MiR-3613-3p from carcinoma associated fibroblasts exosomes promoted breast cancer cell proliferation and metastasis by regulating SOCS2 expression

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Research

Keywords: miR-3613-3p, breast cancer, metastasis, fibroblasts, exosomes, SOCS2

Posted Date: February 27th, 2020

DOI: https://doi.org/10.21203/rs.2.24682/v1

Version of Record: A version of this preprint was published on April 28th, 2020. See the published version at https://doi.org/10.1002/iub.2292.

Abstract

Background Exosomes carrying microRNA (miRNAs) mediate cell to cell communication which are important regulators in cancer growth and progression. However, the roles and molecular mechanism of the miRNAs in the exosomes from carcinoma associated fibroblasts (CAFs) are still not clear.

Methods The targeted gene of miR-3613-3p was predicted by TargetScan and miRanda. The proliferation of cancer cells was conducted by cell counting kit-8 and colony formation assay. Cancer cell migration and invasion were measured by wound healing assay and Transwell assays respectively. Luciferase activity was assayed by dual luciferase assay system. miRNA and mRNA expression was measured by real time RT-PCR. SOCS2 protein levels was assayed by western blotting.

Results It was verified that miR-3613-3p was up-regulated in exosomes from fibroblasts educated by TGF-β1, breast cancer cells and breast cancer tissues. Exosomal miR-3613-3p promoted breast cancer cell proliferation and metastasis. Loss-of-function experiments revealed that miR-3613-3p down-regulation in the CAFs exosomes suppressed cell proliferation and drug resistance in breast cancer by targeting SOCS2 expression. The clinical data showed that miR-3613-3p was negatively related to SOCS2 in breast cancer tissues.

Conclusion These findings demonstrated that activated fibroblasts exosomes with high levels of miR-3613-3p played an oncogenic role in breast cancer cell survival, metastasis and drug responses, which suggested that the oncogenic role of miR-3613-3p in breast cancer progression.

Background

Tumor microenvironment is the niche of tumor growth and progression [1, 2]. Cancer tissues consist of various cells and non-cell components. Carcinoma associated fibroblasts (CAFs) are the most prominent cell type in stroma, which build a permissive and supportive microenvironment for tumor development by interacting with cancer cells and other type of cells such as endothelial cells, macrophages [3–5]. Extracellular vehicles (EVs) shuttling from one cell to another cell is one way to communicate each other in tumor niche [6].

Exosomes, secreting from diverse cell types, are a type of EVs with 30–150 nm diameter and play complicated roles in intercellular communication [6–8]. Stromal cells and breast cancer cells interaction could induce exosomes to transfer from stroma cells into breast cancer cells, and then stimulate the change of cellular functions and facilitate cancer progression [9]. Exosomes function as natural vehicles delivering various molecules to recipient cells [8–10]. One of important molecules in exosomes is microRNA (miRNA). miRNAs from CAFs exosomes play promoting or suppressing roles in tumor. There are some reports verifying that low levels of exosomes miRNAs from CAFs led to cancer progression such as miR-3188, miR-139 [11–12]. There are also higher levels of exosomal miRNAs from CAFs induced to therapy resistance such as miR-196a [13], miR-34a-5p [14]. The previous reports indicated that fibroblasts

exosomes play important roles in cancer development and progression. However, the roles and molecular mechanisms of exosomes from CAFs on breast cancer cells are still largely unraveled.

In this study, based our previous miRNA array, miR-3613-3p was up-regulated in CAFs exosomes significantly. We hypothesized that carcinoma associated fibroblasts exosomes might have important influence on the regulation of breast cancer growth via miR-3613-3p. So, the roles and mechanism of CAFs exosomal miR-3613-3p in regulating breast cancer cell biological functions was investigated. Our results showed that CAFs exosomal miR-3613-3p promoted breast cancer cell proliferation, ROS production and metastasis by SOCS2.

Materials And Methods

Cell culture

Breast cancer cell lines including BT474 and MCF7 used in the study were primarily purchased from ATCC. The cells were cultured according to the standard protocols. Cells were cultured in DMEM-F12 or 1640 with 10% fetal bovine serum with penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), with or without EGF and insulin. Fibroblasts (NHDF) were purchased from Jingkang (Shanghai, China) and cultured in DMEM-F12 with 10% fetal bovine serum with penicillin (100 U/ml), streptomycin sulfate (100 μg/ml). The cells were incubated in a humidified incubator at 37 °C with 5% CO_2.

Exosomes Preparation

Breast cancer tissues were splice into small pieces and digested with collagenase I for overnight, the cells were cultured and the conditioned medium were collected. Fibroblasts educated by TGF-β1 or cancer cells and the cell conditioned medium was collected. The conditioned medium was filtered through a 0.22-μm filter (Merck Millipore, Massachusetts, USA) to remove cellular debris, and then exosomes were extracted using the kit from Invitrogen.

Cell Proliferation Assay

Breast cells were treated with exosomes from CAFs with miR-3613-3p down-regulation and the controls. The cells were seeded in 96-well plates and the cell survival ability were assayed by CCK8 at the indicated time. The survival rates were analyzed. The cells were seeded in 6-well plates for two weeks and the colonies with 50 cells were counted and analyzed.

Western blotting

Cultured cells were harvested and lysed with RIPA buffer containing the protease inhibitors on ice for 30 min. Equal protein was separated by SDS-PAGE. The protein was transferred onto PVDF membrane

using and probed with primary antibodies and then horseradish peroxidase–labeled secondary antibodies. The protein band signals were visualized using an ECL.

