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Original Paper

Exosomal miR-27a Derived from Gastric Cancer Cells Regulates the Transformation of Fibroblasts into Cancer-Associated Fibroblasts

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Key Words
miR-27a • Cancer associated fibroblasts • Exosome • Gastric cancer • CSRP2 • Tumor microenvironment

Abstract
Background/Aims: The malignant biological behavior of gastric cancer(GC) is not only determined by cancer cells alone, but also closely regulated by the microenvironment. Fibroblasts represent a large proportion of the components in the tumor microenvironment, and they promote the development of disease. Currently, accumulating evidence suggests that exosomes can function as intercellular transport systems to relay their contents, especially microRNAs(miRNAs). ***Methods:*** First, we detected the highly-expressed level of miR-27a in exosomes isolated from gastric cancer cells by qRT-PCR. MiR-27a –over-expressed models *in vitro* and *in vivo* were established to investigate the transformation of cancer-associated fibroblasts observed by Western blotting, and the malignant behavior of gastric cancer cells using the methods CCK8 and Transwell. Moreover, the downregulation of CSRP2 in fibroblasts was used to evaluate the promotion of malignancy of gastric cancer using the methods CCK8 and Transwell. ***Results:*** In this study, we found a marked high level of miR-27a in exosomes derived from GC cells. miR-27a was found to function an oncogene that not only induced the reprogramming of fibroblasts into cancer-associated fibroblasts(CAFs), but also promoted the proliferation, motility and metastasis of cancer cells *in vitro* and *in vivo*. Conversely, CAFs with over-expression of miR-27a could pleiotropically increase the malignant behavior of the GC cells. For the first time, we revealed that CSRP2 is a downstream target of miR-27a. CSRP2

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downregulation could increase the proliferation and motility of GC cells. ***Conclusion:*** Thus, this report indicates that miR-27a in exosomes derived from GC cells has a crucial impact on the microenvironment and may be used as a potential therapeutic target in the treatment of GC.

Introduction

Gastric cancer (GC) ranks as the fourth most common type of malignant tumor in morbidity and the second leading cause of cancer death in the world [1, 2]. Although treatment strategies have substantially developed over the years, the prognosis for GC remains to be improved [3-5]. To find new targets and treatments, it is crucial to have insights into the malignant biological characteristics of GC.

Mounting evidence has elucidated that malignant biological behaviors of tumors are determined by the complex structures of malignant cancer cells and their microenvironment [6]. The tumor microenvironment is mainly composed of extracellular matrix(ECM), immune cells, blood vessels, cytokines and, importantly, non-epithelial cells such as fibroblasts [7, 8]. Among these, cancer-associated fibroblasts (CAFs) exert a profound negative impact on the host [9] and create a niche for cancer cells to promote their malignant behaviors [10, 11]. The mutual interactions and formation of communication networks between cancer cells and their microenvironment have already been reported [12, 13], while the exact mechanism of the regulation of GC cells by the microenvironment remains unclear.

Exosomes are vesicles by which cells can communicate with one other exchanging mRNA, DNA, protein, and microRNAs (miRNAs) [14]. Previous studies have demonstrated that each exosome carries approximately 121 miRNAs [15]. MiRNAs derived from exosomes could promotes varieties of malignant behaviors [16]. To date, it has only been a few studies on miRNAs that are transported in exosomes derived from cancer cells and their consequent functions on the transformation into CAFs. In our preliminary studies, we identified that specific miRNAs in GC, such as miR-27a, miR-20a and miR-34a, were abundant in exosomes derived from GC cells, while miR-1 and miR-423-5p showed the opposite trend [17]. Among these miRNAs, the high and stable expression of miR-27a derived from exosomes is closely related to poor prognosis according to our investigation of the clinical information [18].

Therefore, our data showed that the high level of miR-27a in the exosomes released from GC cells could have an impact on the regulation of the microenvironment and transformation of CAFs. Thus, miR-27a could be used as a new potential target molecule for gastric cancer therapy.

Materials and Methods

Cell culture

The gastric cancer cell line SGC-7901 was purchased from the Cell Resource Center, Peking Union Medical College (Beijing, China) and cultured in RPMI-1640 medium (Gibco, USA). The human skin fibroblast cell line CCC-HSF-1 was obtained from Life Science College of Wuhan University and maintained in DMEM medium (Gibco, USA). Both media were supplemented with 10% FBS in a humid atmosphere incubator with 5% CO_2 at 37˚C.

Real-time reverse transcription-polymerase chain reaction and western blotting

For total RNA of exosomes extraction, RNA was isolated using the Total RNA Purification Kit (Norgen, Cat. 17200) in accordance with the directions from 200 μL of frozen exosome in PBS. The volume of the obtained RNA solution was 60 μL. Total RNA, for CSRP2 messenger RNA (mRNA) expression quantification, was extracted from cell lines with Trizol reagent (Invitrogen, Carlsbad, CA) in the light of the manufacturer's instructions. cDNA was prepared from 500 ng of total RNA using the Primescript RT Master Kit (Takara, Otsu, Japan). Applied Biosystems SYBR Green Gene Expression Assays were performed using an Applied

Biosystems 7500 Real-Time PCR System. The $2^{-\Delta\Delta CT}$ method was applied to estimate relative gene transcript levels standardized to the β-actin reference gene. The expression levels of miR-27a and CSRP2 mRNA were normalized to that of the U6 snRNA and β-actin distinctively, by using the comparative CT method. The primer sequences were as follows:

5'CTGACTGAGAAAGAAGGCGAAATC'3 and
5' TGCTGGCTGTTTCACAGTAGTGA'3 for CSRP2, or
5' CCCGCCTCCGACTCAAAATG'3 and
5' CTGGCCATAACCGTAGCCTT'3 for CSRP2

Cells were seeded in 6-well-plate for protein extraction with RIPA buffer (Solarbio, Beijing, China) in the presence of protease inhibitor cocktail. Separated proteins (25 μg/lane) were lysed in 10% SDS–PAGE and transferred to a PVDF membrane on request. After blocked for 1h with 5% BSA, the blots were incubated at 4˚C overnight with the following antibodies: CD63 (Santa Cruz), Rabbit-anti-α-SMA (Abcam, ab5694) (1/1000), mouse-anti-β-actin (ZSGB-BIO, China) (1/1000), CSRP2(Abcam, ab65946) (1/1000), GAPDH (Santa Cruz, sc-20357) (1/1000). Afterwards, the blots were reacted with a peroxidase-conjugated secondary antibody for 1 hour at room temperature, followed by detection of the proteins with ECL reagents (Pierce). And quantification of protein levels was performed using image J software. As secondary antibodies, we used: goat-anti-mouse IgG, goat-anti-rabbit and donkey-anti-goat IgG (all from ZSGB-BIO, China) (1/2000).

Phenotypic assays (cell proliferation, migration, invasion and wound-healing assays)

Cells were seeded in 96-well plates coated with 10 μL of CCK-8 (Dojindo Japan) per well and treated distinctively, incubated at 37°C for 4 h in a humidified CO_2 incubator. Afterwards, the absorbance was measured at a wavelength of 450 nm.

Cell migratory and invasive abilities were assessed by Transwell (Corning Life Sciences, Bedford, MA, USA) and Matrigel invasion (BD Biosciences) respectively. For migration assays, cells were harvested and re-suspended in serum-free 1640 medium and subsequently deposited onto an 8 μm pore size polycarbonate membrane Transwell insert. For invasion assays, cells were placed on the 8 μm pore size polycarbonate membrane Transwell insert coated with 40 μL of Matrigel matrix (1:7 diluted with serum-free medium). Cells migrated to the underside of the membrane were fixed and stained with 0.1% crystal violet and were enumerated by counting four random fields per well.

For wound-healing assays, 3.0×10^5 of fibroblasts were seeded into 6-well plates. An artificial gap was created on the confluent cell monolayer with a plastic tip and the images of the wound area were captured in the following 24h.

