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MicroRNA-155 Functions as an OncomiR in Breast Cancer by Targeting the *Suppressor of Cytokine Signaling 1* Gene

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Abstract

MicroRNA-155 (miR-155) is overexpressed in many human cancers; however, the mechanisms by which miR-155 functions as a putative oncomiR are largely unknown. Here, we report that the tumor suppressor gene *suppressor of cytokine signaling 1* (*socs1*) is an evolutionarily conserved target of miR-155 in breast cancer cells. We found that *mir-155* expression is inversely correlated with *socs1* expression in breast cancer cell lines as well as in a subset of primary breast tumors. We also identified a 24A→G mutation in the miR-155 binding site of the *SOCS1* 3′ untranslated region in a breast tumor that reduced miR-155 repression, implicating a mechanism for miRNA targets to avoid repression. Ectopic expression of miR-155 significantly promoted the proliferation of breast cancer cells, the formation of soft agar foci *in vitro*, and the development of tumors in nude mice. In breast cancer cells, RNA interference silencing of *socs1* recapitulates the oncogenic effects of miR-155, whereas restoration of *socs1* expression attenuates the protumorigenesis function of miR-155, suggesting that miR-155 exerts its oncogenic role by negatively regulating *socs1*. Overexpression of miR-155 in breast cancer cells leads to constitutive activation of signal transducer and activator of transcription 3 (STAT3) through the Janus-activated kinase (JAK) pathway, and stimulation of breast cancer cells by the inflammatory cytokines IFN-γ and interleukin-6 (IL-6), lipopolysaccharide (LPS), and polyriboinosinic:polyribocytidylic acid [poly(I:C)] significantly upregulates *mir-155* expression, suggesting that miR-155 may serve as a bridge between inflammation and cancer. Taken together, our study reveals that miR-155 is an oncomiR in breast cancer and that miR-155 may be a potential target in breast cancer therapy. *Cancer Res; 70(8); 3119–27. ©2010 AACR.*

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Introduction

MicroRNAs (miRNA) are ~22-nucleotide RNAs that negatively regulate gene expression in eukaryotes (1). miRNA genes are one of the most abundant classes of regulatory genes in mammals, and mounting evidence indicates that miRNAs are key regulators of animal development and involved in human diseases such as cancer (2).

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The miR-155 locus is located within a region known as B-cell integration cluster (*BIC*; ref. 3), which was originally thought to be a proto-oncogene associated with lymphoma (4). miR-155 was first implicated in the oncogenesis of hematopoietic malignancies based on the finding that *BIC/mir-155* expression is upregulated in B-cell lymphomas and chronic lymphocytic leukemia (5). Consistent with this observation, transgenic expression of miR-155 in B cells causes acute lymphoblastic leukemia/high-grade lymphoma (6). *mir-155* is also overexpressed in various solid tumors, including breast, lung, colon, pancreatic, and thyroid cancers (7–11). Moreover, high expression levels of miR-155 have been found to correlate with poor prognoses of lung cancer and pancreatic tumor (10, 11). All of these lines of evidence are consistent with the notion that miR-155 functions as an oncogenic miRNA (oncomiR) in human cancers.

OncomiRs that are upregulated in tumors function in tumor development mainly via repressing the expression of tumor suppressor or tumor suppressor–like genes (12, 13). Of note, miR-155 promotes pancreatic tumor development and mammary gland epithelial cell migration and invasion by targeting the tumor suppressors *TP53INP1* and *RhoA*, respectively (8, 14). In addition, miR-155 induces B-cell malignancies by targeting *Ship* and *C/EBPβ* in a transgenic mouse model (6). Most of the validated target genes of miR-155, however, including *Pu.1, c-MAF, AID, interleukin-1*

(*IL-1*), *IKKε*, *Ets-1*, and *Meis1* (15–20), have not been implicated in miR-155–mediated oncogenesis but are relevant to the function of miR-155 in the immune and hematopoietic systems. Given that miRNAs usually regulate a large set of targets, it is speculated that there might be more miR-155 targets that are involved in oncogenesis.

Although miR-155 has been found to be upregulated in breast cancer, its role in breast tumorigenesis has not yet been defined. In the present study, we identified *suppressor of cytokine signaling 1* (*socs1*) as a novel target of miR-155 in breast cancer cells. SOCS1 is a tumor suppressor that normally functions as a negative feedback regulator of Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) signaling (21). Furthermore, we show that overexpression of miR-155 in breast cancer cells promoted cell proliferation, colony formation, and xenograft tumor growth through the repression of *socs1*. Finally, we show that upregulation of miR-155 in breast cancer contributed to the posttranscriptional silencing of *socs1* and led to constitutive activation of STAT3 in breast cancer cells. Together, these results suggest that *mir-155* is an oncomiR for breast cancer.

Materials and Methods

Vector construction. The *mir-155* expression vector pSIF-GFP–miR-155, carrying a ~400-bp human *BIC* sequences driven by H1 promoter, was derived from pSIF-GFP vector (System Biosciences). Flag-fused human *SOCS1* coding sequences with or without its 3′ untranslated region (UTR) were cloned into pcDNA3 vector (Invitrogen) to construct *socs1* expression vectors pF-SOCS1-3′UTR and pF-SOCS1. All the constructs were confirmed by DNA sequencing.

