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Original Article

miR-874 inhibits metastasis-relevant traits via targeting SH2B adaptor protein 1 (SH2B1) in gastric cancer

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Abstract: Gastric cancer (GC) is one of the most common human cancers and the second leading cause of cancer-related mortality worldwide. The major cause of death is metastasis. Elucidating molecular mechanism of metastasis in gastric cancer will help us to further understand the pathogenesis and progression of the disease, and offer new targets for effective therapies. In this study, we found that SH2B1 overexpression promoted invasion, migration and anoilds resistance and silencing it inhibited invasion, migration and anoilds resistance in gastric cancer SGC-7901 cells. However, over-expressing or silencing it did not affect proliferation in the cells. miR-874 could degrade SH2B1 by targeting its 3'UTR and was negatively associated with metastasis traits in SGC-7901 cells. Its overexpression inhibited proliferation in the cells. Thus, we concluded that miR-874 inhibits metastasis-relevant traits via targeting SH2B1 in gastric cancer SGC-7901 cells.

Keywords: Gastric cancer, SH2B1, miR-874, metastasis

Introduction

Gastric cancer (GC) is one of the most common human cancers and the second leading cause of cancer-related mortality worldwide [1]. The major cause of death is metastasis, which greatly hinders treatment success [2].

Src homology 2 (SH2) B adaptor protein 1 (SH2B1; originally named SH2-B) is a member of a family of adaptor proteins that influences a variety of signaling pathways mediated by Janus kinase (JAK) and receptor tyrosine kinases [3]. SH2B1 performs classical adaptor functions, such as recruitment of specific proteins to activated receptors, it also demonstrates a unique ability to enhance the kinase activity of the cytokine receptor-associated tyrosine kinase JAK2, as well as that of several receptor tyrosine kinases [3]. Deletion of the SH2B1 gene results in severe obesity and both leptin and insulin resistance, as well as infertility, which might be a consequence of resistance to insulin-like growth factor I [3]. SH2B1 plays an important role in the progression of lung cancer, oesophageal cancer and neuroblastoma [4-7]. But its roles have not been reported in gastric cancer.

miRNAs are regulatory, non-coding RNAs about 18-25 nucleotides in length and are expressed at specific stages of tissue development or cell differentiation, and have large-scale effects on the expression of a variety of genes at the post-transcriptional level. Through base-pairing with its targeted mRNAs, a miRNA induces RNA degradation or translational suppression of the targeted transcripts [8-12]. Some miRNAs can function either as oncogenes or tumour suppressors [13-15] and expression profiling analyses have revealed characteristic miRNA signatures in certain human cancers [16-18]. But, the precise parts played by the expressed miRNAs in specific steps of malignant progression, including metastasis, are still emerging.

In this study, we found that SH2B1 overexpression promoted invasion, migration and anoilds resistance and silencing it inhibited invasion, migration and anoilds resistance in gastric cancer SGC-7901 cells. However, over-expressing or silencing it did not affect proliferation in the cells. miR-874 could degrade SH2B1 by targeting its 3'UTR and was negatively associated with metastasis traits in SGC-7901 cells. Its overexpression inhibited proliferation in the cells. Thus, we concluded that miR-874 inhibits

metastasis-relevant traits via targeting SH2B1 in gastric cancer SGC-7901 cells.

Materials and methods

The human gastric cancer cell line

SGC-7901 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (WISENT, Canada) and antibiotics (1% penicillin/streptomycin, Gibco, USA). The cell line was grown in a humidified chamber supplemented with 5% CO_2 at 37°C.

SH2B1 expressing plasmids/empty vectors, shSH2B1/scramble, pre-miR-874/control miR and transfection experiments

SH2B1 expressing plasmids/empty vectors and shSH2B1/scramble were purchased from Tiangen (Beijing, China). Pre-miR-874 and control miR were purchased from Ambion, Inc. (Ambion, Austin, TX, USA). For transfection experiments, the cells were cultured in serum-free medium without antibiotics at 60% confluence for 24 h, and then transfected with transfection reagent (Lipofectamine 2000, Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. After incubation for 6 h, the medium was removed and replaced with normal culture medium for 48 h, unless otherwise specified.