Immunofluorescence

GC cells were fixed with 4% paraformaldehyde for 20 mins, permeabilized with 0.1% Triton X-100 for 10 mins at room temperature prior to blocking in 5% BSA for 30 mins. α-SMA was detected by reacting with antibody (1:100) overnight at 4°C, which followed by Alexa Fluor 488-conjugated secondary antibodies at 1:200 for 1 h in the dark. Coverslips were mounted on glass slides using ProLong® Diamond AntifadeMountant with DAPI (Life Technologies, USA) mounting medium prior to imaging with a fluorescence microscope.

miRNA microarray analysis

Microarray analyses were performed on exosomes using miRNA Labeling Reagent and Hybridization Kits (Agilent Technologies) and human miR Microarray Kits (Agilent Technologies). A total of 100 ng of total RNA from each sample was treated with phosphatase and then labeled with Cyanine 3-pCp. The labeled RNA was purified using Micro Bio-spin columns(Bio-Rad) and was subsequently hybridized to a human miRNA microarray slide at 55°C for 20 h. After hybridization, the slides were washed with Gene Expression Wash Buffer(Agilent) and scanned on an Agilent Microarray Scanner using Agilent Scan Control version A.7.0.1

miR-27a expression plasmid construction and stable transfection

According to the sequence of miR-27a (5'-TTCACAGTGGCTAAGTTCCGC-3') documented in the miRNA Registry database, oligonucleotides encoding miR-27a were sub-cloned into BamH I and Xho I restriction sites of pGLVH1/GFP/Puro (GenePharma Co., Ltd, Shanghai, China), as well as the negative control group (5'-UUCUCCGAACGUGUCACGUTT-3'), the inhibitor group (5'-GCGGAACUUAGCCACUGUGAA-3'), and the

inhibitor NC group (5'-CAGUACUUUUGUGUAGUACAA-3'). The transfer plasmid and pGLVH1/miR-27a/GFP/Puro and lentiviral packaging vectors psPAX2 and pMD2.G were transfected into 293T cells, and the lentiviral particles were collected. HSF-1 cells were then stably transfected with the lentiviral particles and then screened for GFP expression using flow cytometry.

CSRP2 knockdown and stable transfection

The loss of CSRP2 expression was achieved by transfecting HSF-1 cells with CSRP2-specific siRNAs (siCSRP2; target sequence:5'-GGGCATCAAACCAGAGAGTGT-3'). Non-targeting siRNAs were used as controls. The transfected cells were maintained in culture for 48 h prior to subsequent analyses.

Isolation and identification of SGC-7901 Exosomes

Exosomes were obtained from cell culturing media with ExoQuick-TC Precipitation Solution (System Bioscience) according to manufacture instruction. Briefly, exosomes from cell cultures were collected by ultracentrifugation at 110, 000 g for 2h at 4 °C after pre-cleaning the media by two successive centrifugations and filtration through a 0.22 μm filter. Afterwards, 10mL of the media, after centrifuged at 3000g for 15mins, were mixed with 2mL of ExoQuick Solution. Subsequently, the pellets were suspended in PBS after centrifugation. Protein concentration in exosomes was estimated through absorbance at 562 nm with a BCA protein assay kit (Pierce). Furthermore, CD63 (Santa Cruz) in exosomes was detected with Western blot analysis as mentioned above.

Transmission electron microscopy.

The exosome-enriched pellets were suspended in 50 μL of PBS, fixed with 4% paraformaldehyde and 4% glutaraldehyde in 0.1M phosphate buffer(pH7.4) at incubation temperature and kept at 4˚C until analysis by TEM. A drop of each exosome sample was placed on a carbon-coated copper grid and immersed in 2% phosphotungstic acid solution(pH7.0) for 30s.The preparations were examined with a transmission electron microscope at an acceleration voltage of 80kV.

Cytokine Array

HSF-1 cells were treated with distinct exosomes from gastric cancer cells or transfected with miR-27a or NC for 24 h. Supernatants were collected and examined for differential cytokine expression using the Human Cytokine Array G5 (RayBiotech) per the manufacturer's instructions using the Image Quant TL software (GE Healthcare Life Sciences).

Luciferase assay

SGC-7901 cells were seeded in a 24-well plate and co-transfected with luciferase reporter constructs encoding the wild-type 3'UTR region of CSRP2 (WT-CSRP2) or a mutated CSRP2 3'UTR region (MU-CSRP2) and miR-27a mimic or miR-27a mimic-NC using Lipofectamine 2000 (Invitrogen, California, USA). Luciferase activities were measured using a Dual-Luciferase Reporter Assay Kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's protocol.

Animal studies

Four- to six-week-old female BALB/c nude mice were maintained in a barrier facility on HEPA-filtered racks. SGC-7901 cells, together with miR-27a- or NC-transfected HSF-1 cells were trypsinized, washed twice in PBS, re-suspended at $2x10^7$ cells/ml in PBS and mixed 1:1, and then injected intraperitoneally into the right flanks of BALB/c nude mice. Primary tumors were measured in 3 dimensions (a, b and c), and tumor volume was calculated as abcx0.52. Tumors were harvested from the flanks of mice. All animal studies were conducted under an approved protocol in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals and was approved by Animal Care. Mice (approval 2016080) were approved by the Animal Care and Use Committee of Tianjin Medical University Cancer Institute and Hospital, China.

Statistical analysis

All the data are representative of 3-6 independent experiments. The data were expressed as the mean ± S.E. of at least three separate experiments. Statistical significance was considered at $P < 0.05$ using Student's *t*-test. In this study, GraphPad Software (San Diego, CA, USA) was used to conduct the analyses.

Results

Characterization and isolation of exosomes

After being isolated from the sera of gastric cancer patients, exosomes were characterized by TEM (Fig. 1A), and the expression of the exosome marker CD63 was estimated by western blotting (Fig. 1B). The significant expression of CD63 was observed in the exosomes. The concentration of exosomal proteins was determined using a BCA kit (Fig. 1C), and there was a remarkable difference between exosomes and PBS. By analyzing the purified exosomes released from gastric cancer cells using qRT-PCR, we observed that miR-27a, miR-20 and miR-34a were present at a high level, while miR-1 and miR-423-5p showed the opposite trend (Fig. 1D). Moreover, the level of miR-27a was significantly high in these exosomes (Fig. 1E), suggesting that exosomes transport miR-27a.

GC cells, exosomes and miR-27a induce transformation into CAFs

Initially, SGC-7901 cells were chosen from 4 of the gastric cell lines, because SGC-7901 cells secret more exosomes comparatively (Fig. 2A). To explore the distinct functions of exosomes, miR-27a and GC cells in the transformation of fibroblasts into CAFs, we conducted a series of experiments on 3 groups of HSF-1 cells by incubating them with GC cells or exosomes derived from GC cells or by transfecting with miR-27a over-expression constructs. Then, we conducted western blotting to analyze the expression of α-SMA, a well-