Cell lines and patient specimens. Breast cancer cell lines MCF-7, MDA-MB-231, SK-BR-3, and BT-474; nontumorigenic human breast epithelial cell line MCF-10A; and embryonic kidney cell line HEK293T cells were obtained from the American Type Culture Collection (ATCC). The medium and serum for the cell cultures were used as ATCC recommended. For inflammatory stimulation, 50 ng/mL IFN-γ, 10 ng/mL IL-6 (eBioscience), 5 ng/mL lipopolysaccharide (LPS; Sigma), or 2 μg/mL polyriboinosinic:polyribocytidylic acid [poly(I:C); Amersham] were contained in the medium, respectively. The cellular morphology of these cell lines is normal in both low and high densities of cultures by microscopy according to the guideline from ATCC (22). Breast tumor specimens and their matching normal breast specimens were collected at the time of surgery from patients in Zhongshan Hospital Affiliated to Fudan University. Samples were snap frozen immediately and stored at −80°C. Approval to collect specimens was granted by the hospital.

RNA oligonucleotide and cell transfection. miR-155 mimics, anti–miR-155, small interfering RNA (siRNA) targeting *SOCS1* mRNA (NM_003745), and their cognate control RNAs were purchased from Ambion. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For transfection of the RNA oligonucleotides, 50 nmol/L of siRNA and 100 nmol/L of miRNA mimics or antisense oligonucleotides were used. The transfection efficiency was estimated to be ~80% for MCF-7 and MDA-MB-231 cells using Cy3 dye-labeled RNA oligonucleotides (Ambion). For plasmid, 4 μg DNA was used in a six-well plate. G418 (200 μg/mL) was added 24 h after transfection. In the rescue experiment, cells were cotransfected with 100 nmol/L of miRNA mimics and 1 μg of plasmid in a six-well plate.

Immunofluorescence assay. MCF-7 cells were tansfected with pSIF-GFP–miR-155 or pSIF-GFP and fixed with 4%

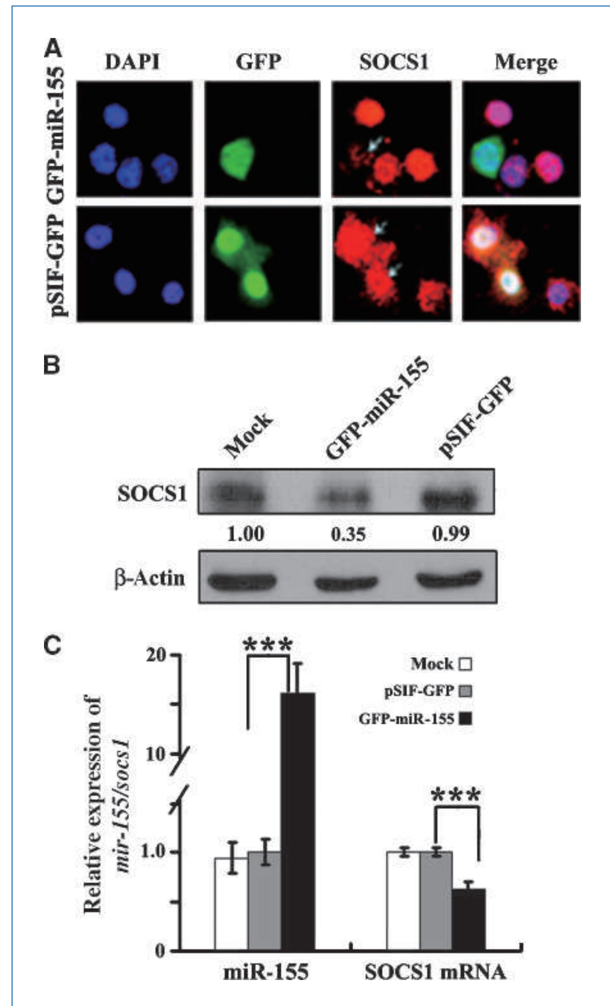

Figure 1. *socs1* is a target of miR-155 in breast cancer cells. A, *socs1* expression was examined by immunofluorescence analysis. GFP–miR-155 and pSIF-GFP showed transfection of miR-155 expression (top) or control vectors (bottom), respectively. Red, SOCS1 protein was immunostained with anti-SOCS1; blue, nuclei were stained with DAPI. GFP images (green) and arrowheads indicate cells with miR-155 expression or control vectors. B, SOCS1 protein levels were examined by Western blot analysis, and β-actin served as an internal reference. C, the levels of miR-155 and *SOCS1* mRNA were quantified by qRT-PCR analysis. U6 and β-actin served as internal normalized references for miR-155 and *SOCS1* mRNA, respectively. Columns, mean of three separate experiments; bars, SE. ***, $P < 0.001$.

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paraformaldehyde 48 h after transfection and then permeabilized with 0.5% Triton X-100 in PBS. Rabbit anti-SOCS1 (Santa Cruz Biotechnology) was used as primary antibody, and Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) was used as secondary antibody to visualize SOCS1. Cells containing the vectors were visualized with ectopic expression of green fluorescent protein (GFP). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

Western blot. Cells were harvested 48 h after transfection, unless otherwise indicated. Antibodies for SOCS1 and proliferating cell nuclear antigen (PCNA) were from Abcam; antibodies for phospho-JAK2, STAT3, and phospho-STAT3 were from Cell Signaling; and antibody for β-actin was from Sigma. The band intensities were quantified with Storm 860 Molecular Imager (GE).

RNA extraction and quantitative reverse transcription-PCR. Total RNA was extracted from cells or tissues with Trizol reagent (Invitrogen). The miR-155 level was quantified by quantitative reverse transcription-PCR (qRT-PCR) using Taqman assay kits (Applied Biosystems), with U6 small nuclear RNA as an internal normalized reference. *SOCS1* mRNA levels were determined using the forward primer 5′-agagcttcgactgcctcttc-3′ and the reverse primer 5′-gatgcgctggcggcacagct-3′, with β-actin as an internal reference. The qRT-PCR results were analyzed and expressed as relative miRNA or mRNA levels of the C_T (cycle threshold) value, which was then converted to fold change.