Western blot analysis

Western blot analysis was performed as described before [19]. Mainly, after incubation with primary anti-body anti-SH2B1 (1:500; Abcam, Cambridge, MA, USA) and anti-β-actin (1:500; Abcam, Cambridge, MA, USA) overnight at 4°C, IRDyeTM-800 conjugated anti-rabbit secondary antibodies (Li-COR, Biosciences, Lincoln, NE, USA) were used for 30 min at room temperature. The specific proteins were visualized by Odyssey™Infrared Imaging System (Gene Company, Lincoln, NE, USA).

Migration and invasion assay

For transwell migration assays, 2.5×10^4 to 5.3×10^4 cells were plated in the top chamber with the non-coated membrane (24-well insert; pore size, 8 mm; BD Biosciences, San Jose, CA, USA). For invasion assays, 1.25×10^5 cells were plated in the top chamber with Matrigel-coated membrane (24-well insert; pore size, 8mm; BD Biosciences, San Jose, CA, USA). In both assays, cells were plated in medium without serum or growth factors, and medium supplemented with serum was used as a chemoattractant in the lower chamber. The cells were incubated for 24 h and cells that did not migrate or invade through the pores were removed by a cotton swab. Cells on the lower surface of the membrane were stained with the Diff-Quick Staining Set (Dade) and counted.

Wound healing assay

Cells (4×10^5) were seeded onto each 35-mm glass bottom dish (MatTek Co., Ashland, MA, USA) and cultured at 37°C with 5% CO_2 for 24 h. The confluent monolayer of cells was wounded. Monolayers of cells were wounded with yellow pipette tips. After washing with warm PBS, the cells were incubated in fresh culture medium. The wounded areas were photographed at the beginning (0 h, top panels) and the end (10 h, bottom panels) of the assay with Nikon inverted microscope (ECLIPSE TE-2000U, Nikon, Japan) equipped with a video camera (DS-U1, Nikon, Japan).

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

Cells seeded on 96-well plates, were stained at indicated time point with 100 ml sterile MTT dye (0.5 mg/ml, Sigma, St. Louis, MO, USA) for 4 h at 37°C, followed by removal of the culture medium and addition of 150 ml of dimethyl sulphoxide (DMSO) (Sigma, St. Louis, MO, USA). The absorbance was measured at 570 nm, with 655 nm as the reference wavelength.

Anoikis assays

Anoikis resistance was evaluated by seeding 7×10^4 cells in ultralow attachment plates (Corning). After 24 hrs of anchorage-independent culture, cells were transfected as indicated and resuspended in 0.4% trypan blue (Sigma, St. Louis, MO, USA) and cell viability was assessed.

Methods of bioinformatics

The analysis of potential microRNA target sites using commonly used prediction algorithms-mRanda (http://www.microrna.org/microrna/home.do).