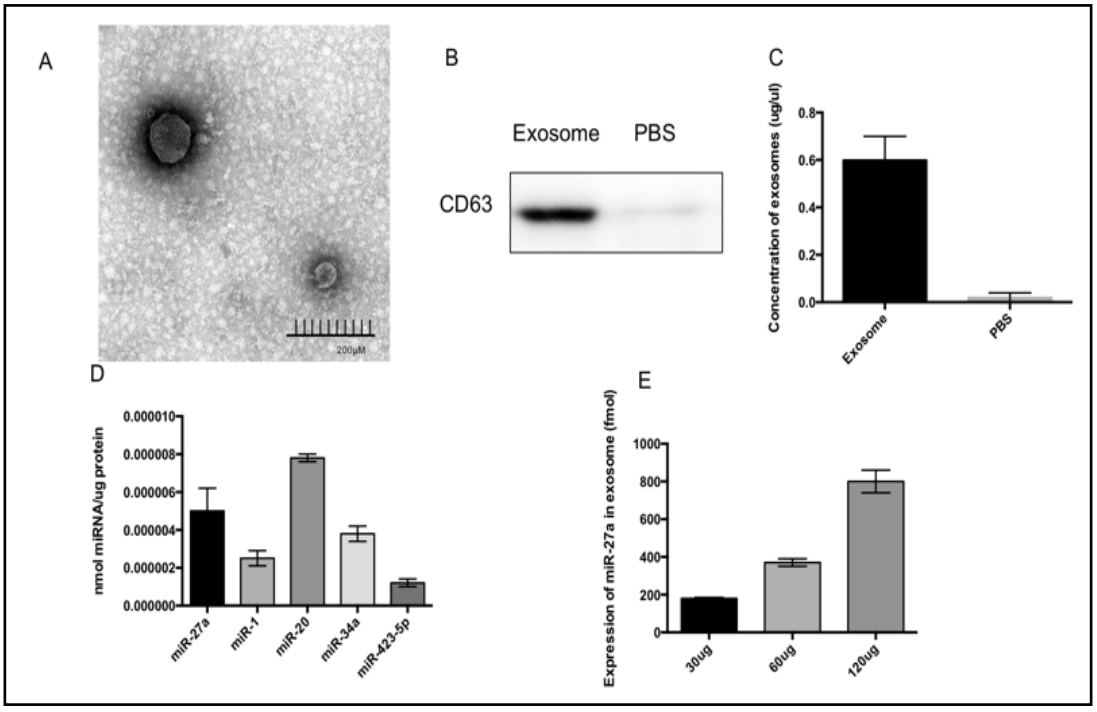

Fig. 1. Characterization and isolation of exosomes. (A) Electron micrograph expressed exosomes isolated from gastric cancer cells. (B) Western blotting analysis showed exosome-enriched medium with high expression of exosome marker CD63. (C) Concentration of exosome was estimated by BCA kit. (D) qRT-PCR showed diverse levels of miRNAs in GC cells. (E)qRT-PCR of miR-27a expression was in different concentrations of exosomes.

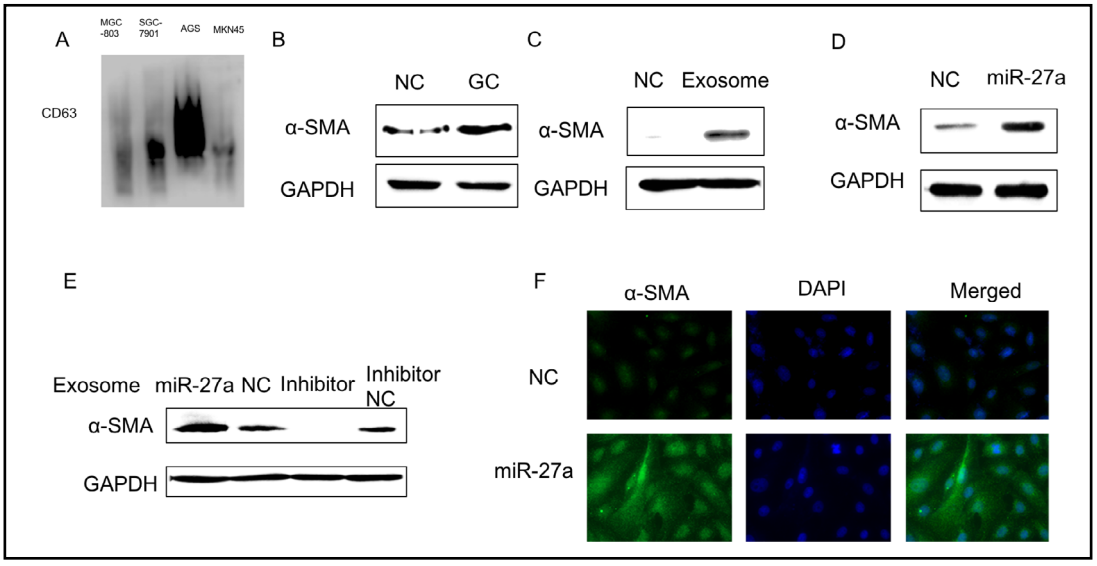

Fig. 2. Cancer cells, exosomes and miR-27a induce transformation into CAFs. (A) Western blotting showed the expression of CD63 in 4 kinds of cell lines. Western blotting analysis of expression levels of α-SMA in HSF-1 cells by incubating them with GC cells (B) or exosomes derived from GC cells (C) or by transfecting with miR-27a over-expression constructs (D). (E) The expression of α-SMA in HSF-1 cells showed by western blotting after co-incubated with miR-27a-over-expressed or miR-27a-lacking-expressed exosomes, respectively.(F)Cell immunofluorescence analysis performed the expression of α-SMA in isolated fibroblasts with miR-27a highly expressed.

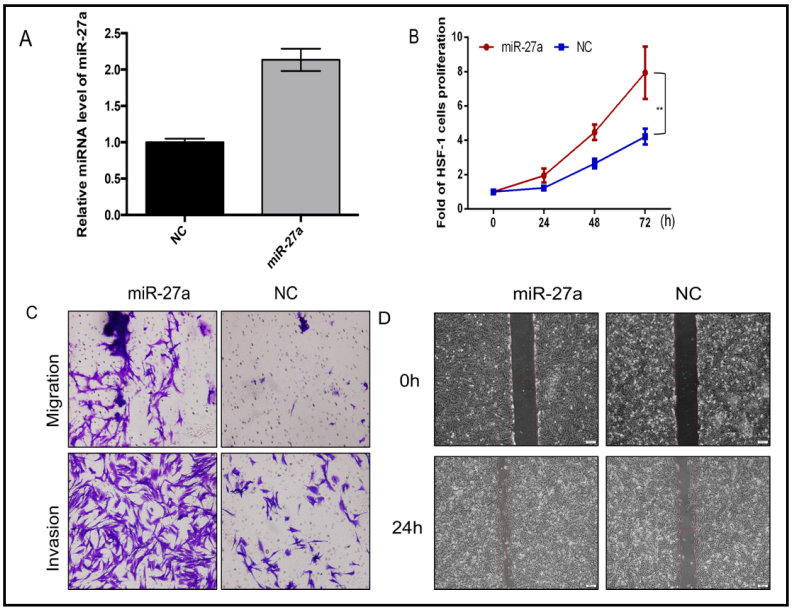

Fig. 3. Upregulation of miR-27a facilitates the proliferation, migration and invasion of fibroblasts in vitro. (A) The expression of miR-27a in HSF-1 cells after transfection verified by qRT-PCR. (B) MiR-27a over-expression significantly accelerated the growth rate of HSF-1 cells as demonstrated by the CCK-8 proliferation assay. P<0.05(C)Fibroblasts with high level of miR-27a promoted the ability of migration and invasion by Transwell migration and invasion assay. (D) Fibroblasts showed stronger ability of migration by wound healing test.

recognized marker of CAFs, in HSF-1 cells. The results revealed that the level of α-SMA was significantly increased in all 3 treatment groups (Fig. 2B-D), compared to that in the negative control group. Furthermore, the expression of α-SMA in miR-27a-over-expressed group was markedly higher compared to that in the negative control group. By down regulating the expression of miR-27a using miR-27a inhibitor transferring lentiviral plasmid, the expression

of α-SMA was much lower than the miR-27a inhibitor group. (Fig. 2E). We further confirmed these results by immunofluorescence to detect the expression pattern of α-SMA in miR-27a-over-expressed HSF-1 cells and negative control cells (Fig. 2F), and our findings showed that GC cells, exosomes, and miR-27a play a vital role in the conversion of fibroblasts to CAFs in gastric cancer.