Gel shift assay of miR-155 duplex formation. The synthesized miR-155 RNA (5′-uuaaugcuaaucgugauaggggg-3′) was ^{32}P labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Then, the labeled RNA was mixed with equal amount of RNAs of miR-155 antisense (5′-cccctatcacgattagcattaa-3′), wild-type *SOCS1* miRNA regulatory element (MRE; 5′-cgccgugcacgcagcauuaa-3′), and A24G MRE (5′-cgccgugcacgcGgcauuaa-3′), respectively. The samples were incubated at 95°C for 5 min and then cooled naturally to room temperature for annealing before being resolved on a 20% native polyacrylamide gel at 4°C. After electrophoresis, the wet gel was wrapped and exposed to X-ray film for autoradiography at −80°C. The RNAs in this assay were provided by RiboBio.

Cell proliferation and soft agar colony formation assays. Breast cancer cells were transfected with RNA oligonucleotide and/or plasmid DNA. Four hours after transfection, equal numbers of viable cells were seeded in 96-well plates for cell proliferation assay. Cell growth was determined by using MTT assay and verified by counting and trypan blue exclusion. In soft agar colony formation, 24 h after transfection, ~5,000 viable cells in 1.5 mL tissue culture medium with 1% glutamine, antibiotics, and 0.4% soft agar were layered onto 0.8% solidified agar in tissue culture medium in a six-well plate. The plates were incubated at 37°C in a humidified incubator with 5% CO_2 for 14 to 21 d, the cells were stained with 0.005% crystal violet (23), and colony foci were counted using a dissecting microscope. Experiments were carried out in triplicate.

Xenograft assays in nude mice. All animal work was done in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication nos. 80-23, revised 1996) and performed according to the institutional ethical guidelines for animal experiment. Twenty-four hours after transfection of RNA oligonucleotide and/or plasmid DNA, $\sim 2 \times 10^6$ cells were suspended in 100 μL PBS and then injected orthotopically into the third mammary gland on either side of the same female BALB/c athymic nude mouse at 6 to 8 wk of age. Six mice were included in one experimental group. Tumor growth rates were examined twice a week for 6 wk. Tumor growth rates were analyzed by measuring tumor length (L) and width (W) and calculating the volume with the formula $\pi LW^2/6$, as previously described (24).

Results and Discussion

socs1 *is a target of miR-155 in breast cancer cells.* In studying the oncogenic function and mechanism of miR-155 in the development of breast cancer, using computational prediction and luciferase reporter assay, we indicated that the tumor suppressor gene *socs1* is an evolutionarily conserved target of miR-155 (Supplementary Fig. S1). We next determined whether overexpression of miR-155 leads to downregulation of endogenous *socs1* expression in human breast cancer cells. We overexpressed miR-155 in MCF-7 cells

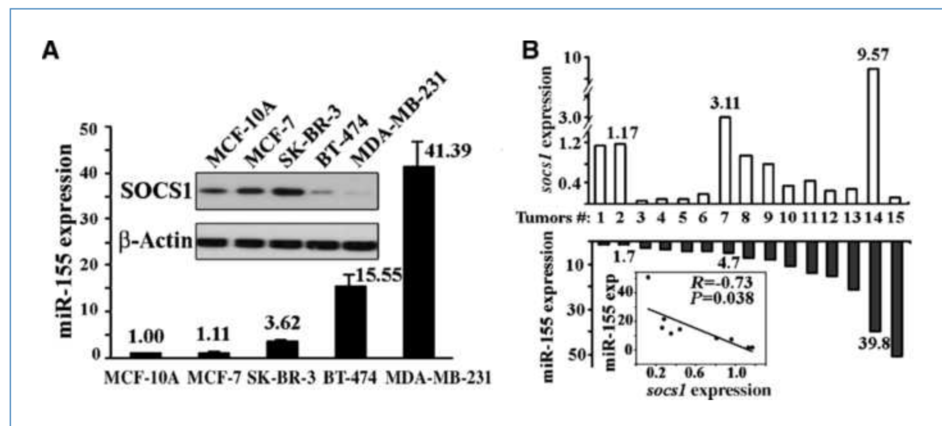

Figure 2. Expression of *socs1* and *mir-155* in breast cancer cells and primary breast tumors. A, expression of *mir-155* and *socs1* in breast cancer cell lines. Top, Western blot analysis of SOCS1 protein levels; bottom, qRT-PCR analysis of miR-155 levels. B, qRT-PCR analysis of miR-155 and *SOCS1* mRNA levels in primary breast tumors. The inset graph indicated a statistically significant inverse correlation (R = −0.73, P = 0.038) in 9 of 15 tumors. The normalized references were the same as that in Fig. 1.

