration and invasion potential, and MCF-7 cells, which show weak migration and invasion potential, for further study. We first performed in vitro loss-of-function analyses by silencing the miR-22 with antisense oligonucleotides in MCF-7 cells. Transfection of miR-22 inhibitor caused increased CD147 protein expression (Fig. 6A) and consequently enhanced cell invasion and migration compared with control cells (P < 0.05; Fig. 6B). The wound-healing assay also indicated that miR-22 downexpression can significantly promote cell motility compared with control group (Fig. 6D). miR-22 inhibitor–treated MCF-7 cells showed higher proliferative capacities than control cells by MTT assay (P < 0.001; Fig. 6F). To further test this, we cotransfected MCF-7 cells with siRNA for CD147 mRNA and As-miR-22 and found that the effect of As-miR-22 was partially attenuated by siRNA for CD147 mRNA (P < 0.05; Fig. 6B, D, and F). These data clearly substantiate that downregulation of miR-22 contributes to enhanced CD147 expression, cell invasion, migration, and proliferation in vitro.

The functional effects of miR-22 overexpression on cell behaviors in vitro in breast cancer cell lines were assessed by transfecting pcDNA3.1-miR-22 into MDA-MB-231 cells. The protein expression of CD147 was reduced in response to miR-22 overexpression (Fig. 6A). The migration chamber assay as well as wound-healing assay indicated that miR-22 overexpression can significantly inhibit cell migration compared with the control group (Fig. 6C and E). In MDA-MB-231 cells, ectopic expression of miR-22 had an inhibitory effect on proliferation in vitro as evidenced by MTT (P < 0.001; Fig. 6F). Strikingly, cotransfection of miR-22 and CD147 overexpression vector almost completely rescued the miR-22-induced inhibition effect (P < 0.05; Fig. 6C, E, and F). These data clearly show that CD147 downregulation is necessary for the miR-22 mediated repression of breast cancer cell invasion, migration, and proliferation in vitro.

miR-22 inhibits the tumor growth and metastatic potential of aggressive breast cancer in vivo

We next asked whether miR-22 overexpression could suppress tumor growth and metastasis in vivo. Using breast cancer tumor models, the negative control and miR-22/CD147 (cotransfected with miR-22 mimics and pcDNA3.1-CD147 vector) mice showed the apparent presence of primary tumor, whereas mice injected with miR-22 mimics exhibited little increase in the GFP fluorescence signal of primary tumor during the same observation period (Fig. 7A, left). Tumors in mice injected with miR-22 and CD147 cells showed no significant difference from control cells (P > 0.005; Fig. 7A and B). Judging from photon count between the controls and miR-22–treated groups at the points of the experiment, miR-22 treatment resulted in a mean reduction in tumor growth (P < 0.05; Fig. 7A, right). We also compared tumor metastasis to important organs in these groups and were surprised to find...
that miR-22 treatment resulted in an inhibition of distant metastasis to the liver, kidney, spleen, stomach, and small intestine (Fig. 7B). The growth curve revealed that the proliferation of miR-22 treated tumors was slower than control. There were significant differences between the fluorescence signals in miR-22 mice and the control mice at days 21, 28, and 35 ($P < 0.05$; Fig. 7C), suggesting that miR-22 exerted significant tumor growth suppression in vivo. These results indicate the inhibition of metastasis by injection with miR-22 in vivo.

Next, we confirmed that synthetic miR-22 was delivered into primary tumor by quantitation of the miR-22 levels in the tumors (Fig. 7D). H&E staining showed that all examined tissues were tumors. GFP was expressed in all tumors group, proving that the detected tumor tissues were derived from stably transfected breast cancer cells with the GFP tag. IHC staining verified the higher expression of CD147 in the negative control and miR-22/CD147 tumors than that in the miR-22-injected tumors (Fig. 7E). Using image optical

Figure 5. The miR-22/Sp1/c-Myc network regulates CD147 expression. A and B, c-Myc inhibits miR-22 transcription and increases Sp1 and CD147 expression by gain- and loss-of-function approaches. Data in A and B are representative of three independent experiments. *$, $P < 0.05$, by Student t test. C, summary diagram describes the miR-22/Sp1/c-Myc network that regulates CD147 expression.