Upregulation of miR-27a facilitates the proliferation, migration and invasion of fibroblasts in vitro

To investigate the impact of miR-27a on the transformation of fibroblasts to CAFs, HSF-1 cells were transfected with lentiviral plasmids containing pre-miR-27a, and the expression of miR-27a was confirmed by means of qRT-PCR (Fig. 3A). The CCK8 assay was used to evaluate the proliferative capacity of the fibroblasts (Fig. 3B, $p<0.05$). miR-27a over-expression significantly promoted the growth of HSF-1 cells, and the difference between the miR-27a-over-expressed and NC-miR groups became increasingly noticeable with time. In addition, we also investigated whether miR-27a influenced the migration and invasion of fibroblasts (Fig. 3C). It was clear that after 6 h of incubation, the fibroblasts over-expressing miR-27a were more migratory than the fibroblasts in the NC group. After 48 h, the extent of invasion of both groups of fibroblasts showed a similar trend as described above. To further verify the ability of migration, we carried out wound healing assays (Fig. 3D). It was found that fibroblasts transfected with miR-27a over-expressing constructs showed a higher degree of motility.

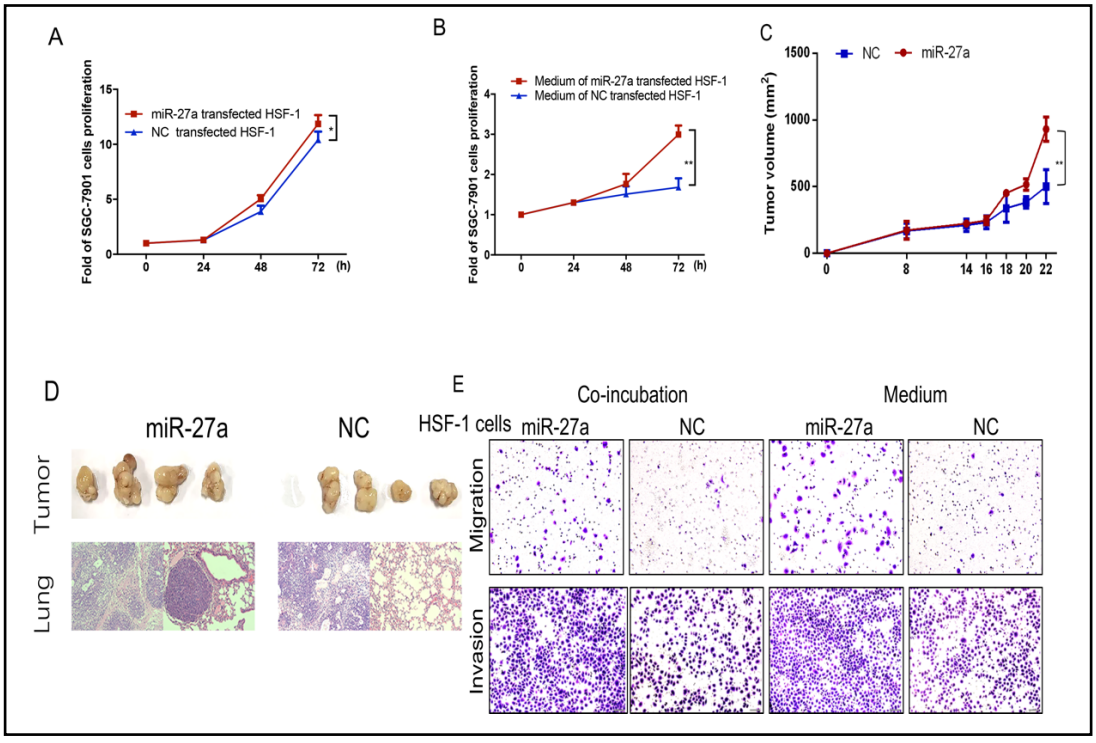

Fig. 4. miR-27a-over-expressed CAFs effect the GC cells over proliferation and motility in vitro and in vivo. (A) SGC-7901 cells co-incubating with miR-27a over-expressed and NC-miR HSF-1 for 24h increasingly promoted the growth rate analyzed by CCK-8 assays. $P<0.05$ (B) SGC-7901 cells cultured in the media of miR-27a transfected and NC-miR HSF-1 for 24h significantly increase the proliferation measured by CCK-8 assays.$p<0.05$ (C, D) HSF-1 cells transfected with lentiviral plasmid containing pre-miR-320a or NC were injected into nude mice. After 22-day period, the tumors were excised. Representative tumors and the variation of tumors were on display. And representative H&E and immune-histochemical staining for lung metastasis showed the group of hyper-expressed miR-27a promoted the capability of metastasis.

miR-27a-over-expressing CAFs effect the proliferation and motility of GC cells in vitro and in vivo

To further investigate the exact effects of CAFs on gastric cancer cells, we carried out a series of experiments to verify the mutual impact of CAFs and SGC-7901 cells. First, SGC-7901 cells seeded in 6-well plates and growing at logarithmic phase were divided into two groups and incubated with miR-27a-transfected HSF-1 cells or conditioned media from these cells for 24 h before subsequent functional analyses. The CCK-8 assay was implemented to measure the proliferation of the treated SGC-7901 cells. It was revealed that SGC-7901 cells co-cultured with miR-27a-over-expressing HSF-1 cells grew more rapidly compared with the cells co-cultured with NC-transfected HSF-1 cells (Fig. 4A). The GC cells cultured in conditioned media from miR-27a-transfected HSF-1 cells exhibited a similar and even more profound trend (Fig. 4B).

Furthermore, we explored the influence of co-culture of the two cell types on motility. The GC cells exhibited a striking increase with regards to migration and invasion after being co-cultured with miR-27a-over-expressing HSF-1 cells or with conditioned media from these cells (Fig. 4E).

In a.ddition, to investigate the effect of miR-27a in vivo on the tumorigenicity of gastric cancer, we injected luciferase-labelled HSF-1 cells transfected with miR-27a or miR-NC combined with SGC-7901 cells into the abdominal cavities of immune-deficient BLAB/c mice. Consequently, after 22 days, the mean size of the tumor nodules of the miR-27a-transfected group was significantly larger than that of the other group (Fig. 4C). Furthermore, as evidence of the effect of miR-27a on the of metastatic capacity of GC cells in vivo, we found that mice injected with miR-27a-transfected fibroblasts experienced lung metastasis as shown by the results of hematoxylin and eosin (H&E) and immune-histochemical staining (Fig. 4D).

CSRP2 is a direct target of miR-27a in GC cells

To understand the underlying mechanism by which miR-27a promotes the progression of gastric cancer, bioinformatics tools were used to predict the potential targets of miR-27a. Among those, CSRP2, which has been found to play a role as a suppress or of oncogenic transformation of fibroblasts [19], was chosen for further investigation. A predicted binding site was confirmed in the 3'untranslated region(UTR) of CSRP2 mRNA (Fig. 5A). To determine whether CSRP2 is a direct target of miR-27a, we performed a luciferase-reporter assay with vectors containing wild-type or mutated 3'UTR of CSRP2(WT-CSRP2 or MU-CSRP2, respectively) (Fig. 5B). It was found that CSRP2 could be directly regulated by miR-27a, and the expression of CSRP2 was inversely correlated with that of miR-27a.

