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-kb 3’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. Additionally, miR-22 overexpression suppressed breast cancer cell invasion, metastasis and proliferation by targeting CD147 in vitro and in vivo.

Furthermore, we found that miR-22 was significantly downregulated in breast cancer tissues and that its expression was inversely correlated with the TNM stage and lymphatic metastasis in patients. Our study provides the first evidence that a miR-22/Sp1/c-Myc network regulates CD147 upregulation in breast cancer and that miR-22 represses breast cancer invasive and metastatic capacities.
Keywords: CD147; miR-22; Sp1; c-Myc; breast cancer; invasion and metastasis

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 breast cancer patients (8-10). Nonetheless, although CD147 plays a critical role in breast cancer progression and prognosis, the mechanisms that underlie the up-regulation of this molecule in breast cancer are not well elucidated.

Recent studies have shown that microRNAs (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 non-coding 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 breast cancer patients 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 biological 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 miR-17–92 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'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 to the expression in localized...
breast tissues and that miR-22 expression levels correlated with the TNM stage and histological 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 immunohistochemical 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) 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 Meniuoke Biopharmaceutical Company, Changzhou, China) 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 American Type Culture Collection (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 lab 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 (Foster City, CA, United States). Expression data were uniformly normalized to either U6 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, and the relative expression levels were evaluated using the $\Delta \Delta Ct$ method (7, 28). Primers were listed in Supplementary table 1.

**Vector construction, siRNA, miRNA, and luciferase reporter assay**

All vectors were constructed as described in supplementary methods. siRNAs targeting Sp1, c-Myc, CD147 and negative control siRNA were purchased from Ambion (Austin, TX, United States). The miRNA mimics, antisense miR-22 (As-miR-22) and negative control were synthesized by Genepharma (Shanghai, China). Cell transfection and dual luciferase reporter assay were performed as described previously (22, 23).

**Chromatin Immunoprecipitation (ChIP)**

ChIP assays were performed using the EZ ChIP Assay kit (Millipore, Billerica, MA,
United States) following the supplied protocol. The antibodies against Sp1 or c-Myc and control IgG were from Santa Cruz (Santa Cruz, CA, United States). The primers specific for CD147 or miR-22 promoter were listed in Supplementary table 1.

**Western blot analysis**

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

**Immunofluorescence**

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

**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 green fluorescent protein (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 in vivo-jetPEI (Polyplus Transfection, New York, NY, United States) 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 weekly for 35 days using Carestream MS FX Pro in-vivo imaging system (Carestream Health, Cheektowaga, NY, United States). For in vivo fluorescence imaging, mice were anesthetized with isoflurane, and a whole-body image was acquired for 20 s 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 × width^2/2. 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.
Each in vitro quantitative test was independently replicated, and all data are presented as means ± SEM. One-way ANOVA or Student’s t test was used to compare the expression levels, luciferase activities, transfiltre cell numbers, fluorescence signals, tumor volumes, and grayscale values of immunohistochemistry 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’s rank correlation coefficient was used as a statistical measure of association. All the statistical tests were two 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 breast cancer patients by immunohistochemistry. 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% (39/50), which was significantly higher than the 12% (6/50) 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 tumourigenic breast cancer cell lines than that in non-tumourigenic cell lines and normal tissues (Fig. 1C and Fig. 1D). In addition, the metastatic cancer cells showed the highest expression level of CD147 (Fig. 1C and Fig. 1D). Notably, the expression level of CD147 was 6-fold higher in the metastatic MDA-MB-231 cell line compared with the non-metastatic 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 high 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 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 two fragments from the CD147 promoter. The transcriptional activities of the two fragments containing the c-Myc (fragment B) or four 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, upper panel). To further assess the biological 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 panel) or siRNA knockdown (Fig. 2D, lower panel), 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. 1). Our results showed that the CD147 expression could be significantly affected by Sp1 and c-Myc expression alteration.
The role 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. So, 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. But 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. 2). Western blot also proved the above results (Supplementary Fig. 3). 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-CD1473’UTR. MDA-MB-231 cells were transiently transfected with pmirGLO-CD1473’UTR and miRNAs mimics or negative control. miR-22 mimics rather than other mimics significantly suppressed the luciferase activity, which indicated that miR-22 participate in the CD147 post-transcriptional 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 were 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). But we could also conclude the possibility that translational repression contributes to the overall repression of CD147 expression. Immunofluorescence also validated 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 to 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 blot 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 were 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. But our results indicated that miR-22 may not participate in the Sp3 and Sp4 post-transcriptional regulations (Supplementary results and Supplementary Fig. 4).
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 mithramycin 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 mutants 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. 5).