Real Time RT-PCR

Total RNA from cells or tissues was extracted using EZ total RNA isolation kit based on the manual. All the primer sequences were ordered from Ruibo (Shanghai, China). The PCR was run on the 7500 Real-Time PCR system using the following thermocycling parameters: 95 °C for 10 minutes, 40 cycles at 95 °C for 10 seconds, 60 °C for 30 seconds, 72 °C for 10 seconds, followed by a melting curve analysis. The expression of miRNA and mRNA was analyzed by comparing Ct values. GAPDH or U6 snRNA was the internal controls.

Lentivirus carrying miRNA or miRNA inhibitor

The lentiviral particles with miR-3613-3p were ordered from Genechem (Shanghai, China). Fibroblasts were transfected with the lentivirus and the knocking down effect in cells was evaluated by real time RT-PCR.

Wound Healing Assay

BT474 and MCF7 cells were treated with exosomes from CAFs infected with lentivirus with miR-3613-3p overexpression and the controls. The cells were seeded in 12-well plates. The cells were made a wound using 200 ul tips. The wound widths were measured and the migration ability was analyzed.

Cell Invasion

BT474 and MCF7 cells were treated with exosomes from CAFs infected with lentivirus with miR-3613-3p overexpression and the controls. The invaded cells were fixed in 100% methanol and then dyed with 0.1% crystal violet. The photos of the invaded cells in three fields were taken and cell invasion ability were analyzed.

ROS Detection

A total ROS detection kit (Enzo Life Sciences) was used to detect intracellular ROS and of BT474 and MCF7 cells according to the manufacturer's protocol [15]. At the end of treatment, cells were stained with ROS detection solution at 37 °C for 1 h and then observed under a fluorescence microscope.

Luciferase Assay

Cancer cells with 3×10^4 cells in every well were grown in a 24-well plate. The cells were co-transfected with luciferase reporter (200 ng per well), miR-3613-3p (200 ng per well), and 10 ng Renilla luciferase vector (pRL-CMV; Genomeditech, China) using Lipofectamine™ 3000 (Invitrogen, USA). The cells were cultured in the regular condition for 48 h and then the luciferase and renilla activity of these samples were measured by a Dual-Luciferase Reporter Assay kit (Promega, USA).

Statistical analysis

All quantitative experiments were repeated at least three independent biological repeats and are presented as the means ± SD (standard deviation). Quantitative data were analyzed by either one-way analysis of variance (ANOVA) (multiple groups or parametric generalized linear model with random effects. p value less than 0.05 was considered statistically significant.

Results

MiR-3613-3p levels increased in exosomes from CAFs

MiR-3613-3p was overexpressed in the exosomes from fibroblasts educated in TGF-β from our previous miRNA array analysis. In order to know the roles and molecular mechanism of miR-3613-3p in cancer progression, exosomes from the TGF-β educated fibroblasts, breast cancer cell educated, and exosomes from carcinoma associated fibroblasts of breast cancer tissues were extracted for verification. The real time RT-PCR analysis showed that miR-3613-3p was up-regulated in exosomes from educated fibroblasts by TGF-β with time dependent (Fig. 1A). miR-3613-3p was also significantly increased in fibroblasts educated by breast cancer cells and CAFs exosomes (Fig. 1B and 1C). It was also up-regulated in the exosomes of breast cancer tissues compared to the adjacent normal tissues (Fig. 1D). These data suggested that miR-3613-3p play an oncogenic role.

Inhibiting miR-3613-3p suppressed breast cancer cell proliferation, ROS production and metastasis

To know the roles of miR-3613-3p in breast cancer cells, firstly, carcinoma associated fibroblasts were transfected with miR-3613-3p inhibitors mediated by lentivirus, and its expression was suppressed in BCFs (breast cancer cell educated fibroblasts), CAFs and their exosomes (Fig. 2A). When MCF7 and SKBR3 cells were treated with the CAF-exo with miR-3613-3p down-regulation, the cell proliferation was assayed by CCK8. It was shown that CAF-exo with miR-3613-3p inhibition suppressed cell proliferation of MCF7 and SKBR3 cells (Fig. 2B and 2C). The ROS production also decreased in breast cancer with CAF-exo miR-3613-3p down-regulation (Fig. 2D). To evaluate the impact of miR-3613-3p on cancer cell metastasis, we measured the migration of cancer cells treated with the CAF-exo with or without miR-3613-3p inhibition. It was shown miR-3613-3p down-regulation in fibroblasts led to a marked decrease of migrating ability of breast cancer cells (Fig. 2E). It was also shown that invasion was suppressed in MCF7 and SKBR3 cells treated with CAF-exo with miR-3613-3p (Fig. 2F).

MiR-3613-3p suppressed SOCS2 expression in breast cancer cells

To find the target genes of miR-3613-3p which are involved in cell proliferation and metastasis, miRNA target gene prediction tools including Targetscan and miRDB were used to predict the target genes of miR-3613-3p. The potential target genes were predicted (Fig. 3A). SOCS2 was down-regulated significantly in MCF7 and SKBR3 cells with miR-3613-3p overexpression (Fig. 3B). Next, wild-type 3'UTR SOCS2 (WT-3'UTR) or mutants 3'UTR SOCS2 (Mut1-3'UTR and Mut2-3'UTR) were constructed using the reporter vector carrying luciferase. To know whether miR-3613-3p directly binds the 3'UTR region of SOCS2, miR-3613-3p mimics and the SOCS2 WT-3'UTR or Mut-3'UTR, the result showed that the luciferase activity of SOCS2 WT-3'UTR was inhibited by miR-3613-3p in breast cancer cells (Fig. 3C). It was also verified that the luciferase of SOCS2 WT-3'UTR was inhibited in breast cancer cells (Fig. 3D). To further know whether miR-3613-3p regulates SOCS2 expression on post-transcriptional levels, breastcancer cells were transfected with miR-3613-3p and the data showed that both SOCS2 mRNA were down-regulated (Fig. 3D). The protein levels of SOCS2 from the cells with miR-3613-3p transfection decreased by western blotting (Fig. 3E and 3F).