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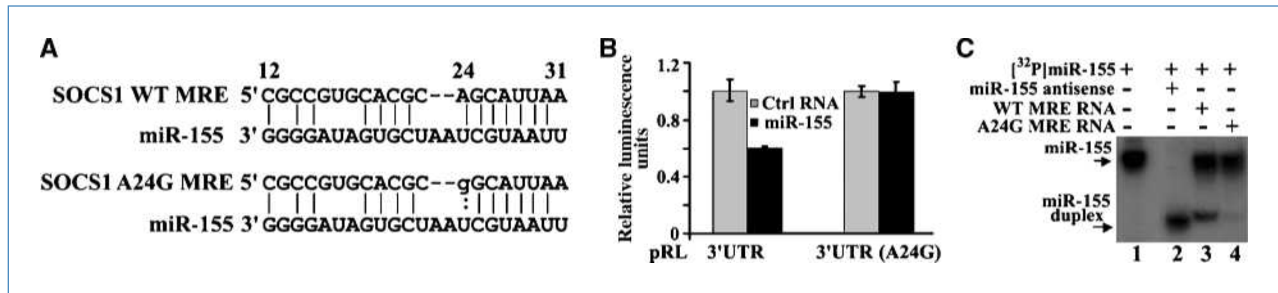

Figure 3. The 24A→G mutation abolishes *socs1* regulation by miR-155. A, identification of the A24G mutation in *socs1* 3′UTR in a breast tumor. B, A24G abolished miR-155 regulation, as assessed by a luciferase reporter assay. The reporter pRL-3′UTR (A24G) was created by site-directed mutagenesis, and the reporter pRL-3′UTR (WT) containing a wild-type 3′UTR served as a control in the reporter assay. C, A24G impaired miR-155 binding. ^{32}P-labeled miR-155 RNA was annealed to equal amount of RNAs of miR-155 antisense (lane 2), WT *SOCS1* MRE (lane 3), and A24G *SOCS1* MRE (lane 4).

using a vector coexpressing *mir-155* and GFP. As shown by immunofluorescent staining, SOCS1 protein was largely reduced in cells with a strong GFP signal and miR-155 overexpression (Fig. 1A, top), whereas it was unaffected in controls (Fig. 1A, bottom). Western blot assays indicated that the SOCS1 protein level was reduced by ~3-fold in MCF-7 cells overexpressing miR-155 compared with the control (Fig. 1B). Moreover, qRT-PCR analyses showed that the *SOCS1* mRNA level was downregulated by ~30% when miR-155 was overexpressed ~16-fold ($P < 0.001$; Fig. 1C). Furthermore, in the breast cancer cell line MDA-MB-231, we knocked down *mir-155* expression using anti–miR-155 and found that the SOCS1 protein level was increased ~5-fold (Supplementary Fig. S2). In these cells, the expression of *mir-155* was reduced ~5-fold, whereas the *SOCS1* mRNA level was upregulated ~2-fold ($P < 0.001$).

These results show that *socs1* is a direct target of miR-155 in breast cancer cells and that mRNA degradation is involved in miR-155–suppressing *socs1*. While we were preparing this article, an independent article reported that *socs1* is a target of miR-155 in T regulatory cells (25), confirming that *socs1* is an authentic target of miR-155. That report showed that miR-155 plays an important role in the function of the immunocyte by targeting *socs1*, whereas we are uniquely interested in *socs1*–miR-155 interaction as it pertains to miR-155 as an oncomiR and *socs1* as a tumor suppressor. Given that miR-155 is overexpressed in many cancers and that it negatively regulates *socs1* expression, we speculate that targeting *socs1* could be a mechanism by which miR-155 acts as an oncomiR in cancers.

Inverse correlation of* mir-155 *and* socs1 *expression in breast cancer cells and breast tumors. To address the biological significance of the *socs1*–miR-155 interaction in breast cancer, we initially examined *socs1* and *mir-155* expression levels in breast cancer cell lines. *socs1* is downregulated in various human tumors, including breast cancer (21). We hypothesized that reduced *socs1* expression in breast cancer cells could be a result of elevated miR-155 expression. Indeed, SOCS1 protein levels were higher in nonmalignant human breast epithelial MCF-10A cells and less malignant breast cancer MCF-7 and SK-BR-3 cells but relatively lower in aggressive breast cancer BT-474 and MDA-MB-231 cells (Fig. 2A, top). In contrast, miR-155 levels were relatively lower in MCF-10A, MCF-7, and SK-BR-3 cells but higher in BT-474 and MDA-MB-231 cells (Fig. 2A, bottom). These results indicate that *mir-155* expression is negatively related to *socs1* expression in breast cancer cell lines.

To test whether this observation could be extrapolated to primary breast tumors, we examined *socs1* and *mir-155* expression in 15 sets of breast tumor and paired normal tissue specimens. We found that *SOCS1* mRNA was reduced by >2-fold in ~60% of breast tumors compared with the normal tissues (Fig. 2B, top). As expected, miR-155 levels were commonly elevated in these tumors, with an increase of >5-fold found in 8 of 15 tumors (Fig. 2B, bottom). Using Pearson's correlation analysis of *socs1*–*mir-155* expression, we obtained a statistically significant inverse correlation ($R = -0.73$, $P = 0.038$) in a total of 9 of 15 tumors (Fig. 2B, bottom, inset).

Taken together, these results indicate that *mir-155* expression is inversely correlated with *socs1* expression in breast cancer cell lines and a subset of primary breast tumors, suggesting that the *socs1*–miR-155 interaction may be biologically significant in breast cancers. Given that knockdown of miR-155 expression in MDA-MB-231 cells upregulated *socs1* expression (Supplementary Fig. S2), we propose that increased *mir-155* expression is likely responsible for the reduced *socs1* expression in breast cancer cells.