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, while 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 panel) and upregulation of the miR-22 target gene Sp1 (Fig. 5A, middle panel), thereby resulting in higher levels of CD147 expression (Fig. 5A, right panel). 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 down-regulation**

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, 6D and 6F). These data clearly substantiate that down-regulation 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 was 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 to the control group (Fig. 6C and 6E). 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, co-transfection of miR-22 and CD147 overexpression vector almost completely rescued the miR-22-induced inhibition.
effect ($P < 0.05$; Fig. 6C, 6E and 6F). 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 (co-transfected 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 7B). 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. Immunohistochemistry 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 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 inhibiting the proliferation and metastasis in breast cancer in vivo by downregulating CD147.

**Correlations of miR-22 with CD147 expressions, 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 non-neoplastic tissues from 50 breast cancer patients. 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 non-neoplastic tissues (Supplementary Fig. 6A). Furthermore, correlation analysis showed that the miR-22 expression level was reversely correlated to lymphatic metastasis ($P < 0.001$), with lower expression in lymphatic metastasis patients (Supplementary Fig. 6B). Besides, the relationship between the relative miR-22 expression and patients' clinical characteristics is shown in Supplementary table 2. 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 histological grade ($P < 0.05$). Combined with the immunohistochemical 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. 6C). 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.

Discussion

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 a 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 is 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 down-regulating CD147. Moreover we analyzed the correlation of the expression level of miR-22 and the clinical characteristics of 50 breast cancer patients. 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, two 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.

So, 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 a 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 posttranscriptional 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 phosphatase and tensin homolog-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 \textit{in vitro} and \textit{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 a 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 progress and might aid in the development of novel cancer therapeutic targets.

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Figure legends

Figure 1. CD147 is overexpressed in breast cancer tissues and cell lines. (A) Immunohistochemical analysis of CD147 protein expression in breast cancer and paired adjacent normal tissues. Pictures of representative areas were 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 immunohistochemistry. Stages I–IV, TNM stages. Statistical analyses were performed using Student’s 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 blot. (E) Analysis for correlation of CD147 and Sp1/c-Myc protein expression level in human breast cancer cell lines and normal tissues by Spearman’s rank correlation coefficient.

Figure 2. Sp1 and c-Myc upregulate CD147 expression in breast cancer cells. (A) A dual-luciferase reporter assay was performed by co-transfection of the CD147 promoter fragment (CD147-pGL3) with overexpression of c-Myc or Sp1. (B) The different length of CD147 promoter fragment were co-transfected with c-Myc or Sp1 expression vectors. (C) Reporter assay in cells transfected with various CD147 promoter constructs with mutations in different binding elements for c-Myc and Sp1. WT, wild type; mu, mutation type. Luciferase activity was expressed as relative to that of the pGL3 vector (a promoter-less vector) (D) ChIP assay demonstrated endogenous c-Myc and Sp1 binding to the CD147 gene promoter (upper panel). ChIP assay showed c-Myc/Sp1 enrichment on CD147 promoter transfected with c-Myc or Sp1 overexpression vector (middle panel) or siRNAs (lower panel). * P<0.05, ** P < 0.01. Statistical test: one-way ANOVA followed by Dunnett’s test using in Fig. 2A, B, C and D (upper); Student’s t-test using in Fig. 2D (middle and lower). (E) c-Myc, Sp1
and CD147 protein expression in cells transfected with corresponding overexpression vector (left panel) or siRNAs (right panel). (F) Expression of c-Myc, Sp1 and CD147 in breast cancer cells transfected with overexpression vector or siRNA, as detected by confocal laser-scanning microscopy. Cells were transfected with snc-RNA as a control. Scale bars: 50 μm.

**Figure 3.** CD147 is a direct target gene of miR-22. (A) Luciferase activities of full-length of CD147 3’UTR and miRNAs mimics or negative control in MDA-MB-231 cells. *P < 0.05, by one-way ANOVA followed by Dunnett’s test. (B) The relative luciferase activities in MDA-MB-231 and BT-549 cells were determined after the CD147 3’UTR or mutant plasmids were co-transfected with miR-22. Upper panel, predicted duplex formation between human CD147 3’UTR and miR-22. The target site mutations are boxed. *P < 0.05, by Student’s t-test. (C) CD147 expression in breast cancer cells treated with miR-22 or antisense miR-22 (As-miR-22) by Western blot. (D) Real time quantitative PCR analysis of CD147 mRNA levels after the miR-22 or As-miR-22 were transfected. Bars represent each sample performed in triplicate, and the error bars represent the standard deviations. Statistical test: Student’s t-test. (E) Immunofluorescence detection of CD147 protein expression after miR-22 or As-miR-22 transfection in MDA-MB-231 and MCF-7 cells, respectively. Scale bars: 50 μm.