CAFs exosomes with miR-3613-3p inhibition led to proliferation and metastasis suppression via SOCS2-STAT3 in breast cancer cells

To know the role of miR-3613-3p-SOCS2-STAT3 in breast cancer cells, MCF7 and SKBR3 cells were treated with CAF-exo, and it was found that miR-3613-3p could be transferred into SUM102 cells (Fig. 4A). Cell survival ability was assayed by colony formation, and colonies ewere restored in the cells with SOCS2 down-regulation in cells with CAF-exo-miR-3613-3p treatment (Fig. 4B and 4C). It was also observed that ROS were restored in the cells with SOCS2 down-regulation in cells with CAF-exo-miR-3613-3p treatment in MCF7 and SKBR3 cells (Fig. 4D). The transwell assay showed that SOCS2 down-regulation could enhanced the miR-3613-3p led to a marked decrease in the migration of SKBR3 and MCF-7 cells treated with CAFs-exo with miR-3613-3p or SOCS2 overexpression than the controls (Fig. 4E and 4F). There was the same result in invasion analysis (Fig. 4G and 4H).

MiR-3613-3p was negatively related to SOCS2 expression in breast cancer tissues

SOCS2 was confirmed as a target gene of miR-3613-3p. SOCS2 levels were higher in many cancer tissues like BLCA, BRCA, CESC, COAD, KICH, KIBP, LUIAD, LUSC, OV, READ, THCA, UCEC and UCS (Fig. 5A). The box plot showed that SOCS2 expression was lower significantly in breast cancer tissues compared with normal tissues (Fig. 5B). To know whether miR-3613-3p is related to SOCS2 in breast cancer tissues, miR-3613-3p and SOCS2 mRNA was measured and the data showed that miR-3613-3p was up-regulated and

SOCS2 levels were lower in breast cancer tissues compared to their adjacent normal tissues (Fig. 5C and 5D). The relationship between miR-3613-3p and SOCS2 showed that miR-3613-3p expression was negatively related to SOCS2 levels in breast cancer tissues (Fig. 5E).

Discussion

Fibroblasts integrate into the tumor stroma and function in a paracrine manner to promote breast cancer progression. The activated carcinoma associated fibroblasts in tumor microenvironment are from fibroblasts or other stroma cells. The secreting paracrine molecule is a main functional way in tumor stroma [5–8]. Although there are studies indicating the important roles of fibroblasts in breast cancer, the roles of fibroblasts in cell proliferation and metastasis is still unclear. The molecular mechanisms mediating breast cancer cell progression need to be investigated. In this study, our purpose is to investigate the effects of CAFs exosome miR-3613-3p on breast cancer cellular functions. We found that CAFs exosome could promote breast cancer survival ability and metastasis by miR-3613-3p-SOCS2.

MiRNAs play great important roles in fibroblasts derived exosomes on cancer progression. The reported miRNAs in CAFs including miR-139, miR-1247-3p and others [16–19]. A report showed that miR-320a from CAF-derived exosomes could function as an antitumour miRNA by binding to its direct downstream target PBX3 to suppress HCC cell proliferation and metastasis by suppressing the activation of the MAPK pathway, which could induce the epithelial-mesenchymal transition and upregulate cyclin-dependent kinase 2 (CDK2) and MMP2 expression to promote cell proliferation and metastasis [6]. CAFs exposed to gemcitabine significantly increase the release of extracellular vesicles called exosomes, which increased chemoresistance-inducing factor, Snail, in recipient epithelial cells and promote proliferation and drug resistance [8]. Down-regulation of miR-1, miR-206 and up-regulation of miR-31 expression contribute to conversion of NFs to CAFs. Exosomal miR-21 can confer chemoresistance and an aggressive phenotype in ovarian cancer cells through its transfer from neighbouring stromal cells, suggesting that preventing the exosomal transfer of miR21 from stromal cells is a new strategy for suppressing ovarian cancer growth [13]. We found that miR-3613-3p was up-regulated in CAFs and their exosomes, which was associated breast cancer cell growth acceleration, stress responses and metastasis.

SOCS2 gene expression is lower in breast cancers, which is induced by diverse kinds of cytokines [20–23]. SOCS2 has been shown to be able to regulate several signalling pathways such as growth hormone (GH) signaling which involves in cell growth [16]. The reports showed that SOCS2 is regulated by miRNAs like miR-194, miR-196a/-196b, miR-500a-3p, miR-191[22–27]. Here, we found miR-3613-3p was higher in CAFs exosomes, transferred into breast cancer cells to promote cell growth and metastasis by down-regulating SOCS2 expression. SOCS2 was lower in breast cancer tissues and cells, which suppressed cell biological functions. The clinical study showed that miR-3613-3p was negatively related to SOCS2 levels in breast cancer tissues.

There is a natural defense system to make damage caused by oxidative stress reduce in the body. ROS is a character of the tumor microenvironment. It is known that ROS is associated with tumor progression

[29–29]. ROS production in cells can be regulated by the tumor microenvironment including the exosomes from CAFs. Our data indicated that down-regulating miR-3613-3p in CAFs exosomes led to less ROS production in breast cancer cells and down-regulating SOCS2 expression recovered ROS production.

These findings demonstrated that CAFs exosomes with low levels of miR-3613-3p played an important role in breast cancer cell survival and drug resistance, which suggested that an anti-oncogenic role of miR-3613-3p in breast cancer progression. MiR-3613-3p could act as a non-specific diagnostic biomarker for breast cancers and a potential biomarker for prognosis prediction of breast cancer.