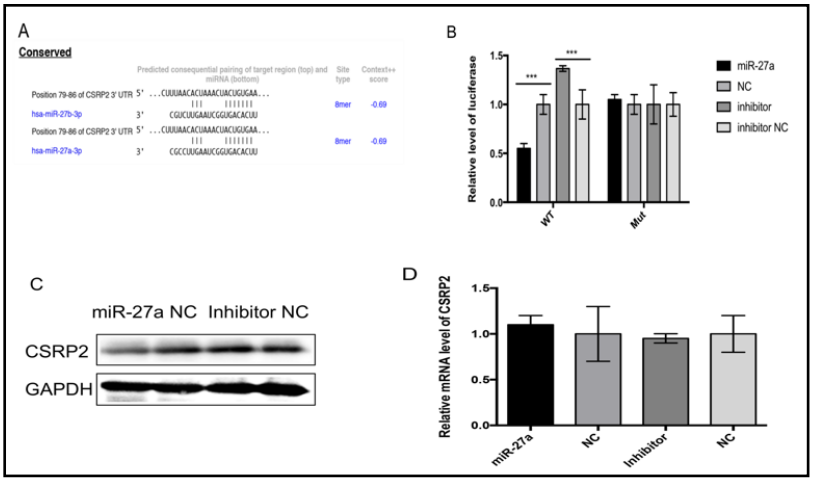

Fig. 5. CSRP2 is a direct target of miR-27a in GC cells. (A) Predicted binding site in the 3'-UTR region of CSRP2. (B) An miR-27a mimic or inhibitor and a luciferase vector encoding the wild-type or mutant CSRP2 3'UTR region were co-transfected into HSF-1 cells, and the relative luciferase acitivity was measured.***p<0.01. (C) CSRP2 protein expression after transfected with miR-27a, NC, inhibitor and inhibitor NC was analyzed by western blotting. (D) Relative mRNA level of CSRP2 performed by qRT-PCR after transfected with miR-27a, NC, inhibitor and inhibitor NC.p>0.05.

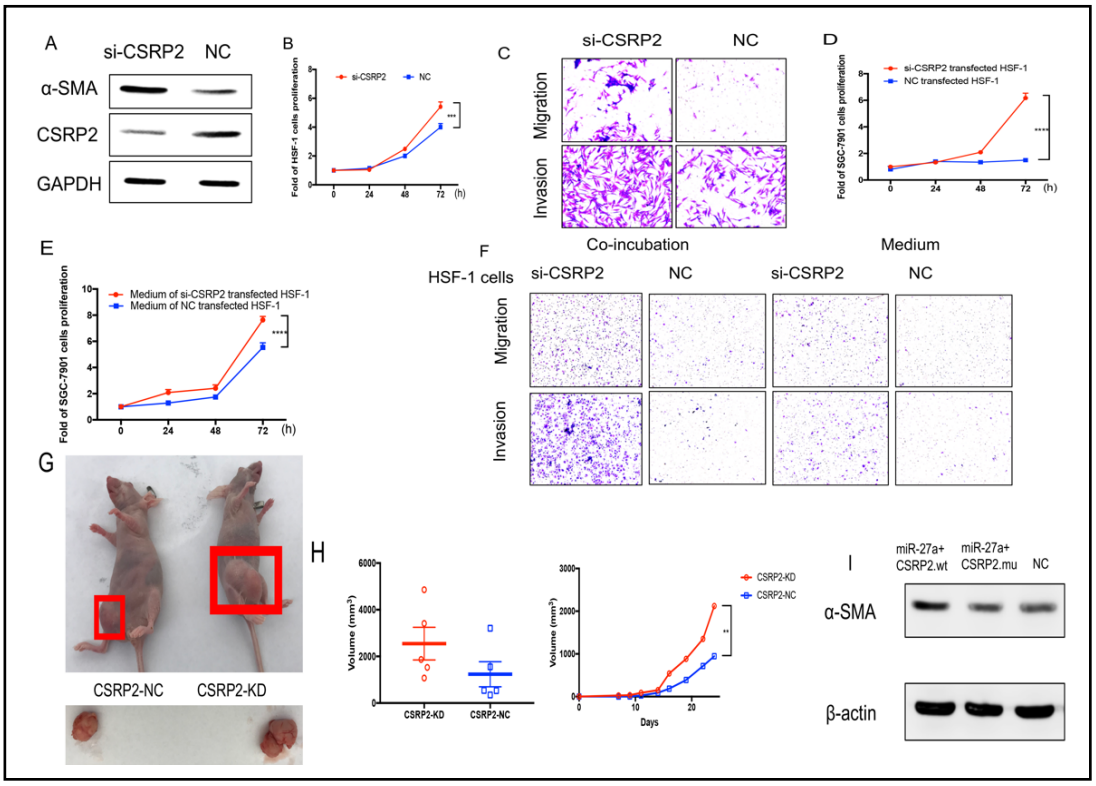

Fig. 6. Downregulation of CSRP2 induces the transformation of fibroblasts into CAFs and promotes the progression of gastric cancer. (A) Western blotting showed the expression of α-SMA and CSRP2 after transfected with si-RNA of CSRP2 and the NC group. (B-C) CSRP2-knockdown promoted HSF-1 cells proliferation, migration and invasion as demonstrated by the CCK-8 proliferation assays (B) and Transwell assays (C) (D)CCK-8 proliferation assays revealed the discrepancy between SGC-7901 cells co-incubated with the HSF-1 cells in a loss of CSRP2 expression and NC group. ****p<0.01(E) CCK-8 proliferation assays showing the difference after co-culturing with the medium of si-CSRP2 transfected HSF-1 cells or negative control. ****p<0.01 (F) The motility of GC cells affected by CAFs, with CSRP2 knocking down, not only with the CAFs, but the medium containing varieties of cytokines performed by transwell assays. (G-H) Volume of tumors in CSRP2-knockdown/NC groups. p<0.05 (I) Western blotting showed the transformation of CAFs by overexpressed miR-27a targeting wild type/mutation type CSRP2.

Moreover, western blot analysis confirmed the downregulation of CSRP2 in the miR-27a-over-expressing HSF-1 cells. In contrast, the inhibitor-transfected group with a loss of miR-27a expression exhibited an upregulation of CSRP2 (Fig. 5C). However, the mRNA level of CSRP2 stayed almost constant after the cells were transfected with miR-27a or inhibitor as seen from the results of qRT-PCR (Fig. 5D).

Downregulation of CSRP2 induces the transformation of fibroblasts into CAFs and promotes the progression of gastric cancer

To explore whether the downregulation of CSRP2 has an impact on the transformation of fibroblasts into CAFs, we knocked down the expression of CSRP2 with short interfering RNA(siRNA) along with a NC group for comparison. We conducted western blotting to confirm that the expression of CSRP2 was downregulated in the si-CSRP2 group compared with that in the NC group (Fig. 6A). Meanwhile, we observed an elevated expression of α-SMA by western blot analysis in the si-CSRP2 group (Fig. 6A).

In addition, we investigated the proliferation of HSF-1 cells by means of the CCK-8 assay after knocking down the expression of CSRP2 (Fig. 6B). It was revealed that the growth rate

of the fibroblasts was promoted by the downregulation of CSRP2 in comparison with growth rate in the NC group.

Furthermore, to verify the effect on the motility of HSF-1 cells by downregulation of CSRP2, we performed the experiment by Transwell assays. The results showed an obvious difference in the migration and invasion of cells in the si-CSRP2 group compared to those in the control group (Fig. 6C).

Furthermore, we investigated the effect of CAFs on GC cells. After incubating SGC-7901 cells with HSF-1 cells with CSRP2 downregulation or the conditioned medium from these cells, it was observed that the treatment group and the negative control group were strikingly different in the results obtained from CCK-8 proliferation (Fig. 6D, E) and Transwell assays (Fig. 6F), which suggested that CAFs could in turn affect the proliferation, migration and invasion of GC cells.

In addition, we performed in vivo experiments that combines SCG-7901 cells and HSF-1 cells with CSRP2-knockdown/NC. Each group included 5 nude mice. Finally, the group with CSRP2-knockdown resulted in more malignant behaviors in the tumor volume (Fig. 6G-H) which explained the down-expression of CSRP2 in fibroblast could promote the progression of gastric cancer.

Moreover, we conduct Western blotting illustrating that miR-27a directly targeted CSRP2. It was indicated that the overexpression of miR-27a could down-regulating the expression of wild type CSRP2, resulting in the promotion of transformation of CAFs. But this phenomenon was not found in mutation type CSRP2 (Fig. 6I).