**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 co-transfected with miR-22. Upper panel, predicted duplex formation between human Sp1 3’UTR and miR-22. The target site mutations are boxed. * \( P < 0.05 \), by Student’s \( t \)-test. (B) Western blot 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 Dunnett’s test. (D) Upper, schematic diagram showing the locations of Sp1 binding sites on miR-22 regulatory region on chromosome 17. Lower, 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 Student’s \( t \)-test. (E) A dual-luciferase reporter assay was performed, which were co-transfected 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. * \( P < 0.05 \), ** \( P < 0.01 \), by Student’s \( t \)-test.

**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 (A-B) are representative of three independent experiments. * \( P < 0.05 \), by Student’s \( t \)-test. (C) Summary diagram describes the miR-22/Sp1/c-Myc network that regulates CD147 expression.
**Figure 6.** miR-22 decreases CD147 expression and suppresses cell invasion, metastasis, and proliferation *in vitro* by targeting CD147. (A) CD147 expression in MCF-7 and MDA-MB-231 cells treated with miR-22 or As-miR-22 by western blot. (B) and (C) Effects of miR-22 on the invasion and migration of breast cancer cells. Morphologic comparison of cells penetrating the artificial basement membrane was shown. * P < 0.05, by one-way ANOVA followed by Dunnett’s test. Scale bars: 200 μm. (D) and (E) Wound healing assay of MCF-7 and MDA-MB-231 cells transfected with miR-22 inhibitor or miR-22 mimics. * P < 0.05, by one-way ANOVA followed by Dunnett’s test. (F) Cell proliferation of breast cancer cells transfected as above was measured in the indicated time periods using MTT assay. ** P < 0.01, two-way repeated measures ANOVA followed by Bonferroni test. Scale bars: 500 μm.

**Figure 7.** Synthetic miR-22 delivery inhibits breast tumor growth and metastasis *in vivo*. (A) *In vivo* fluorescence images of the pat orthotopic breast cancer model in nude mice. The colored region represents the GFP fluorescence signal of breast cancer cells in nude mice. Right, signal intensity scale. Quantitation of GFP emitted from the primary tumor of mice was presented as means ± SEM (n = 6). Increasing red color indicates increasing signal strength, while weaker signal strengths are represented by increasing blue color. After the final imaging, the mice were sacrificed, and the tumors were excised. The tumor size was measured in the resected pat tumor. Lower panel, quantitative analysis of the fluorescence intensities and tumor volumes in the
three groups. * $P < 0.05$, ** $P < 0.001$, by one-way ANOVA followed by Dunnett’s test. (B) Selected organ images of mice on day 35 after inoculation. LU, lung; KI, kidney; LI, liver; SP, spleen; ST, stomach IN, intestine; ** $P < 0.001$, by one-way ANOVA followed by Dunnett’s test. Lower panel, quantitative analysis of fluorescence intensities in the three groups. (C) Effect of miR-22 on breast cancer proliferation detected using *in vivo* imaging. * $P < 0.05$, ** $P < 0.001$, by two-way repeated measures ANOVA followed by Bonferroni test. (D) Relative quantitation of miR-22 level in primary tumor tissues was analyzed by qRT-PCR. U6 was used as an internal normalization control. * $P < 0.05$, by one-way ANOVA followed by Dunnett’s test. (E) H&E staining and immunohistochemistry showed the CD147, GFP and PCNA expression. (F) Staining intensity of CD147 in three groups. The expression of CD147 was calculated as optical density relative to the NC group. * $P < 0.05$, compared to the NC group, as determined using one-way ANOVA followed by Dunnett’s test. (G) miR-22 inhibits the expression of PCNA in mice. The PCNA labeling index was calculated as the percentage of positively stained nuclei. * $P < 0.05$, compared to the NC group, as determined using one-way ANOVA followed by Dunnett’s test.
Figure 1
Figure 2
A)

Luciferase

miR-22 binding sites of 3'UTR

Sp1 3'UTR WT

5'...UCUACACACCAAGUCGCCGACGUC...3'

miR-22

Sp1 3'UTR mu

5'...UCUACACACCAAGUGUGUCGCCGAC...3'

Relative luciferase activity (fold to pGL3)

pDNA3.1

pDNA3.1-miR-22

pRNA/GL/O 3'UTR

pRNA/GL/O 3'UTR-mut

MDA-MB-231

BT-549

C)

Mithramycin A (ng/mL)

0 10 30 100 300 500

Sp1

Tubulin

Relative mIR-22 expression

Mithramycin A (ng/mL)

D)

Sp1-element 2-607

GGGGCGGG

Sp1-element 1-783

CCCAGCAGCC

Sp1-element 3-544

GGGCCG

(-1)17p13

miR-22

Mock

pGL3

miR-22-pGL3

F)

DNA binding (% of Input DNA)

Scramble

Sp1 siRNA

WT

m1

m2

m3

Relative luciferase activity (fold to pGL3)

Empty vector

Sp1 construct

**

Empty vector

Sp1 construct

*
Figure 6

A

B

C

D

E

F

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
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