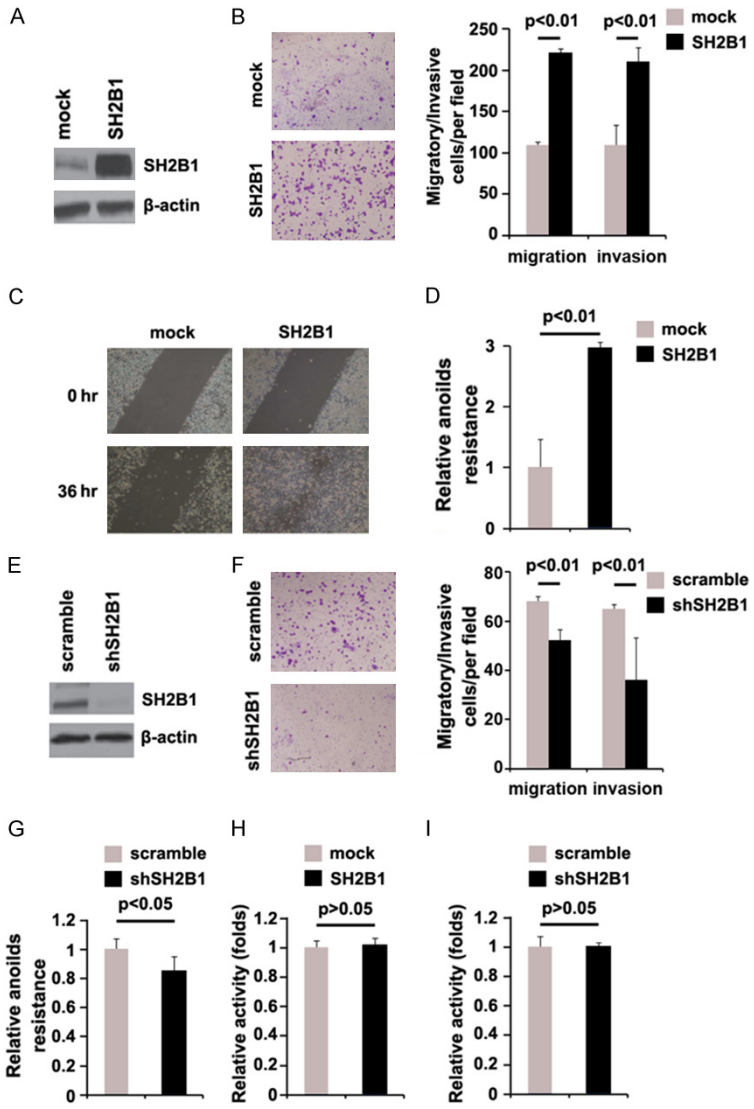

Figure 1. SH2B1 promotes metastasis-relevant traits in gastric cancer SGC-7901 cells. A: Western Blot for SH2B1 in SGC-7901 cells transfected with SH2B1 expressing plasmids. Mock groups were transfected with empty vector. β-actin was a loading control. n=3. B: Matrigel invasion assay and Transwell invasion assay for SGC-7901 cells transfected with SH2B1 expressing plasmids and empty vector (mock). n=3. C: Wound-healing assays for SGC-7901 cells transfected with SH2B1 expressing plasmids and empty vector (mock). n=3. D: Anoikis assays for SGC-7901 cells infected as indicated. n=3. E: Western Blot for SH2B1 in SGC-7901 cells transfected as indicated. β-actin was a loading control. n=3. F: Matrigel invasion assay and Transwell invasion assay for SGC-7901 cells transfected with shSH2B1 or scramble. n=3. G: Anoikis assays for SGC-7901 cells infected as indicated. n=3. H: MTT assay for SGC-7901 cells infected as indicated. n=3. I: MTT assay for SGC-7901 cells infected as indicated. n=3.

Immunofluorescence analyses

For immunofluorescence analyses, cells were plated on glass coverslips in six-well plates and transfected with 30 nM pre-miR-874 or control miR. At 36 h after transfection, coverslips were stained with the mentioned anti-SH2B1 antibodies. Alexa Fluor 488 goat anti-rabbit IgG antibody was used as secondary antibody (Invitrogen, Carlsbad, CA, USA). Coverslips were counterstained with DAPI (Invitrogen-Molecular Probes, Eugene, Oregon, USA) for visualization of nuclei. Microscopic analysis was performed with a confocal laser-scanning microscope (Leica Microsystems, Bensheim, Germany). Fluorescence intensities were measured in a few viewing areas for 200-300 cells per coverslip and analyzed using ImageJ 1.37v software (http://rsb.info.nih.gov/ij/index.html).

Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR (qRT-PCR) for SH2B1

Total RNA was isolated from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from the total RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and random hexamer primers (Sangon, Shanghai, China). The thermal cycle profile was as follows: denaturation for 30 sec at 95°C, annealing for 45 sec at 52-58°C depending on the primers used, and extension for 45 sec at 73°C. PCR products were visualized on 2% agarose gels stained with ethidium bromide under UV transillumination. qRT-PCR was done with a Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's protocol. The primer sequences for SH2B1: forward primer 5'-TTCGAT-

ATGCTTGAGCACTTCCGG-3' and reverse 5'-GCCTCTTCTGCCCCAGGATGT-3'.

Real-time PCR for miRNA

Total RNA from cultured cells, with efficient recovery of small RNAs, was isolated using the mirVanamiRNA Isolation Kit (Ambion, Austin, TX, USA). Detection of the mature form of miRNAs was performed using the mirVanaqRT-PCR miRNA Detection Kit, according to the manufacturer's instructions (Ambion, Austin, TX, USA). The U6 small nuclear RNA was used as an internal control.

Statistical analysis

Data are presented as mean ± s.e.m. Student's t test (two-tailed) was used to compare two groups ($P<0.05$ was considered significant).

Results

SH2B1 promotes metastasis-relevant traits in gastric cancer SGC-7901 cells

In an attempt to identify the role of SH2B1 in regulating migration and invasion of SGC-7901 cells, the cells were transfected with SH2B1 expressing plasmids. After stable transfection, SH2B1 protein expression was detected by western blot and the results showed that SH2B1 protein was increased by SH2B1 expressing plasmids in the cells (**Figure 1A**). Next, we performed migration and invasion assay to detect migration and invasion of SGC-7901 cells transfected with SH2B1 expressing plasmids and empty vectors. Ectopic SH2B1 did promote motility and invasion by about 2-folds in the cells (**Figure 1B**). To confirm the results, would healing assay was performed. Would healing assay showed that SH2B1 significantly promoted motility in the cells (**Figure 1C**). To further show the effects of SH2B1 on metastasis, we performed anoikis assays to analyze its effects on anoikis resistance. SH2B1-transfected cells exhibited about 200% increased resistance to anoikis-mediated cell death (**Figure 1D**).

Having demonstrated that SH2B1 overexpression promoted migration and invasion in SGC-7901 cells, to provide further evidence that the roles of SH2B1 were involved in migration and invasion, we studied the effects of an inhibitor of SH2B1-shSH2B1. After stable transfection, SH2B1 expression was examined by western blot. The results showed that shSH2B1 significantly downregulated SH2B1 expression in SGC-7901 cells (**Figure 1E**). Next, we performed migration and invasion assay to detect migration and invasion of SGC-7901 cells transfected with shSH2B1 plasmids and scramble. Silencing SH2B1 did inhibit motility and invasion by about 0.2-folds (**Figure 1F**). To further confirm that the roles of shSH2B1 on metastasis, we performed anoikis assays to analyze its effects on anoikis resistance. Contrary to SH2B1 overexpression, shSH2B1-transfected cells exhibited about 20% decreased resistance to anoikis-mediated cell death (**Figure 1G**). We also performed MTT assay to detect whether it affected proliferation in SGC-7901 cells. Overexpressing SH2B1 and silencing it did not affect cells viability (**Figure 1H**, **1I**).

miR-874 degrades SH2B1 in SGC-7901 cells

Having demonstrated that SH2B1 promoted metastasis-relevant traits in gastric cancer SGC-7901 cells, next we studied the mechanisms regulating SH2B1 expression in SGC-7901 cells. MicroRNAs (miRs) are a class of small noncoding RNAs (~22 nucleotides) and negatively regulate protein-coding gene expression by targeting mRNA degradation or translation inhibition [9, 10, 20].

To further confirm whether SH2B1 could be regulated by microRNA, we used the commonly used prediction algorithm-miRanda (http://www.microrna.org/microrna/home.do) to analyze 3'UTR of SH2B1. A dozen of microRNAs were found by the algorithm. But we are interested in miR-874, because miR-874 was significantly down-regulated in gastric cancer [21].