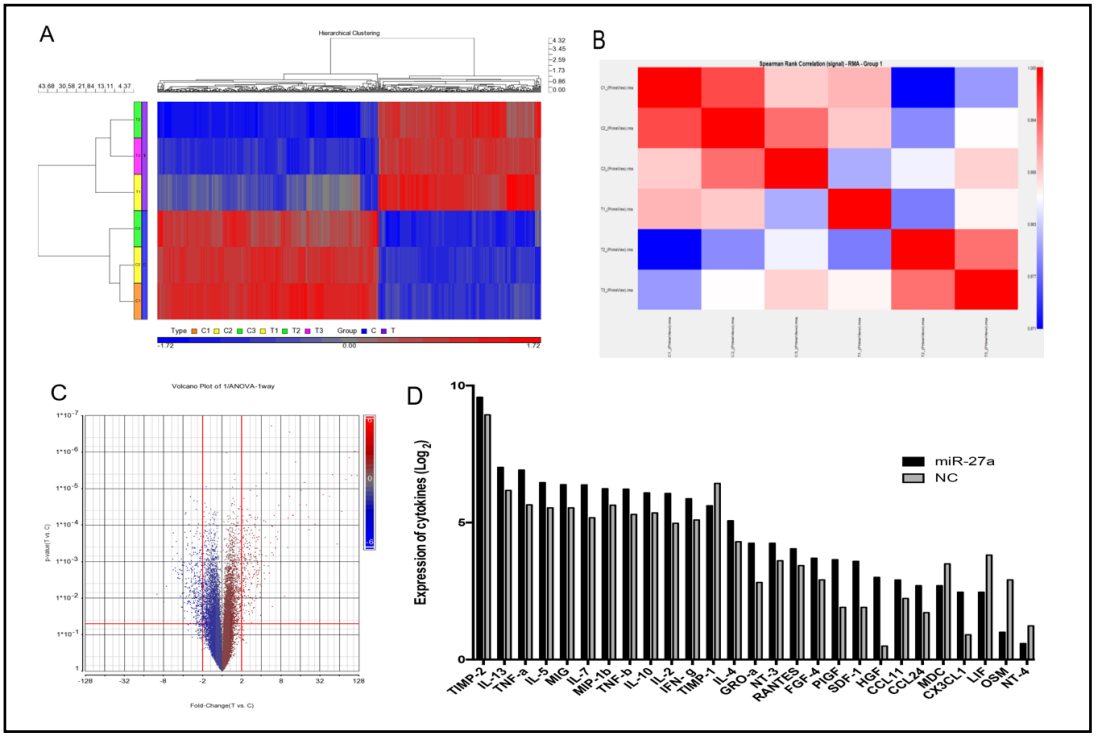

Fig. 7. Differentially expressed genes between fibroblasts co-incubating with or without exosomes containing miR-27a. (A) Heat map presentation of the expression profile of genes. Each row represents a sample while each column represents a gene. The above 3 rows were fibroblasts cultured with exosomes containing miR-27a, while the below 3 rows were negative control. (B) Expression of each gene was validated by qRT-PCR and shown by heat map. (C) Volcano plot analysis of microarray data on differentially expressed genes between treatment and control group. The vertical red lines correspond to a 2.0-fold up and down regulation, while the horizontal red line represents a P-value of 0.05. Statistical significance was defined as fold change≥2.0 and P-value≤0.05. (D) Difference expression of some cytokines between treatment and control group. $p<0.05$.

Differentially expressed genes between fibroblasts incubated with or without exosomes containing miR-27a

We next performed microarray analysis to reveal the gene expression profiles of CAFs incubated with exosome carrying miR-27a. First, the SGC-7901 cells were divided into two groups and transfected with miR-27a-over-expression or miR-NC constructs. Heat maps (Fig. 7A, B) showed that there was a significant difference in gene expression between treatment and control groups. Volcano plot analysis (Fig. 7C) was used to visualize the fold change and statistical difference in the expression profiles for the fibroblasts cultured with exosomes after normalization to the control group. Afterwards, Kyoto Encyclopedia of Genes and Genomes(KEGG) analysis was performed to determine the role of exosomes containing miR-27a in the transformation to CAFs (Table 1). It was found that the interaction between cytokines and their corresponding receptors represented the largest proportion of all pathways at approximately 6.1%, which was followed by the NOD-like receptor signaling pathway and pathways in cancer, among others. To validate and further explore the findings, cytokine arrays were used, and the results showed that many cytokines, including TNF-β, SDF-1 and FGF-4, markedly differentially expressed between the treatment and control groups, suggesting that cytokines play a critical role in the tumor microenvironment (Fig. 7D).

Table 1. KEGG analysis on the role of pathways in fibroblasts surrounded by exosomes with up-regulation of miR-27a

Term	Count	%	P Value	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
Cytokine-cytokine receptor interaction	25	6.097560976	1.46E-07	262	5085	3.416871293	1.79E-05	1.79E-05	1.69E-04
NOD-like receptor signaling pathway	12	2.926829268	7.63E07	62	5085	6.930940482	9.31E-05	4.66E-05	8.79E-04
Pathways in cancer	20	4.87804878	0.001602614	328	5085	2.183527997	0.177721188	0.063143589	1.82973014
Chemokine Signaling pathway	14	3.414634146	0.001881305	187	5085	2.680952022	0.205256141	0.055815655	2.144777795
Toll-like receptor signaling pathway	8	1.951219512	0.021392051	101	5085	2.836424488	0.928506567	0.409998344	22.03994435
ECM-receptor interaction	7	1.707317073	0.028315338	84	5085	2.98415493	0.969932842	0.442366663	28.15922743
Hematop-oi etic cell lineage	7	1.707317073	0.031323923	86	5085	2.914755978	0.979403848	0.425735066	30.67901923
ABC transporters	5	1.219512195	0.032632106	44	5085	4.069302177	0.982534394	0.397061581	31.74927519
TGF-beta Signaling pathway	7	1.707417073	0.032903776	87	5085	2.881253035	0.983122743	0.364619999	31.96963383
Complement and coagulation cascades	6	1.463414634	0.041606262	68	5085	3.113900796	0.99439773	0.404562199	38.69394479
Focal adhesion	11	2.682926829	0.051410247	201	5085	1.959743536	0.998402031	0.443097794	45.5386196
Cytosolic DNA-sensing pathway	5	1.219512195	0.065115273	55	5085	3.255441741	0.999729303	0.49567907	53.94062152
Arachidonic acid metabolism	5	1.219512195	0.68680034	56	5085	3.197308853	0.999830149	0.487132487	55.92268222
Hypertrophic cardiomyopathy(HCM)	6	1.463414634	0.086018933	85	5085	2.5227754764	0.99982847	0.543337242	64.49872975

Discussion

The aim of the present study was to clarify the crucial functions of exosomes carrying miRNAs in the transformation of CAFs as well as in the progression of gastric cancer.

It is already well recognized that the occurrence and progression of cancer are not simply dependent on cancer cells but also rely to a large extent on the tumor microenvironment [20, 21]. The components of the tumor microenvironment may also affect patients survival [22]. It had already demonstrated that demonstrated the new roles of hedgehog signaling pathway in the hBMSCs-derived exosomes induced tumor progression [23]. Among the predominant factors in the tumor microenvironment, are fibroblasts that can be transformed into CAFs under certain conditions [24]. CAFs play an important role in promoting cancer by secreting cytokines such as PDGF, TGF-β, VDGF and CXCL12[25-29]. Therefore, it is crucial to elucidate the precise mechanisms of the transformation of fibroblasts into CAFs by cancer cells.

While most studies on miRNA have placed emphasis on tumor cells, little is known about the role of miRNAs in the tumor-associated microenvironment. Previous studies have demonstrated that exosomes, which retain miRNAs in a stable form, serve as efficient transport mechanisms to deliver signals between cells [30]. In our preliminary experiments, we detected the expression of certain miRNAs that was altered in the exosomes derived from GC cells and finally chose miR-27a, which was abundant in these exosomes and was associated with prognosis in GC, for subsequent experiments. Thus, we hypothesized that exosomes rich in miR-27a can affect the tumor microenvironment in gastric cancer, especially by

reprogramming fibroblasts into CAFs. Nevertheless, there could be other miRNAs infecting the transformation together, which need further exploration in our current study.