24A→G mutation in the SOCS1 mRNA 3′UTR abolishes miR-155 repression. Unexpectedly, in tumors T7 and T14, we found that *socs1* expression was upregulated ~3- and 10-fold, respectively, whereas *mir-155* expression was ~5- and 40-fold higher than in controls (Fig. 2B). To understand this discrepancy, we sequenced the *socs1* gene and identified a 24A→G mutation in the miR-155 binding site of the *SOCS1* 3′UTR from tumor T14 (Fig. 3A). Using a reporter assay, we found that the reporter fused to the A24G-mutated *SOCS1* 3′UTR was not repressed by miR-155 (Fig. 3B), showing that the mutation prevents miR-155 regulation. Furthermore, a RNA duplex formation assay revealed that the *SOCS1* A24G MRE RNA could not form a stable duplex with the miR-155 strand (Fig. 3C, lane 4), whereas miR-155 antisense and wild-type *SOCS1* MRE RNA could form a complete or partial RNA duplex with miR-155 (lanes 2 and 3). These results indicate

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that the A24G mutation impairs miR-155 binding to the miR-155 binding site of *SOCS1* 3′UTR and, consequently, abolishes miR-155 regulation.

This mutation breaks the seventh A-U base pair between the *SOCS1* MRE and miR-155 seed sequences and changes a canonical 7-mer A1 seed-matched site to a 6-mer site, which may have reduced the efficacy for miRNA target recognition (26). Similar to our findings, single-nucleotide polymorphisms in miRNA target sites have been claimed to prevent the miRNA regulation of target genes, which are associated with many human diseases (27). Widespread shortening of 3′ UTRs and, consequently, fewer miRNA regulatory sites are observed in cancer cells and proliferating T cells, decreasing the negative effect of miRNAs in these cells (28, 29). Therefore, mutation or loss of miRNA sites may be a common mechanism for targets to avoid miRNA repression.

We have identified a few *socs1* 3′UTR mutations at potential miRNA binding sites. The details of these mutations and their biological significance in breast carcinogenesis will be discussed in other studies.

***miR-155 acts as an oncomiR in breast cancer cells* in vitro *and* in vivo.** Given that miR-155 is overexpressed in breast cancers and that it may act as an oncomiR, we decided to examine whether miR-155 has oncogenic functions in breast cancer cells *in vitro* and *in vivo*. The results of the *in vitro* assays indicated that miR-155 mimics significantly promoted the proliferation of MDA-MB-231 cells ($P < 0.001$; Fig. 4A) and stimulated MCF-7 cells to grow more (>3-fold) and larger colonies on soft agar ($P < 0.001$; Fig. 4B, left). Conversely, anti–miR-155 in MDA-MB-231 cells significantly reduced cell proliferation ($P < 0.001$; Fig. 4A) and resulted in ~2.5-fold fewer colony foci ($P < 0.001$; Fig. 4B, right). Western blot analyses confirmed that *socs1* in these cells was modulated by miR-155, and the expression of the proliferation biomarker PCNA was enhanced ~3-fold by miR-155 mimics and reduced ~10-fold by anti–miR-155 (Fig. 4A, right). These results show that miR-155 promotes the proliferation and anchorage-independent growth of breast cancer cells *in vitro*.

To determine whether miR-155 regulates tumor growth *in vivo*, we used tumor xenografts by inoculating MDA-MB-231 cells with modulated *mir-155* expression in nude mice. We first injected MDA-MB-231 cells with anti–miR-155 into nude mice and found that these mice bore smaller tumors compared with those expressing control RNA (Fig. 4C, left). At the end of the observation (6 weeks), the average volume (105 ± 30 mm^3) of tumors expressing anti–miR-155 was only about one third of that (294 ± 81 mm^3) of the control group ($P < 0.01$). When miR-155 mimics were used to upregulate *mir-155* expression in MDA-MB-231 cells, we found that tumor growth was significantly accelerated (Fig. 4C, right), and by the end of 6 weeks, the average tumor volume for miR-155 mimic–transfected and control RNA–transfected cells was 566 ± 132 mm^3 and 238 ± 68 mm^3, respectively ($P < 0.01$). These results show that miR-155 stimulates the tumor growth of breast cancer cells *in vivo*.

Taken together, our results show that miR-155 acts as an oncomiR in breast cancer cells. This is the first time that miR-155 is directly shown to act as an oncomiR in cultured breast cancer cell lines and in immunodeficient mice xenografted with human breast cancer cells.

Targeting* socs1 *is a mechanism of miR-155 as an oncomiR in breast cancer cells. To investigate whether miR-155