Figure 4. The miR-22 and Sp1 regulatory loop. A, Sp1 was a direct target of miR-22. The relative luciferase activities in MDA-MB-231 and BT-549 cells were determined after the Sp1 3' UTR or mutant plasmids were cotransfected with miR-22. Top, predicted duplex formation between human Sp1 3' UTR and miR-22. The target site mutations are boxed. *$, $P < 0.05$, by Student t test. B, Western blotting showed the effect of miR-22 on Sp1 expression in breast cancer cells. C, Sp1 inhibition by mithramycin A increases miR-22 RNA expression in MDA-MB-231 cells by real-time quantitative PCR. *$, $P < 0.05$, by one-way ANOVA followed by the Dunnett test. D, top, schematic diagram showing the locations of Sp1-binding sites on miR-22 regulatory region on chromosome 17. Bottom, the effect of Sp1 on miR-22 transcriptional regulatory is distinguished by detecting the relative luciferase activity transfected with Sp1 expression plasmid and miR-22 promoter report plasmid. *$, $P < 0.05$, by the Student t test. E, a dual-luciferase reporter assay was performed, which was cotransfected with the miR-22 WT or mutation promoters and Sp1 expression plasmid. *$, $P < 0.05$, by one-way ANOVA. F, the Sp1 regulates the miR-22 expression through binding to the miR-22 promoter using ChIP and real-time quantitative PCR. *$, $P < 0.01$, by Student t test.
density analysis with Image-Pro Plus, we determined that the staining intensity of CD147 in the miR-22-treated group was significantly lower than that in the NC group ($P < 0.05$; Fig. 7F). Proliferating cell nuclear antigen (PCNA) localizes to the nucleus and reflects cell proliferation status. In our work, we found that PCNA was expressed in the breast cancer cell nuclei (Fig. 7E). The PCNA labeling index was calculated as the percentage of positively stained nuclei. There was a significant difference between the miR-22-treated group and NC group ($P < 0.05$; Fig. 7G). Together, these findings suggest that miR-22 significantly inhibited proliferation and metastasis in breast cancer in vivo by downregulating CD147.

**Correlations of miR-22 with CD147 expression, tumor grade, and metastatic status in breast cancer patients**

To further investigate the role of miR-22 in progression of human breast cancer, we compared the expression levels in clinical breast cancer tissues and paired adjacent nonneoplastic tissues from 50 patients with breast cancer. By real-time quantitative RT-PCR, we showed that the expression levels of miR-22 were reduced in 44 of 50 cases of breast cancer tissues ($P < 0.001$), compared with those of adjacent nonneoplastic tissues (Supplementary Fig. S6A). Furthermore, correlation analysis showed that the miR-22 expression level was reversely correlated to lymphatic metastasis ($P < 0.001$), with lower expression in patients with lymphatic metastasis (Supplementary Fig. S6B). Besides, the relationship between the relative miR-22 expression and patients' clinical characteristics is shown in Supplementary Table S2. As evident from the analyzed data, miR-22 expression did not appear to be related to age, tumor size, estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor 2 (HER2) status ($P > 0.05$). We found that miR-22 expression was significantly associated with TNM stage and histologic grade ($P < 0.05$). Combined with the IHC results of CD147, we found that there was a negative relationship between the miR-22 expression level and the CD147 protein level by Spearman correlation analysis ($r = -0.7177$ and $P < 0.0001$; Supplementary Fig. S6C). These data verify that miR-22 regulates CD147 expression at the post-transcriptional level. Reduced miR-22 expression could be a cause of high CD147 expression and could thus be involved in breast carcinoma progression.
In our study, we showed that CD147 was overexpressed in breast cancer tissues and cell lines, and the high expression correlated with tumor invasion and metastasis. Our investigation led to the identification of an miR-22/Sp1/c-Myc network that regulates CD147 gene transcription. Sp1 and c-Myc bind to the CD147 promoter and enhance its expression. The miR-22 could repress CD147 expression through directly binding to its 3’UTR. Furthermore, we provided the first evidence of an indirect but pivotal role of miR-22 in modulating CD147 expression in breast cancer. Sp1, a transactivator of the CD147 gene, binds to the miR-22 regulatory elements and represses miR-22 expression, whereas miR-22 blocks Sp1 translation and in turn upregulates its own transcription. c-Myc, a transcription factor that physically interacts with Sp1 to upregulate CD147 expression, inhibits miR-22 expression, whereas miR-22...
suppresses c-Myc partner expression and in turn downregulates c-Myc regulatory effect. Our study also demonstrated that miR-22 dramatically repressed the migration, invasion, and proliferation of breast cancer cells in vitro and tumor metastasis in vivo by downregulating CD147. Moreover, we analyzed the correlation of the expression level of miR-22 and the clinical characteristics of 50 patients with breast cancer. Our analytical results showed that the expression level of miR-22 was inversely correlated with TNM stages and lymphatic metastasis in patients. To the best of our knowledge, this study is the first to examine the transcriptional mechanism of CD147 upregulation in breast cancer and to reveal that miR-22 plays an important role in the breast cancer invasive and metastatic potential.

Invasion and metastasis, 2 of the most important hallmarks of malignant tumors, are the prominent fatal factors for human cancers (31). Our work showed that CD147 was more strongly upregulated in breast cancer tissues than in the adjacent tissues and that this overexpression correlated with tumor metastasis and advanced histologic grades. Combined with the previous results, CD147 might serve as a breast cancer biomarker detection (6, 8, 32, 33). Elucidation of this transcriptional regulation mechanism could provide new targets for breast cancer clinical therapy.