Conclusion

The present study indicated that CAFs exosomes promoted breast cancer cell proliferation, drug resistance by miR-3613-3p. Our study for the first time verified that miR-3613-3p functioned as a suppressing miRNA in breast cancer cell by targeting SOCS2 expression in breast cancer cells (Fig. 6). There needs further research on the mechanisms of cancer cell educated fibroblasts exosomal miR-3613-3p in breast cancer progression.

Abbreviations

ATCC: American Type Culture Collection; NFs: normal fibroblasts; CAFs: Carcinoma associated fibroblasts; EVs: extracellular vehicles; TEM: transmission electron microscope; SDS: sodium dodecyl sulfate; PBS: phosphate-buffered saline; ECM: extracellular matrix.

Declarations

Ethics approval and consent to participate

No ethical issues.

Consent for publication

All authors agree to publish the paper.

Competing interests

The authors declare that they have no conflict of interests.

Funding

This work was supported by grands from National Natural Science Foundation of China (No. 81502571), Shanghai Science and Technology Committee (No. 19140901000) and Hospital Key Project (No. QYZ2019-05).

Authors' contributions

FL designed and conceived the research, YL and JD performed all the experiments work and drafted the manuscript, YY analyzed and interpreted the data. FL reviewed the manuscript.

Acknowledgements

We sincerely thank for all persons' help in the study.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

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Figures

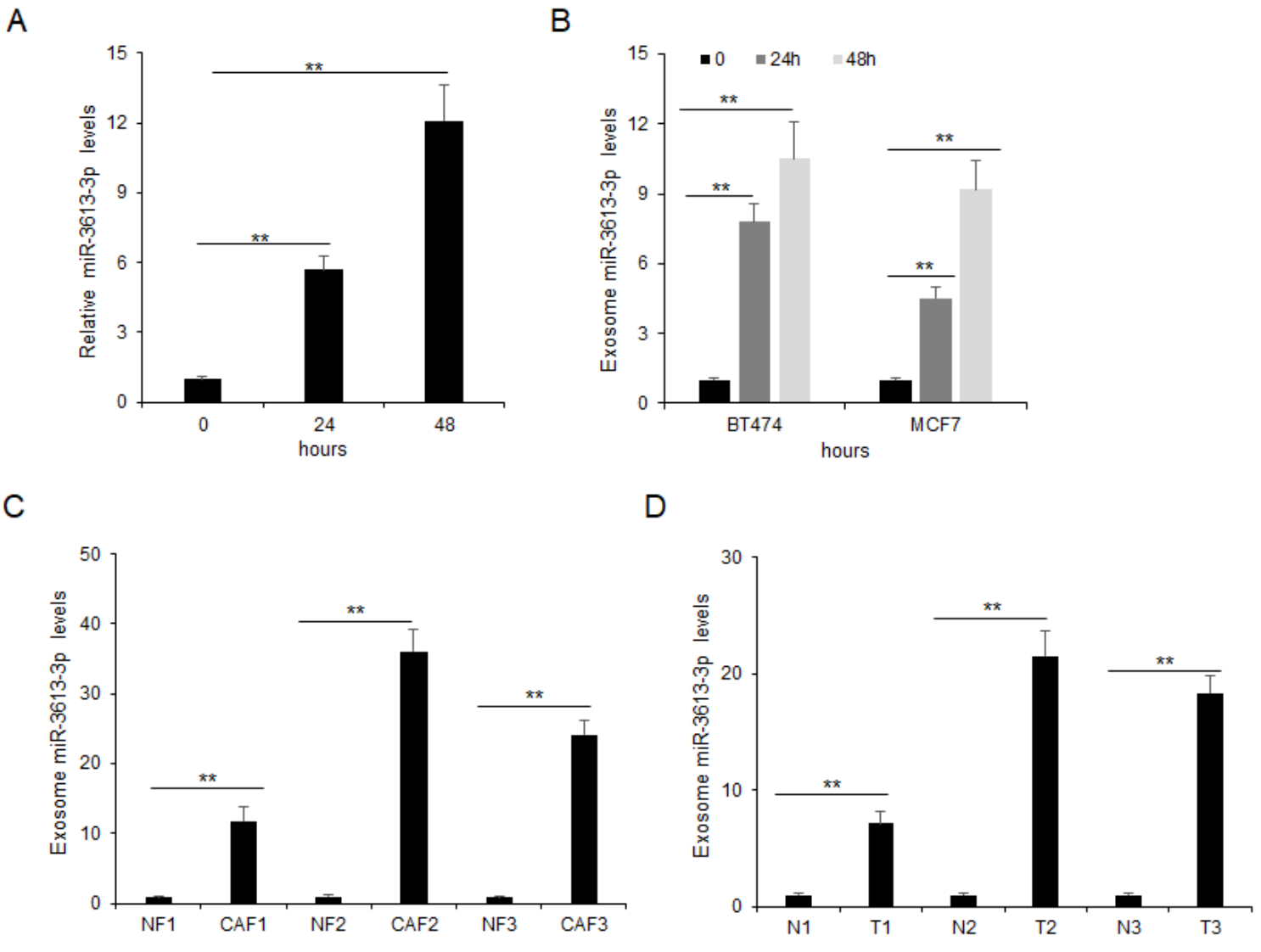

Figure 1

MiR-3613-3p levels increased in exosomes from CAFs. (A) miR-3613-3p expression was fibroblasts in the present of TGF-β. Fibroblasts were treated with or without TGF-β (10ng/ml) for 24h and 48h, and then the exosomes were collected for miR-3613-3p analysis. (B) miR-3613-3p expression was increased in the exosomes from cancer cell educated fibroblasts. Fibroblasts were co-cultured with breast cancer cells in the transwell system for 24h and 48h, and then exosomes were extracted for RNA isolation and real time RT-PCR was used for miR-3613-3p level analysis. (C) miR-3613-3p expression was increased in the exosomes from cancer cell educated fibroblasts. Normal fibroblasts and CAFs were co-cultured 48h, and then exosomes were extracted for RNA isolation and real time RT-PCR was used for miR-3613-3p level analysis. (D) miR-3613-3p expression was increased in the exosomes from breast cancer tissues. Fresh breast cancer tissues were cut into small pieces and digested in collagenase I overnight and then the exosomes were collected for RNA extraction. RNA was used for miR-3613-3p level analysis. **p<0.01.