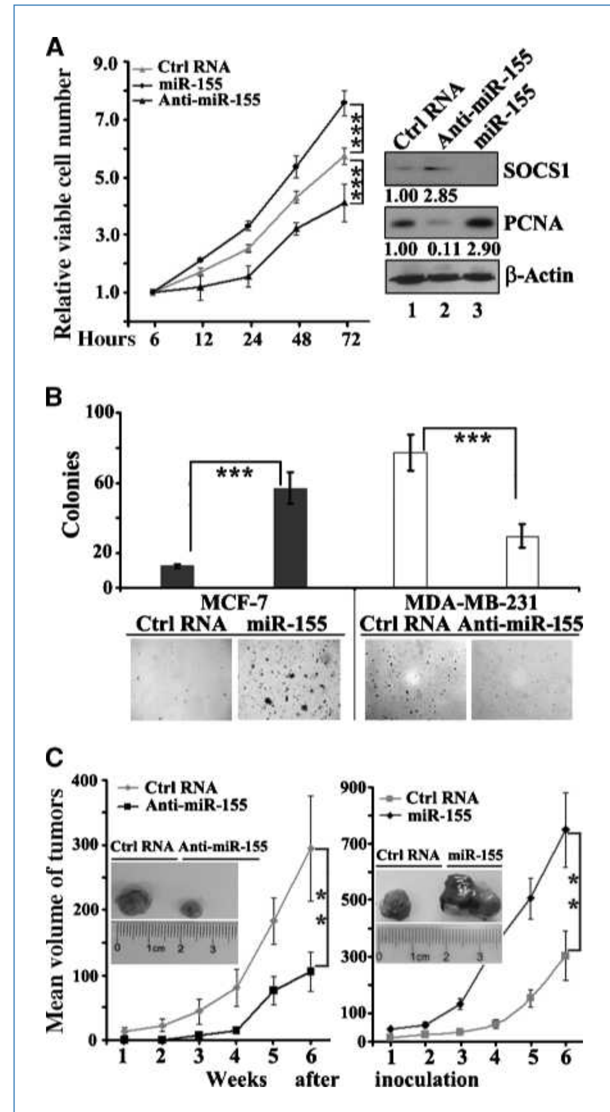

Figure 4. miR-155 acts as an oncomiR in breast cancer cells *in vitro* and *in vivo*. A, miR-155 promotes breast cancer cell proliferation. MDA-MB-231 cells were transfected with miR-155 mimics, anti–miR-155, or control RNA (Ctrl RNA). MTT assays were performed at the indicated time points after transfection. Western blot analysis was performed 72 h after transfection. B, miR-155 promotes anchorage-independent growth of breast cancer cells. MCF-7 cells were transfected with miR-155 mimics or control RNA; MDA-MB-231 cells were transfected with anti–miR-155 or control RNA. Soft agar colony formation assays were performed 24 h after transfection. C, miR-155 stimulates tumor growth of breast cancer cells in nude mice. MDA-MB-231 cells were transfected with miR-155 mimics, anti–miR-155, or control RNA. Twenty-four hours after transfection, 2×10^6 cells were injected orthotopically into the mammary fat pads of female nude mice. The curves show the time course of tumor growth, and the inset photographs are representative xenografted tumors 6 wks after inoculation. Points, average of three separate experiments; bars, SD. **, $P < 0.01$; ***, $P < 0.001$.

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exerts its oncogenic function by targeting *socs1*, we examined whether RNA interference (RNAi) knockdown of *socs1* expression could recapitulate the oncogenic effects of miR-155 in breast cancer cells. We found that siRNA knockdown of *socs1* in MCF-7 cells significantly promoted cell proliferation ($P < 0.001$; Fig. 5A, left) and anchorage-independent growth ($P < 0.001$; Fig. 5A, right). Western blot analysis confirmed that *SOCS1* siRNA reduced SOCS1 protein levels ~5-fold and increased PCNA protein levels ~4-fold (Fig. 5A, right). These results indicate that a reduction of *socs1* expression can mimic miR-155 in promoting breast cancer cells to proliferate and form soft agar foci, suggesting that targeting *socs1* may be a mechanism of the oncogenic function of miR-155 in breast cancer cells. However, in MDA-MB-231 with high *mir-155* and low *socs1* expression, we found that *SOCS1* siRNA was less efficient than miR-155 mimics to induce cell growth (Supplementary Fig. S4), suggesting that other targets might be required for miR-155 oncogenic function in breast cancer cells.

We then performed rescue experiments to further validate that *socs1* targeting is involved in miR-155–mediated oncogenesis in breast cancer cells. Two *socs1* expression vectors, pF-SOCS1 and pF-SOCS1-3′UTR, were used in the experiments. Both constructs could similarly overexpress SOCS1 protein in HEK293T cells with low *mir-155* expression (Supplementary Fig. S3). Overexpression of SOCS1 protein with pF-SOCS1 greatly suppressed the proliferation of MDA-MB-231 cells overexpressing miR-155 (Fig. 5B, left). Intriguingly, this growth inhibition was significantly attenuated when the *SOCS1* 3′UTR was included in the expression construct. Consistently, cells transfected with pF-SOCS1 expressed ~8-fold more SOCS1 protein and 3-fold less PCNA protein than those transfected with pF-SOCS1-3′UTR (Fig. 5B, left, lanes 2 and 3). These results show that the *SOCS1* 3′UTR in the expression vector dramatically inhibited the production of SOCS1 protein in miR-155–overexpressing cells, and therefore, it was less efficient in rescuing miR-155–promoted cell proliferation. As expected, cotransfection of pF-SOCS1 and miR-155 mimics into MCF-7 cells significantly rescued miR-155–stimulated anchorage-independent growth ($P < 0.001$; Fig. 5B, middle), and MDA-MB-231 cells cotransfected with miR-155 mimics and pF-SOCS1 produced tumors much more slowly than miR-155 mimics and pcDNA3Flag cotransfectants (Fig. 5B, right). At the end of 6 weeks, the average tumor volume in the rescue experimental group was 172 ± 50 mm^3, whereas that in the control group was 494 ± 130 mm^3, which represented a >2.5-fold decrease ($P < 0.01$). These findings indicate that reintroduction of SOCS1 protein may override the effects of miR-155 overexpression on the tumor growth of