Target sites on 3'UTR of SH2B1 were showed in **Figure 2A**. We hypothesized that miR-874 could down-regulate SH2B1 expression by targeting its 3'UTR in gastric cancer. To identify the role of miR-874 in regulating SH2B1 expression in gastric cancer, we transfected SGC-7901 cells with pre-miR-874 and control miR. After transfection, miR-874 expression was detected by real-time PCR and the results showed that miR-874 was significantly increased by pre-miR-874 in the cells (**Figure 2B**).

To confirm the hypothesis, we performed western bolt to detect SH2B1 expression in SGC-7901 cells transfected with pre-miR-874 and control miR. The results showed that SH2B1

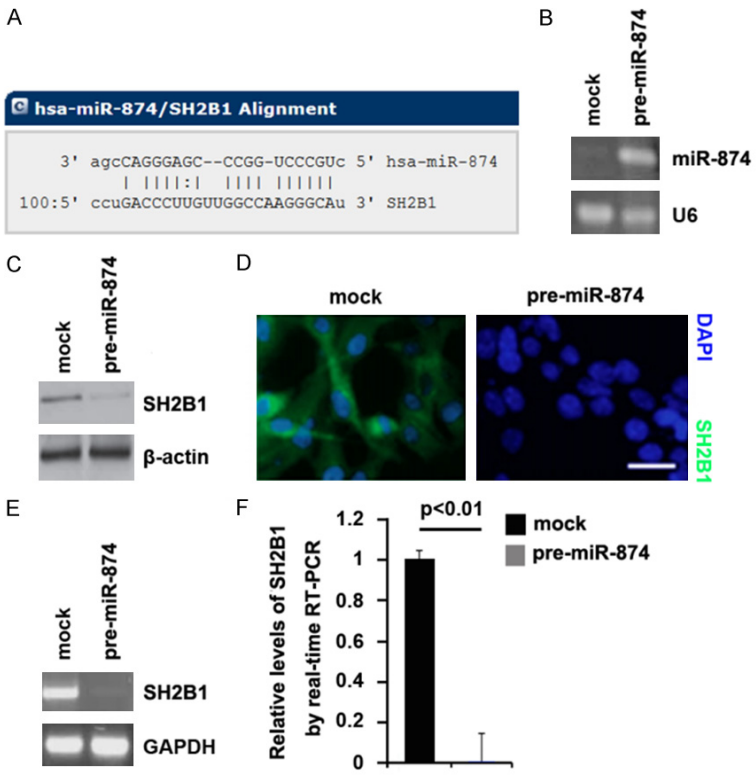

Figure 2. miR-874 degrades SH2B1 in SGC-7901 cells. A: Schematic of predicted miR-874-binding sites in the 3'UTR of SH2B1 mRNA by miRanda. B: Real-time PCR for miR-874 in SGC-7901 cells transfected with pre-miR-874 and control miR. U6 was a loading control. n=3. C: Western Blot for SH2B1 in SGC-7901 cells transfected with pre-miR-874 and control miR (mock). β-actin was a loading control. n=3. D: Immunofluorescence analyses for SH2B1 in SGC-7901 cells transfected with pre-miR-874 and control miR (mock). n=3. E: RT-PCR for SH2B1 in SGC-7901 cells transfected with pre-miR-874 and control miR (mock). GAPDH was a loading control. n=3. F: Real-time PCR for SH2B1 in SGC-7901 cells transfected with pre-miR-874 and control miR (mock). GAPDH was a loading control. n=3.

protein was significantly inhibited by miR-874 (**Figure 2C**). We next performed immunofluorescence analyses in SGC-7901 cells transfected with pre-miR-874 and control miR. The results showed that SH2B1 protein was evidently inhibited in the cells transfected with pre-miR-874 (**Figure 2D**). To determine whether miR-874 could degrade SH2B1 mRNA, we performed RT-PCR and real-time PCR. We found that SH2B1 mRNA can be degraded by miR-874 (**Figure 2E** and **2F**).