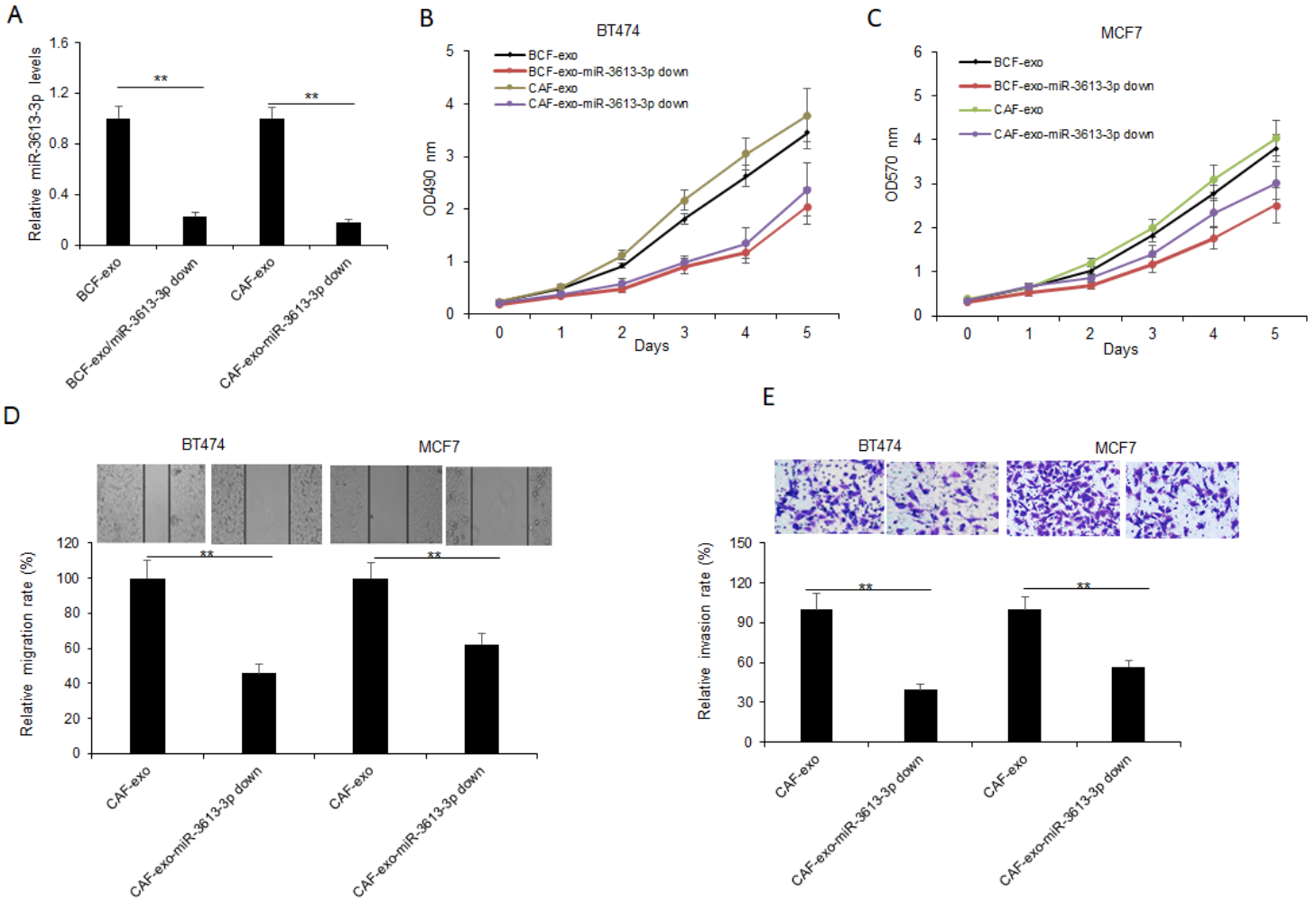

Figure 2

Inhibiting miR-3613-3p suppressed breast cancer cell drug resistance, ROS production and metastasis. (A) miR-3613-3p expression increased in fibroblasts or TGF-β treated fibroblasts in the exosomes. Fibroblasts or TGF-β treated fibroblasts were transfected with miR-3613-3p mimics or the miRNA controls for 48h and the RNA was extracted for real time RT-PCR. (B-C) cell proliferation was assayed by CCK8 method. MCF7 and SKBR3 cells were treated with CAF-exo with miR-3613-3p overexpression by the

miRNA mimics transfection. Cell growth was measured at day 1, 2, 3, 4 and 5. (D) ROS production in breast cancer cells with CAF-exo treatment. (E) MCF7 and SKBR3 cell migration ability was assayed by transwell assay. MCF7 and SKBR3 cells were treated with the CAF-exo with miR-3613-3p up-regulation for 24h. The migrated cells were counted and the relative migration was analyzed. (F) MCF7 and SKBR3 cell invasion ability was assayed by transwell assay. MCF7 and SKBR3 cells were treated with the CAF-exo with miR-3613-3p over-expression for 24h. The invaded cells were counted and the relative migration was analyzed. **p<0.01; *p<0.05.