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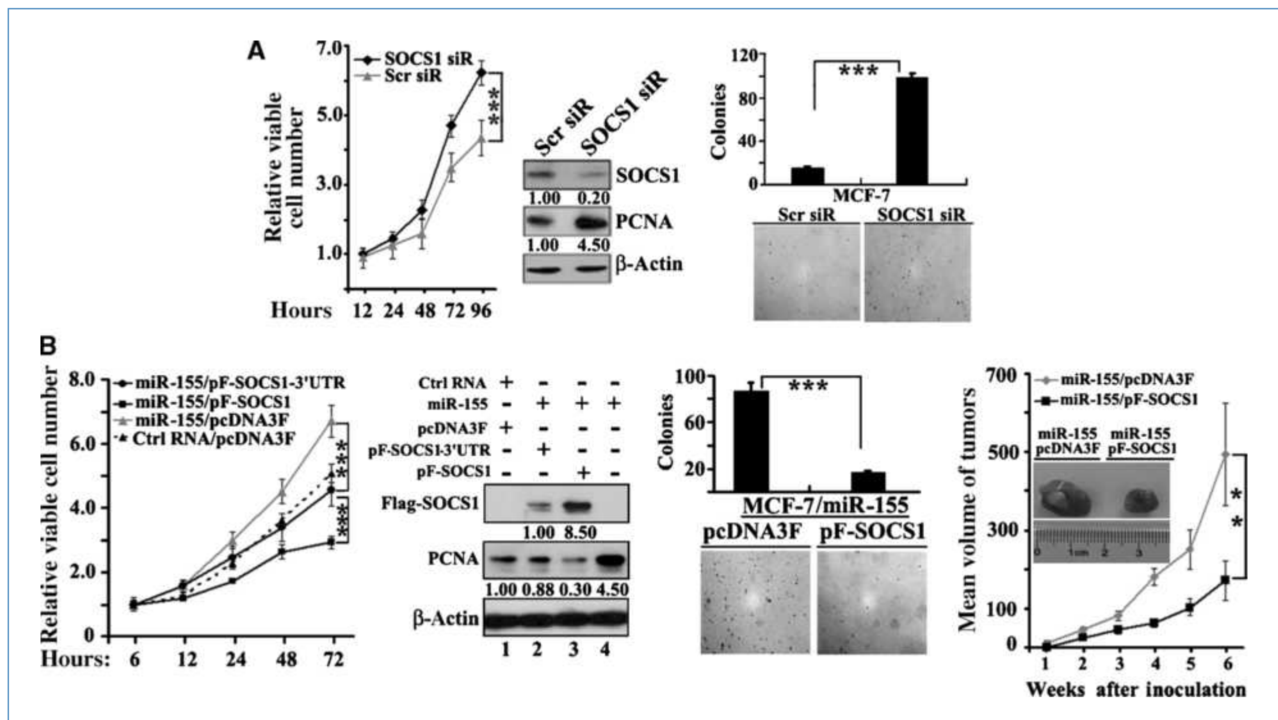

Figure 5. Targeting *socs1* is involved in the oncogenic function of miR-155 in breast cancer cells. A, RNAi knockdown of *socs1* recapitulates the oncogenic effect of miR-155. MCF-7 cells were transfected with *SOCS1* siRNA (SOCS1 siR) or scrambled siRNA (Scr siR). Left, cell proliferation assay; right, soft agar colony formation assay. B, *socs1* expression inhibits the oncogenic function of miR-155. Left, cell proliferation assay. MDA-MB-231 cells were cotransfected with miR-155 mimics and pF-SOCS1, pF-SOCS1-3′UTR, or control. Middle, soft agar colony formation assay. MCF-7 cells were cotransfected with miR-155 mimics and pF-SOCS1 or control. Right, xenograft assay in nude mice. MDA-MB-231 cells were cotransfected with miR-155 mimics and pF-SOCS1 or control. Points, mean of three separate experiments; bars, SD. **, $P < 0.01$; ***, $P < 0.001$.