miR-874 overexpression inhibits metastasis-relevant traits and proliferation in gastric cancer SGC-7901 cells

Next, we performed migration and invasion assay to detect migration and invasion of SGC-7901 cells transfected with pre-miR-874 and control miR. Ectopic miR-874 did inhibit motility and invasion by about 30%-40% (**Figure 3A**). To confirm the results, would healing assay was performed. Would healing assay showed that miR-874 significantly inhibited motility in the cells (**Figure 3B**).

To further examine the effects of miR-874 on metastasis, we performed anoikis assays to analyze its effects on anoikis resistance. miR-874-transfected cells exhibited about 40% decreased resistance to anoikis-mediated cell death (**Figure 3C**). We also performed MTT assay to study whether it affected proliferation in SGC-7901 cells. Its overexpression inhibited proliferation in SGC-7901 cells (**Figure 3D**).

Discussion

miRNAs are short (20-24 nt), stable, non-coding RNA molecules that regulate 60% of coding genes by binding to mRNA molecules to prevent translation and/or promote degradation. To date, over 1,000 miRNAs have been identified, and they have been shown to participate in nearly all biological processes, including cell proliferation and metastasis. Indeed, novel functions and mechanisms by which miRNAs regulate their target genes are regularly discovered [22, 23]. Recently, it has been reported that miR-874 was significantly down-regulated in gastric cancer [21]. miR-874 can inhibit proliferation, migration, invasion and tumor angiogenesis by targeting STAT3 and AQP3 in gastric cancer (**Figure 3E**) [21, 24]. Consistent with the reports, we found that overexpressing miR-874 inhibits proliferation, migration, invasion and anoilds resistance. The results further confirmed the tumor suppressive roles of miR-874 in gastric cancer (**Figure 3E**).

In addition, we found another target gene of miR-874 in gastric cancer cells. miR-874 can