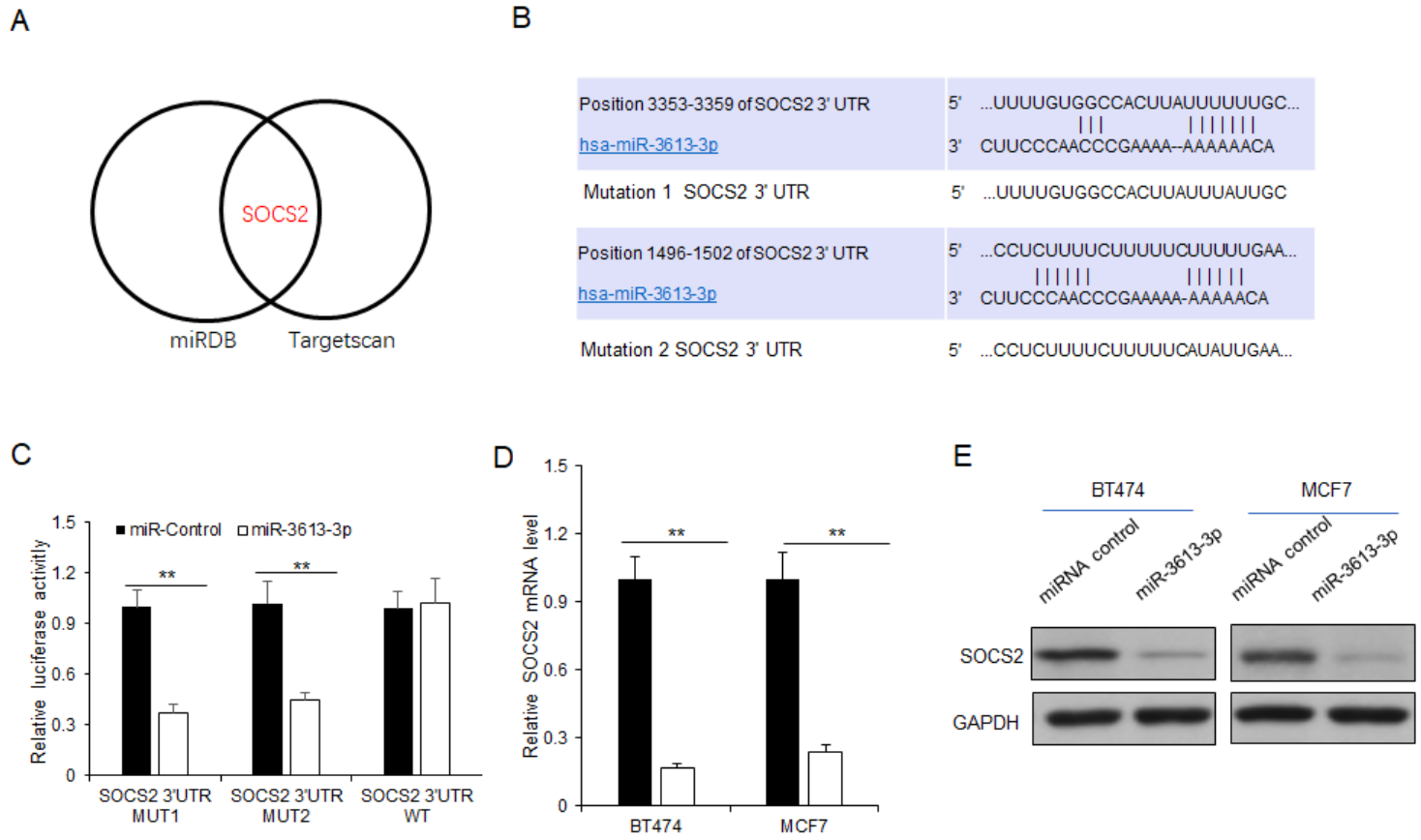

Figure 3

MiR-3613-3p suppressed SOCS2 expression in breast cancer cells. (A) Schematic representation of 3'-UTR of mRNA reporter with the miR-3613-3p seed-binding sites. (B-C) Luciferase activity assay of MCF7 and SKBR3 cells transfected with luciferase constructs containing WT-3'UTR and Mut-3'UTR of SOCS2. (D) SOCS2 mRNA was determined by qPCR in MCF7 and SKBR3 cells transfected with miR-3613-3p mimics or their controls. (E) SOCS2 protein was determined by western blotting in MCF7 and SKBR3 cells transfected with miR-3613-3p mimics or their controls. (F) SOCS2 protein was determined by western blotting in breast cancer cells transfected with miR-3613-3p mimics or their controls. **p<0.01; *p<0.05.