Therefore, we explored the molecular mechanism underlying CD147 overexpression in breast cancer and identified a critical regulatory miR-22/Sp1/c-Myc network that modulates CD147 expression. Sp1 is usually recognized as a transcriptional activator of various genes involved in almost all cellular processes in mammalian cells (34). Sp1 also participates in cancer development and progression (35, 36). Myc is one of the most frequently activated oncogenes in a wide variety of human cancers with a broad influence on cell proliferation, survival, differentiation, and genetic stability (37). It is intriguing that binding of Myc alone is generally insufficient to activate its target genes, and other transcriptional factors are often required for a full effect on transcription (38, 39). In our study, we confirmed that the Sp1 and c-Myc served as transcriptional factors to activate CD147 transcription and upregulate its expression in breast cancer.

Our study also investigated the potential involvement of an miRNA-mediated mechanism in increased expression of CD147 in breast cancer. Luciferase activity assay confirmed that miR-22 directly could target the 3'UTR of CD147 and then suppress its expression. Because a single miRNA potentially can target many genes, our study added CD147 as one more bona fide target of miR-22. Moreover, our results showed that Sp1 was negatively regulated by miR-22 at the postranscriptional level, via a specific target site within the 3'UTR. Therefore, we concluded that miR-22 could indirectly participate in modulating CD147 level by regulating expression of Sp1.

miRNAs have been shown to be regulated by the upstream transcription factors (40–42). Our results verified that Sp1 suppressed the transcription activity of miR-22 by binding directly to the promoter of miR-22, leading to downregulation of miR-22 expression and upregulation of CD147. Hence, miR-22 forms an autoregulatory loop with Sp1 to regulate its own expression. Recent studies demonstrate that miR-22 constitutes a feedback loop with c-Myc and Myc partner and forms a regulatory loop in the PTEN–AKT pathway (14, 16, 19, 43). So we concluded that aberrant activation of CD147 results in c-Myc–dependent miR-22 downregulation and an increase in Sp1 expression. The Sp1 further inhibits miR-22 expression and with c-Myc alone to transactivate CD147.

Because of the central role of miR-22 in mechanisms of CD147 regulation, we investigated its role in breast cancer progression. Gain-of-function and loss-of-function assays were performed to assess the effects of miR-22 on breast cancer invasion and metastasis. Results showed that silencing of miR-22 upregulated CD147 and strengthened cell proliferation, invasion, and metastasis in vitro, whereas overexpression of miR-22 inhibited CD147 expression as well as cell proliferation, invasion, and metastasis in vitro. Synthetic miR-22 injection significantly suppressed tumor growth and metastasis in a mouse model of breast cancer metastasis, indicating the therapeutic potential of miR-22 in breast cancer metastasis. Our previous study identified CD147 as a positive regulator of cell invasion and metastasis of human hepatocellular carcinoma (44). The in vitro and in vivo CD147 rescue experiments proved that miR-22 regulated invasion and metastasis of breast cancer cells mainly by targeting CD147. miR-22 plays a tumor-suppressive role by downregulating oncogenic target genes in many kinds of cancer, including breast cancer (18, 19, 21, 45). However, on the other side, miR-22 was recently suggested to have an oncogenic role by targeting PTEN or TET family (16, 46). The reported paradoxical functions of miR-22 imply that miR-22 might act as a tissue/cell-specific or context-dependent tumor suppressor microRNA and the function diversely depending on its target genes and related regulatory networks. Perhaps, miR-22 may play more complex roles that exceed our perception in cancer, which needs us to explore it more deeply.

Finally, we detected the expression correlations of miR-22 and CD147 in the 50 paired cases of human breast cancer tissues. Expressions of CD147 were upregulated, whereas miR-22 was downregulated in breast cancer tissues. There was a negative relationship between miR-22 and CD147 expression levels. This evidence was consistent with that miR-22 suppresses its target CD147 in breast cancer tissues and then inhibits breast cancer progression. Hence, miR-22 is an important suppressor in breast cancer invasion and metastasis, and CD147 seems to be a major downstream effector of miR-22 in its target network. Currently, the emergence of new technologies that use synthetic miRNA mimics or anti-miRNA oligonucleotides holds great promise for clinical miRNA therapy (47). Synthetic miR-22 mimic treatments for cancer will become a significant scientific and therapeutic challenge.

In conclusion, to the best of our knowledge, this is the first study to examine the transcriptional mechanism of CD147 upregulation in breast cancer and to reveal that an miR-22/Sp1/c-Myc network plays an important role in CD147 transcriptional regulation. miR-22 negatively regulates CD147 expression, thus suppressing the migration and invasion capacities of breast cancer cells in vitro and in vivo and...
consequently suppressing tumor progression. These findings will contribute to our understanding of the molecular mechanism by which CD147 promotes breast cancer progression and might aid in the development of novel cancer therapeutic targets.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: L.-M. Kong, C.-G. Liao
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Analysis and interpretation of data (e.g., statistical analysis, biosciences, computational analysis): C.-G. Liao, Y. Zhang, J. Xu, Y. Zhang, H. Bian

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