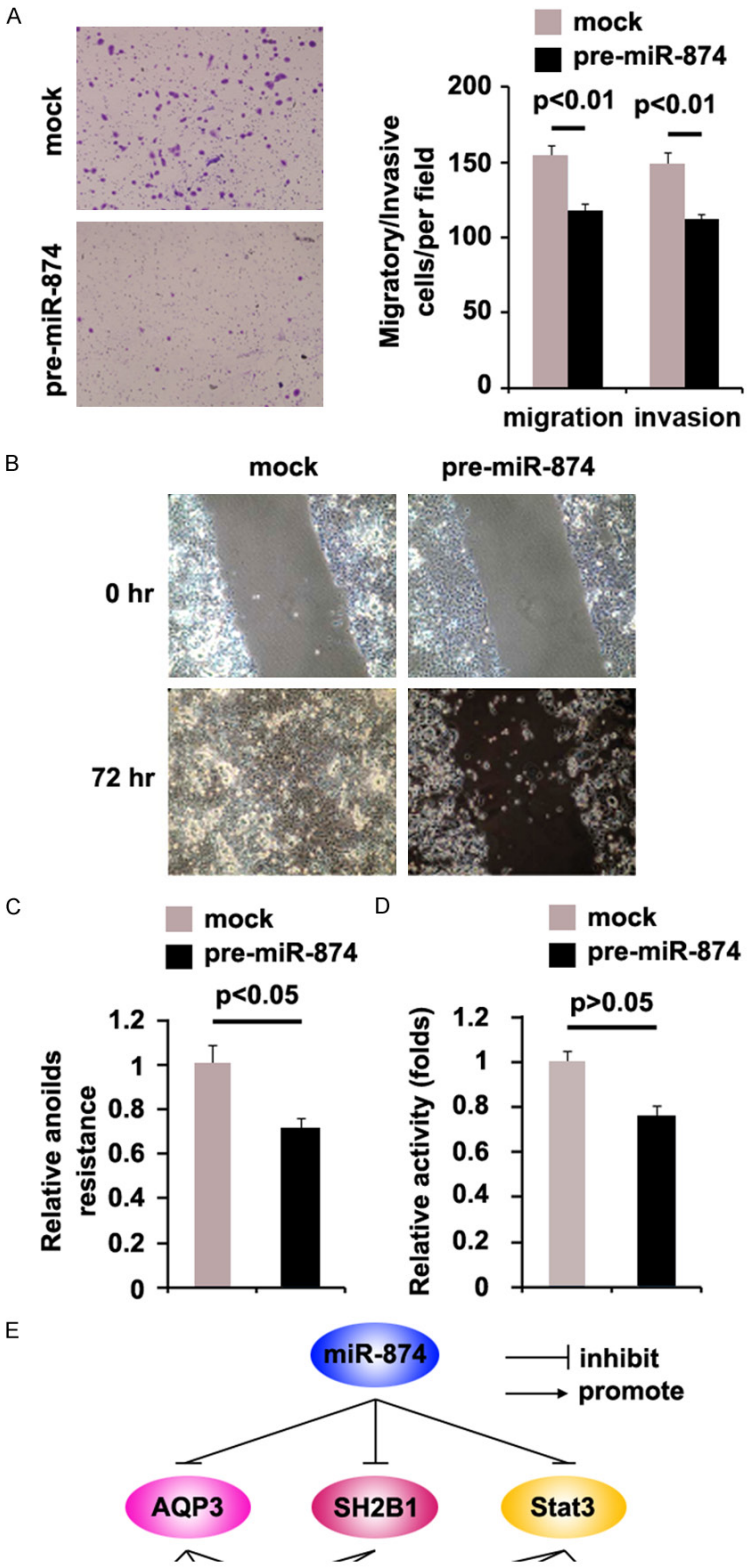

Figure 3. miR-874 overexpression inhibits metastasis-relevant traits and proliferation in gastric cancer SGC-7901 cells. A: Matrigel invasion assay and Transwell invasion assay for SGC-7901 cells transfected with pre-miR-874 and control miR (mock). n=3. B: Wound-healing assays for SGC-7901 cells transfected with pre-miR-874 and control miR (mock). n=3. C: Anoikis assays for SGC-7901 cells infected as indicated. n=3. D: MTT assay for SGC-7901 cells infected as indicated. n=3. E: miR-874 inhibits proliferation, migration, invasion and angiogenesis by targeting AQP3, SH2B1 and Stat3 in gastric cancer.

degrade SH2B1 in SGC-7901 cells. It has been reported that SH2B1 functions as an oncogene lung cancer, oesophageal cancer and neuroblastoma [4-7]. Yet, its roles have not been reported in gastric cancer. In line with previous reports that over-expression of SH2B1 closely correlates with malignant progression of invasion and metastasis of esophageal cancer [6] and contribute to the malignant progression of non-small cell lung cancer (NSCLC) [7], we found that SH2B1 overexpression promoted invasion, migration and anoilds resistance and silencing it inhibited invasion, migration and anoilds resistance in gastric cancer SGC-7901 cells. But different with the report that SH2B1 overexpression can promote proliferation and silencing it can inhibit proliferation in NSCLC [4], we showed that its over-expression and silencing did not affect proliferation in SGC-7901 cells. We hypothesized that miR-874 can inhibit proliferation by regulating other genes rather than SH2B1 (**Figure 3E**).

All in all, we confirmed that miR-874 inhibits metastasis-relevant traits via targeting SH2B1 in gastric cancer. Elucidating the mechanism that miR-874 inhibits metastasis-relevant traits via targeting SH2B1 in gastric cancer will help us to better understand the molecular mechanism of

metastasis. Thus, targeting SH2B1 and restoration of miR-874 may represent a promising therapeutic way to suppress SH2B1-mediated metastasis.

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Disclosure of conflict of interest

None.

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