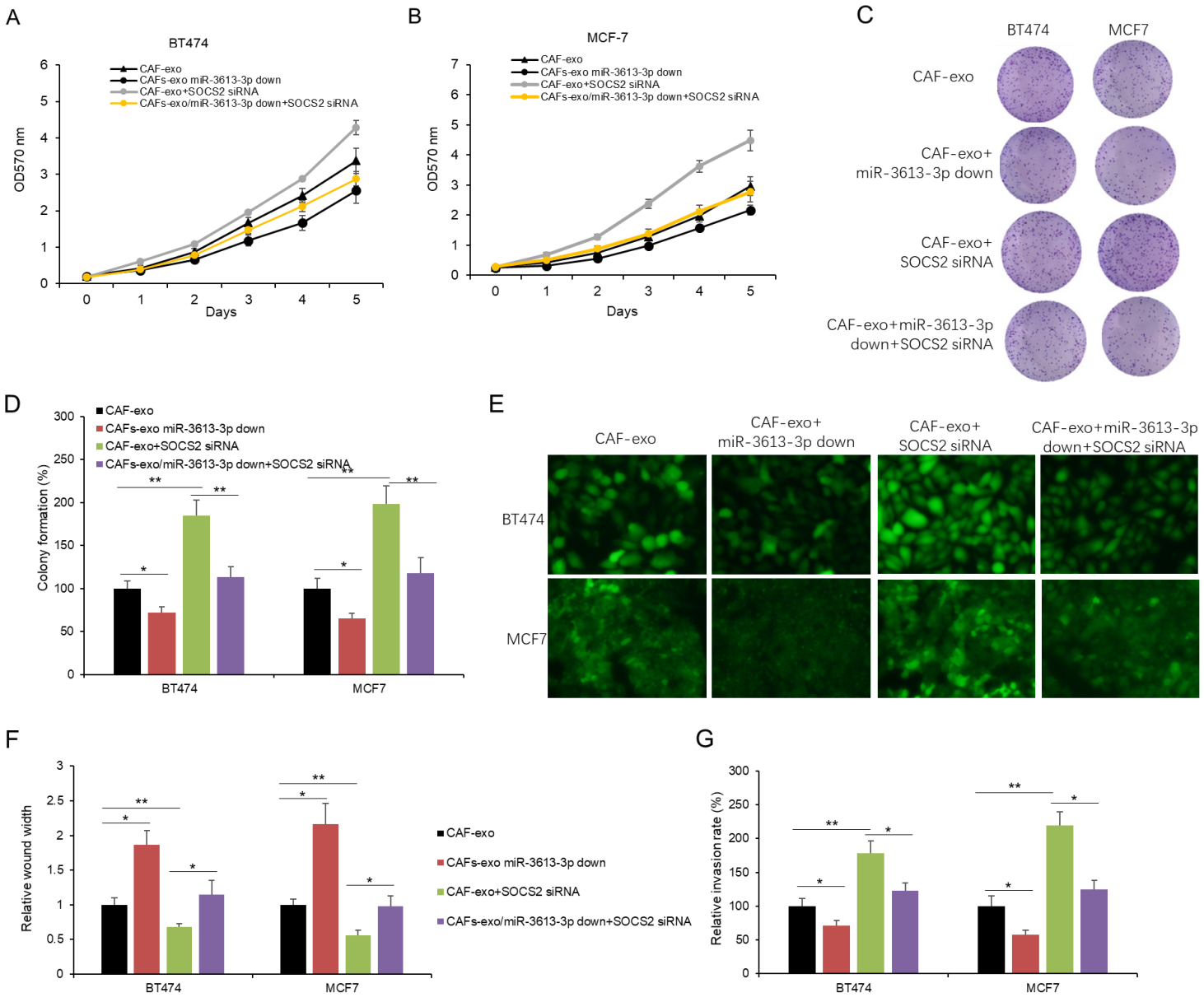

Figure 4

CAFs exosomes with miR-3613-3p inhibition led to overcome drug resistance via SOCS2-STAT3. (A) Exosomal miR-3613-3p transferred from CAFs to breast cancer cells. (B-C) MCF7 and SKBR3 cell proliferation was assayed by CCK8 method. MCF7 and SKBR3 cells were treated with CAF-exo with miR-3613-3p down-regulation combining with SOCS2 or without SOCS2 overexpression. Cell growth was measured at day 1, 2, 3, 4 and 5. (D) ROS production in breast cancer cells. MCF7 and SKBR3 cells were treated with CAF-exo with miR-3613-3p down-regulation combining with SOCS2 or without SOCS2 overexpression for 24h, and then ROS production was observed under a fluorescence microscope. (E-F) MCF7 and SKBR3 cell invasion ability was assayed by transwell assay with Matrigel coated chambers. MCF7 and SKBR3 cells were treated with CAF-exo with miR-3613-3p down-regulation combining with SOCS2 or without SOCS2 overexpression. **p<0.01; *p<0.05.