In line with the genetic heterogeneity between normal fibroblasts and CAFs [31, 32], we first examined the effect on the transformation of fibroblasts to CAFs by miR-27a, exosomes and conditioned media from GC cells. We demonstrated that the acquisition of a CAF-like phenotype in fibroblasts could be induced by tumor cells through exosome-mediated delivery of miR-27a, which not unexpected since exosomes have been shown to deliver miRNAs into cells and play a role in their transformation [33]. Furthermore, various properties of the fibroblasts were affected by miR-27a over-expression including proliferation and motility, suggesting that miR-27a is probably used as a signal by tumor cells to convert the microenvironment into a pre-tumoral niche. To further investigate the underlying mechanism, microarray and cytokine array analyses were performed, and the results suggested that cytokines may have a regulatory role in the reprogramming of fibroblasts affected by exosomes carrying miR-27a, which require further validation. As shown in the graph presented in Fig. 4E, we observed a sharp contrast between the two groups regarding the expression of certain cytokines, indicating their crucial functions in the transformation of fibroblasts into CAFs. Among those cytokines, hepatocyte growth factor(HGF) was the most differentially expressed. By binding with its tyrosine kinase receptor MET, HGF promotes cell proliferation, angiogenesis and invasion in various cancer types [34-37]. It has been reported that HGF is an important factor secreted by CAFs, and it acts as an important mediator of tumor-promoting effects of gastric CAFs [38], suggesting that gastric cancer is probably an excellent model for studying the therapeutic effect of HGF/MET. Moreover, Stromal cell-derived factor-1(SDF-1), which signals via its cognate receptor CXCR4 and is expressed on the surface of carcinoma cells, directly boosts the proliferation of the carcinoma cells and can stimulate neoangiogenesis by recruiting circulating endothelial progenitor cells (EPCs) into the tumor stroma [39, 40]. In addition, the establishment of the self-sustaining TGF-β and SDF-1 autocrine signaling gives rise to tumor-promoting CAFs during tumor progression. This autocrine-signaling mechanism may be an attractive therapeutic target to block the evolution of tumor-promoting CAFs [41].We also observed a reduced expression of oncostatin M(OSM) in the group over-expressing miR-27a. OSM, an interleukin-6-family cytokine, induces cell growth inhibition, differentiation, and apoptosis in a variety of cancers including lung cancer, breast cancer and melanoma [42-44].It has been shown that the loss of OSMR expression occurs frequently in peptic cancers such as colorectal, gastric and pancreatic cancer, but rarely in cancers of other organs. In addition, this phenomenon has been shown to be an early event in colorectal carcinogenesis [45], which makes OSM/OSMR a potential target to be used in diagnosis and therapy.

Furthermore, we investigated the existence of a positive feedback loop to see whether CAFs could in turn affect the GC cells. In our tests, it was revealed that miR-27a-over-expressed fibroblasts as well as conditioned medium derived from these cells could increase the proliferation and motility of GC cells *in vivo* and *in vitro*.

Intriguingly, to elucidate the mechanism of miR-27a, we discovered a sequence in the 3'-UTR region of CSRP2 mRNA that is complementary to miR-27a. Recently, it has been reported that CSRP2, which is highly expressed in breast cancer [46]and B-cell acute lymphoblastic leukemia(ALL)[47], promotes the progression and metastasis of cancer and functions as an oncogene. Nevertheless, there are few studies on the relationship between CSRP2 and miRNAs as well as their effects on tumorigenesis in gastric cancer. To the best of our knowledge, we reported for the first time that miR-27a could directly target to the CSRP2 by binding to its 3'-UTR and reducing its protein levels without affecting the level of mRNA. Furthermore, our results demonstrated that the reprogramming of fibroblasts into CAFs and the capacity of proliferation and motility of these cells were improved by the knockdown of CSRP2. These results were further confirmed by co-incubation of GC cells with CSRP2-silenced fibroblasts or their medium, suggesting that CAFs secrete cytokines that can affect the progression of GC cells, which required further exploration.

Therefore, GC cell-derived exosomes with high levels of miR-27a can transform fibroblasts into CAFs. Additionally, CSRP2 may perform as a downstream target of miR-27a, and its expression is inversely proportional to that of miR-27a in gastric cancer. Additionally, miR-27a-over-expressed or CSRP2-silenced fibroblasts can accelerate the progression of GC.

Abbreviations

miRNA (microRNA); CAFs (cancer-associated fibroblasts); GC (gastric cancer); ECM (extracellular matrix); SDF-1 (Stromal cell-derived factor-1); EPCs (endothelial progenitor cells); OSM (oncostatin M); ALL (acute lymphoblastic leukemia).

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All animal studies were conducted under an approved protocol in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals and was approved by Animal Care. Mice (approval 2016080) were approved by the Animal Care and Use Committee of Tianjin Medical University Cancer Institute and Hospital, China.

Disclosure Statement

The authors declare that they have no competing interests.

References

1 Shmulevich I: Large-scale molecular characterization and analysis of gastric cancer. Chin J Cancer 2014;33:369-370.
2 Hamashima C: Current issues and future perspectives of gastric cancer screening. World J Gastroenterol 2014;20:13767-13774.
3 Hohenberger P, Gretschel S: Gastric cancer. Lancet 2003;362:305-315.
4 Nashimoto A, Akazawa K, Isobe Y, Miyashiro I, Katai H, Kodera Y, Tsujitani S, Seto Y, Furukawa H, Oda I, Ono H, Tanabe S, Kaminishi M: Gastric cancer treated in 2002 in Japan: 2009 annual report of the JGCA nationwide registry. Gastric Cancer 2013;16:1-27.
5 Zhang WH, Chen XZ, Liu K, Chen XL, Yang K, Zhang B, Chen ZX, Chen JP, Zhou ZG, Hu JK: Outcomes of surgical treatment for gastric cancer patients: 11-year experience of a Chinese high-volume hospital. Med Oncol 2014;31:150.
6 Kessenbrock K, Plaks V, Werb Z: Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 2010;141:52-67.
7 Liotta LA, Kohn EC: The microenvironment of the tumour-host interface. Nature 2001;411:375-379.
8 Challagundla KB, Wise PM, Neviani P, Chava H, Murtadha M, Xu T, Kennedy R, Ivan C, Zhang X, Vannini I, Fanini F, Amadori D, Calin GA, Hadjidaniel M, Shimada H, Jong A, Seeger RC, Asgharzadeh S, Goldkorn A, Fabbri M: Exosome-mediated transfer of microRNAs within the tumor microenvironment and neuroblastoma resistance to chemotherapy. J Natl Cancer Inst 2015;107.