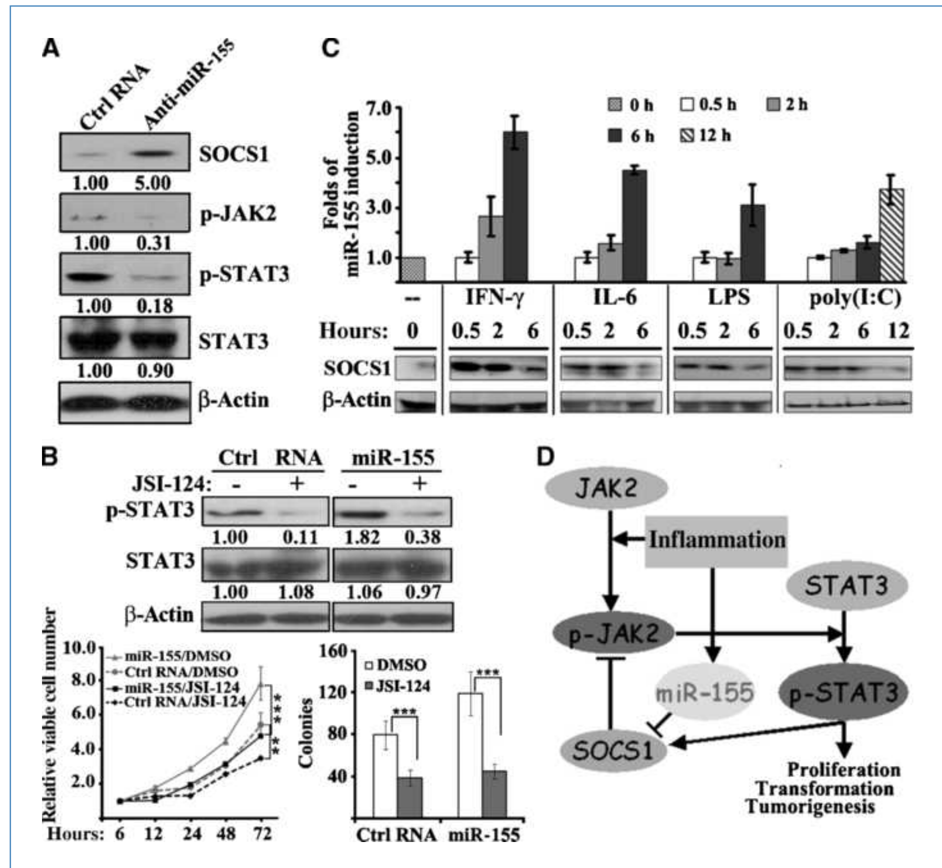

Figure 6. miR-155 may act as a bridge between inflammation and cancer. A, knockdown of *mir-155* reduced the phosphorylated forms of JAK2/STAT3 in MDA-MB-231 cells. Western blot analysis was performed 72 h after transfection. B, the STAT3 inhibitor JSI-124 reversed miR-155–promoting STAT3 activation and cell growth. Assays of Western blot, cell proliferation, and anchorage-independent growth performed after miR-155 mimic–transfected or control RNA–transfected MDA-MB-231 were treated with 10 μmol/L JSI-124 for 24 h. C, effects of inflammatory stimulation on *mir-155* and *socs1* expression. After MCF-7 cells were treated with IFN-γ, IL-6, LPS, and poly(I:C) at the indicated time points, *mir-155* and *socs1* expression was examined by qRT-PCR and Western blot assays, respectively. D, model of miR-155 as a bridge between inflammation and tumorigenesis. **, $P < 0.01$; ***, $P < 0.001$.

MDA-MB-231 cells. These results show that restoration of *socs1* expression attenuates the tumorigenic function of miR-155 in breast cancer cells, further suggesting that targeting *socs1* is a mechanism by which miR-155 exerts its oncogenic function.

Taken together, we have shown that miR-155 acts as an oncomiR in breast cancer cells and that it may do so by downregulating *socs1* expression. *socs1* has been reported to be a tumor suppressor that negatively regulates cell growth and transformation of several cancer cells (30, 31). In addition, as shown in Fig. 2, *socs1* is downregulated in breast cancer, whereas miR-155 is commonly overexpressed in breast cancer as an oncomiR. It is likely that the downregulation of *socs1* by miR-155 is an authentic mechanism of miR-155–mediated oncogenesis in breast tumors.

Enhancement of JAK2/STAT3 signaling in breast cancer cells by miR-155. SOCS1 has been known to be a negative feedback regulator of JAK/STAT signaling, and SOCS1 antitumor activity is through inhibiting STAT3 signaling via the JAK pathway (21, 31). STAT3 is an oncoprotein that is constitutively activated in breast cancer cell lines and human breast tumors, regulating cell survival, proliferation, and metastatic potential (32). Given that *socs1* is repressed by miR-155, we speculated that miR-155 overexpression in breast tumors may play a role in STAT3 signaling. In MDA-MB-231 cells in which *mir-155* was highly expressed (Fig. 2A) and STAT3 was constitutively activated, we found that anti–miR-155 expression resulted in increased levels of SOCS1 protein up to 5-fold and decreased levels of phospho-JAK2 and phospho-STAT3 about 3- and 5-fold, respectively. In contrast, the level of STAT3 protein was not affected by anti–miR-155 (Fig. 6A). Furthermore, we used JSI-124, a highly selective and potent inhibitor of STAT3, to validate miR-155–SOCS1–STAT3 axis functions (33). As shown in Fig. 6B, miR-155 activated STAT3 ~80% in MDA-MB-231 and JSI-124 reversed miR-155–promoting activation of STAT3 and cell growth. These results show that miR-155 overexpression enhances oncogenic STAT3 signaling via the JAK pathway in breast cancer cells. Given that the expression of miR-155 is significantly enhanced and that STAT3 signaling is constitutively active in malignant breast cancer cell lines and a subset of primary breast tumors, we propose that miR-155 may function as an oncomiR in breast cancer development by boosting STAT3 signaling in breast epithelial cells.

miR-155 may act as a bridge between inflammation and malignancy. Inflammation is an important promoter of cancer. Interestingly, STAT3 signaling, which is one of the most important inflammatory pathways recognized to contribute to inflammation-associated malignancy (34), is activated by miR-155 (Fig. 6A and B). Even more interestingly, miR-155 is induced by inflammatory mediators in macrophages, by pathogens in gastric epithelial cells, and by retroviruses in

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chicken embryo fibroblasts (35–37). We found that the inflammatory stimulation [e.g., IFN-γ, IL-6, LPS, and poly(I:C) treatment] regulated the expression of *mir-155* and *socs1* in breast cancer MCF-7 cells. The level of miR-155 expression was about 3- to 7-fold higher at 6 to 12 hours after IFN-γ, IL-6, LPS, or poly(I:C) stimulation (Fig. 6C, top). In contrast, *socs1* expression was rapidly induced 30 minutes after each stimulation but was significantly attenuated 6 to 12 hours later (Fig. 6C, bottom). This result is consistent with the idea that inflammation-induced miR-155 expression is likely a mechanism that contributes to inflammation-related cancers. Therefore, we propose that miR-155 may act as a bridge between inflammation and tumorigenesis (Fig. 6D). As miR-155 is being induced during an inflammatory response, it blocks the negative feedback loop of JAK/STAT signaling via posttranscriptional silencing of *socs1* and boosts the tumor-promoting inflammatory signaling of STAT3. Eventually, the persistent activation of STAT3 leads to cell survival, transformation, and tumor growth.

In conclusion, we have shown that miR-155 functions as an oncomiR in breast carcinomas by targeting *socs1* and consequently contributing to constitutive STAT3 activation in breast cancer. Importantly, the cross talk between miR-155, *socs1*, and STAT3 signaling may provide a new mechanism for inflammation-associated tumorigenesis and suggests the potential use of miR-155 and SOCS1 in cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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