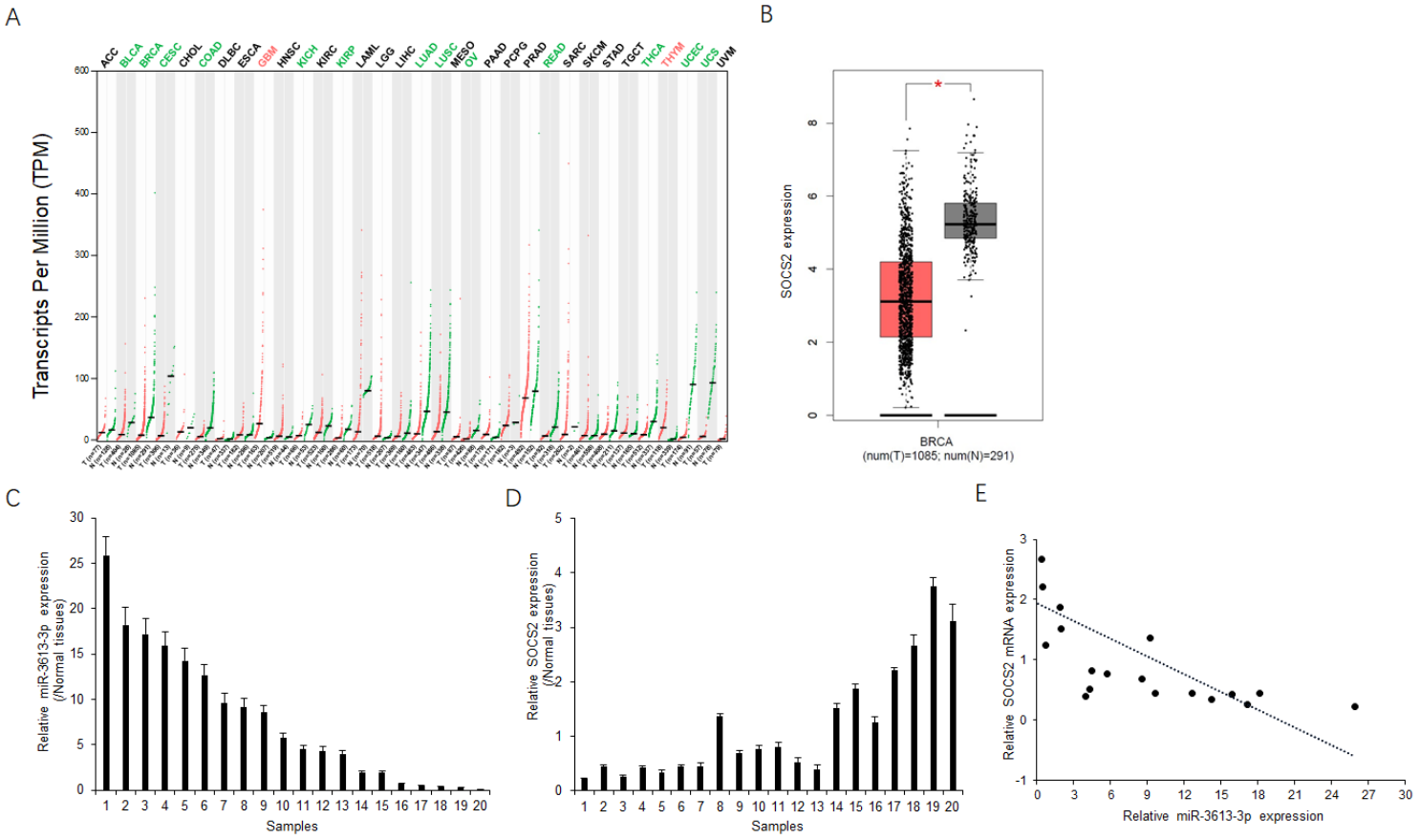

Figure 5

MiR-3613-3p was negatively related to SOCS2 expression in breast cancer tissues. (A) SOCS2 expression in multiple cancer types. The data was from GEPIA2. (B) The average SOCS2 expression from breast cancer tissues and adjacent normal tissues. Data was shown in box plot. (C) miR-3613-3p expression was up-regulated in breast cancer tissues. Total RNA was extracted from breast cancer tissues and their adjacent normal tissues. RNA was used for real time RT-PCR. (D) SOCS2 mRNA expression in breast cancer tissues. Total RNA was extracted from breast cancer tissues and their adjacent normal tissues. RNA was used for real time RT-PCR. (E) The relationship between miR-3613-3p and SOCS2 expression was negatively in breast cancer tissues. **p<0.01. BLCA: bladder urothelial carcinoma, BRCA: breast invasive carcinoma, CESC: cervical and endocervical cancers, COAD: colon adenocarcinoma, KICH: kidney chromophobe, KIRP: kidney renal papillary cell carcinoma, LUAD: lung adenocarcinoma, LUSC: lung squamous cell carcinoma, OV: ovarian serous cystadenocarcinoma, READ: rectum adenocarcinoma, THCA: thyroid carcinoma, UCEC: uterine corpus endometrial carcinoma and UCS: uterine carcinosarcoma.

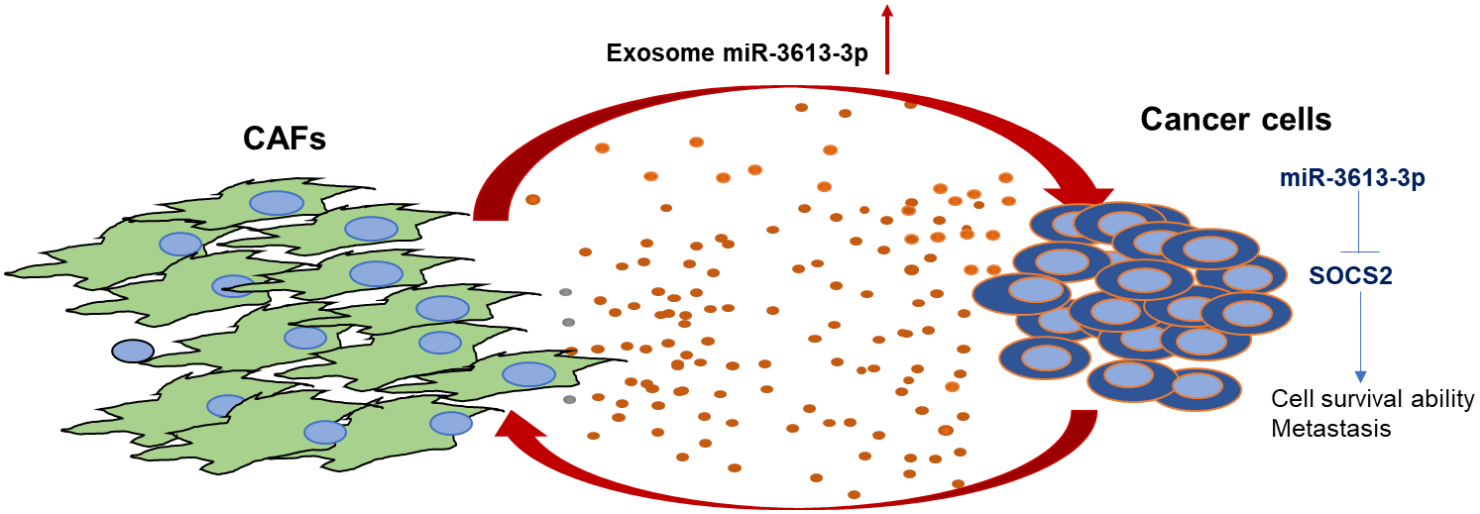

Figure 6

CAFs exosomal miR-3613-3p promoted breast cancer cell proliferation and metastasis by SOCS2. MiR-3613-3p from CAFs exosomes could transferred into breast cancer cells, down-regulated SOCS2 expression. SOCS2 down-regulation in breast cancer cells could increase cell grown then metastatic ability.