9 Hwang RF, Moore T, Arumugam T, Ramachandran V, Amos KD, Rivera A, Ji B, Evans DB, Logsdon CD: Cancer-associated stromal fibroblasts promote pancreatic tumor progression. Cancer Res 2008;68:918-926.
10 Spaeth EL, Dembinski JL, Sasser AK, Watson K, Klopp A, Hall B, Andreeff M, Marini F: Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression. PLoS One 2009;4:e4992.
11 Haubeiss S, Schmid JO, Murdter TE, Sonnenberg M, Friedel G, van der Kuip H, Aulitzky WE: Dasatinib reverses cancer-associated fibroblasts (CAFs) from primary lung carcinomas to a phenotype comparable to that of normal fibroblasts. Molecular Cancer 2010;9.
12 Junttila MR, de Sauvage FJ: Influence of tumour micro-environment heterogeneity on therapeutic response. Nature 2013;501:346-354.
13 Hui L, Chen Y: Tumor microenvironment: Sanctuary of the devil. Cancer Lett 2015;368:7-13.
14 Cocucci E, Racchetti G, Meldolesi J: Shedding microvesicles: artefacts no more. Trends Cell Biol 2009;19:43-51.
15 Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO: Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007;9:654-U672.
16 Li XJ, Ren ZJ, Tang JH, Yu Q: Exosomal MicroRNA MiR-1246 Promotes Cell Proliferation, Invasion and Drug Resistance by Targeting CCNG2 in Breast Cancer. Cell Physiol Biochem 2017;44:1741-1748.
17 Liu R, Zhang CN, Hu ZB, Li G, Wang C, Yang CH, Huang DZ, Chen X, Zhang HY, Zhuang R, Deng T, Liu H, Yin JJ, Wang SF, Zen K, Ba Y, Zhang CY: A five-microRNA signature identified from genome-wide serum microRNA expression profiling serves as a fingerprint for gastric cancer diagnosis. Eur J Cancer 2011;47:784-791.
18 Huang DZ, Wang HY, Liu R, Li HL, Ge SH, Bai M, Deng T, Yao GY, Ba Y: miRNA27a is a biomarker for predicting chemosensitivity and prognosis in metastatic or recurrent gastric cancer. J Cell Biochem 2014;115:549-556.
19 Weiskirchen R, Bister K: Suppression in transformed avian fibroblasts of a gene (crp) encoding a cysteine-rich protein containing LIM domains. Oncogene 1993;8:2317-2324.
20 Koontongkaew S: The tumor microenvironment contribution to development, growth, invasion and metastasis of head and neck squamous cell carcinomas. J Cancer 2013;4:66-83.
21 Soysal SD, Tzankov A, Muenst SE: Role of the tumor microenvironment in breast cancer. Pathobiology 2015;82:142-152.
22 Finak G, Bertos N, Pepin F, Sadekova S, Souleimanova M, Zhao H, Chen H, Omeroglu G, Meterissian S, Omeroglu A, Hallett M, Park M: Stromal gene expression predicts clinical outcome in breast cancer. Nat Med 2008;14:518-527.
23 Qi J, Zhou YL, Jiao ZY, Wang X, Zhao Y, Li YB, Chen HJ, Yang LX, Zhu HW, Li YM: Exosomes derived from human bone marrow mesenchymal stem cells promote tumor growth through hedgehog signaling pathway. Cell Physiol Biochem 2017;42:2242-2254.
24 Kalluri R, Zeisberg M: Fibroblasts in cancer. Nat Rev Cancer 2006;6:392-401.
25 Baglole CJ, Ray DM, Bernstein SH, Feldon SE, Smith TJ, Sime PJ, Phipps RP: More than structural cells, fibroblasts create and orchestrate the tumor microenvironment. Immunol Invest 2006;35:297-325.
26 Egeblad M, Littlepage LE, Werb Z: The fibroblastic coconspirator in cancer progression. Cold Spring Harb Symp Quant Biol 2005;70:383-388.
27 Feig C, Jones JO, Kraman M, Wells RJ, Deonarine A, Chan DS, Connell CM, Roberts EW, Zhao Q, Caballero OL, Teichmann SA, Janowitz T, Jodrell DI, Tuveson DA, Fearon DT: Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. Proc Natl Acad Sci U S A 2013;110:20212-20217.
28 Murata T, Mizushima H, Chinen I, Moribe H, Yagi S, Hoffman RM, Kimura T, Yoshino K, Ueda Y, Enomoto T, Mekada E: HB-EGF and PDGF mediate reciprocal interactions of carcinoma cells with cancer-associated fibroblasts to support progression of uterine cervical cancers. Cancer Res 2011;71:6633-6642.
29 Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D, Hammer DA, Weaver VM: Tensional homeostasis and the malignant phenotype. Cancer Cell 2005;8:241-254.
30 Simons M, Raposo G: Exosomes--vesicular carriers for intercellular communication. Curr Opin Cell Biol 2009;21:575-581.
31 Bauer M, Su G, Casper C, He R, Rehrauer W, Friedl A: Heterogeneity of gene expression in stromal fibroblasts of human breast carcinomas and normal breast. Oncogene 2010;29:1732-1740.

32 Peng Q, Zhao L, Hou Y, Sun Y, Wang L, Luo H, Peng H, Liu M: Biological characteristics and genetic heterogeneity between carcinoma-associated fibroblasts and their paired normal fibroblasts in human breast cancer. PLoS One 2013;8:e60321.
33 Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO: Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007;9:654-659.
34 Chen P, Mo Q, Wang B, Weng D, Wu P, Chen G: Breast cancer associated fibroblasts promote MCF-7 invasion *in vitro* by secretion of HGF. J Huazhong Univ Sci Technolog Med Sci 2012;32:92-96.
35 Gao CF, Vande Woude GF: HGF/SF-Met signaling in tumor progression. Cell Res 2005;15:49-51.
36 Ren Y, Cao B, Law S, Xie Y, Lee PY, Cheung L, Chen Y, Huang X, Chan HM, Zhao P, Luk J, Vande Woude G, Wong J: Hepatocyte growth factor promotes cancer cell migration and angiogenic factors expression: a prognostic marker of human esophageal squamous cell carcinomas. Clin Cancer Res 2005;11:6190-6197.
37 Saito S, Morishima K, Ui T, Hoshino H, Matsubara D, Ishikawa S, Aburatani H, Fukayama M, Hosoya Y, Sata N, Lefor AK, Yasuda Y, Niki T: The role of HGF/MET and FGF/FGFR in fibroblast-derived growth stimulation and lapatinib-resistance of esophageal squamous cell carcinoma. BMC Cancer 2015;15:82.
38 Wu X, Chen X, Zhou Q, Li P, Yu B, Li J, Qu Y, Yan J, Yu Y, Yan M, Zhu Z, Liu B, Su L: Hepatocyte growth factor activates tumor stromal fibroblasts to promote tumorigenesis in gastric cancer. Cancer Lett 2013;335:128-135.
39 Balkwill F: Cancer and the chemokine network. Nat Rev Cancer 2004;4:540-550.
40 Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA: Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 2005;121:335-348.
41 Kojima Y, Acar A, Eaton EN, Mellody KT, Scheel C, Ben-Porath I, Onder TT, Wang ZC, Richardson AL, Weinberg RA, Orimo A: Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. Proc Natl Acad Sci U S A 2010;107:20009-20014.
42 Tanaka M, Miyajima A: Oncostatin M, a multifunctional cytokine. Rev Physiol Biochem Pharmacol 2003;149:39-52.
43 Grant SL, Begley CG: The oncostatin M signalling pathway: reversing the neoplastic phenotype? Mol Med Today 1999;5:406-412.
44 Douglas AM, Grant SL, Goss GA, Clouston DR, Sutherland RL, Begley CG: Oncostatin M induces the differentiation of breast cancer cells. Int J Cancer 1998;75:64-73.
45 Deng G, Kakar S, Okudiara K, Choi E, Sleisenger MH, Kim YS: Unique methylation pattern of oncostatin m receptor gene in cancers of colorectum and other digestive organs. Clin Cancer Res 2009;15:1519-1526.
46 Hoffmann C, Mao X, Dieterle M, Moreau F, Al Absi A, Steinmetz A, Oudin A, Berchem G, Janji B, Thomas C: CRP2, a new invadopodia actin bundling factor critically promotes breast cancer cell invasion and metastasis. Oncotarget 2016;7:13688-13705.
47 Wang SJ, Wang PZ, Gale RP, Qin YZ, Liu YR, Lai YY, Jiang H, Jiang Q, Zhang XH, Jiang B, Xu LP, Huang XJ, Liu KY, Ruan GR: Cysteine and glycine-rich protein 2 (CSRP2) transcript levels correlate with leukemia relapse and leukemia-free survival in adults with B-cell acute lymphoblastic leukemia and normal cytogenetics. Oncotarget 2017;8:35984-36000.