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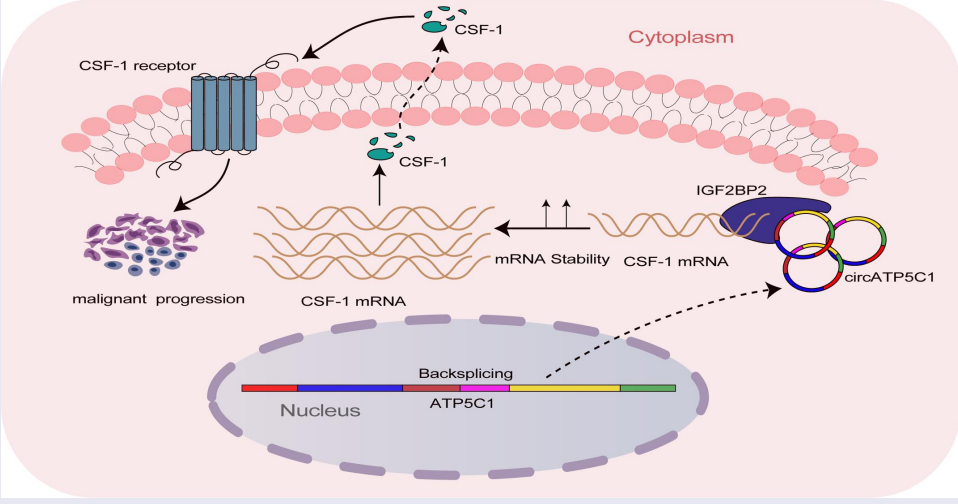
CircATP5C1 promotes triple-negative breast cancer progression by binding IGF2BP2 to modulate CSF-1 secretion

Hongbo Liu[a], Haoqi Wang[a], Wei Gao[a], Yang Yuan[a], Tiantian Tang[a], Meixiang Sang[b], Fei Liu[b], and Cuizhi Geng[a]

[a]Department of Breast Center, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei Province, China; [b]Research Center and Tumor Research Institute, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei Province, China

ABSTRACT

Triple-negative breast cancer (TNBC) is a common malignant disease among females and severely threatens the health of women worldwide. Nowadays, circular RNAs (circRNAs) aroused our interest for their functions in human cancers, including TNBC. However, the mechanism of most circRNAs in the progression of TNBC remains unclear. We found a novel circRNA named circATP5C1, whose function in TNBC remains uncovered. Tissue microarray was used to analyze the association between the expression of circATP5C1 and the prognoses of TNBC patients. Gain-and loss-of-function experiments were performed to validate the biological functions of circATP5C1 in different TNBC cell lines. RNA-seq analyses were conducted to find out the target genes regulated by circATP5C1. RNA pull-down assay and mass spectrometry were used to select the proteins associated with circATP5C1. RNA FISH-immunofluorescence and RNA immunoprecipitation (RIP) were complemented to validate the interaction between circATP5C1 and its binding protein. CircATP5C1 was identified to have predictive function in prognosis of TNBC patients. CircATP5C1 advanced the progression of TNBC cells. Mechanistically, Colony stimulating factor 1 (CSF-1) is a vital downstream gene regulated by circATP5C1. The alteration of CSF-1 expression level was validated due to the interaction between circATP5C1 and insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP2). Rescue experiments demonstrated that circATP5C1 accelerates the progression of TNBC partly via binding with IGF2BP2 to increase the secretion of CSF-1. This study uncovers a novel mechanism of circATP5C1/IGF2BP2/CSF-1 pathway in regulating progression of TNBC.



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Introduction

It was reported that there were 2.3 million new cases of breast cancer (BC) in 2020, accounting for nearly 12% of all new cancer cases, with 685 000 deaths in the same year.[1] Due to improvements in therapy and screening techniques, the mortality rate of patients with BC has declined in recent years.[2] However, BC is still the leading cause of cancer-related deaths for women worldwide.[3] Accounting for generally 15% of all breast cancers, triple-negative breast cancer (TNBC) is characterized by the absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor

CONTACT Fei Liu kyzxlf@hebmu.edu.cn Research Center and Tumor Research Institute, The Fourth Hospital of Hebei Medical University, No. 169 Tianshan Street, Yuhua District, Shijiazhuang, Hebei Province 050017, China; Cuizhi Geng 46300349@hebmu.edu.cn Department of Breast Center, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei Province 050017, China

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receptor 2 (HER2) expression.[4] For lack of effective therapeutic targets, TNBC patients are insensitive to endocrine therapies, molecularly targeted therapies and current chemotherapeutic targeted therapies. Therefore, it has a high degree of aggressiveness and metastatic potential which leads to a poor prognosis and shorter overall survival.[5] Therefore, it is imperative to research on the pathogenesis of TNBC thoroughly and explore more prognostic biomarkers and novel therapeutic targets for clinical diagnosis and therapy.

Circular RNAs (circRNAs) are a sort of covalently closed RNA molecules. Without 5 ' cap and 3' terminal poly (A) tail, circRNAs cannot be hydrolyzed by exonuclease, which makes them more stable than linear RNAs.[6,7] It was confirmed by high-throughput sequencing technology that circRNAs exist widely in human cells and has histological specificity.[8,9] Nowadays, emerging investigations pointed out that circRNAs could be associated with many tumors, such as BC, lung adenocarcinoma, hepatocellular carcinoma, prostate cancer and renal cell carcinoma.[10–14] A growing number of studies have demonstrated that circRNAs have various functions in governing gene expression. CircRNAs can play an important role as competitive endogenous RNAs (ceRNAs). They can competitively bind with microRNAs (miRNAs) preventing specific miRNAs from interacting with and repressing their target mRNAs.[15] In ovarian cancer tissues and cells, circ_0061140 is elevated and promotes proliferation, migration, invasion and angiogenesis through the miR-761/leucine zipper-EF-hand containing transmembrane protein 1 axis.[16] Circ_PPAPDC1A was significantly upregulated and exerts an oncogenic role in NSCLC with Osimertinib resistance by sponging miR-30a-3p to active IGF1R/PI3K/AKT/mTOR pathway.[17] Besides, circRNAs can bind with proteins to affect cancer-related phenotypes.[18] It is reported that there are five patterns according to the direct effects which circRNAs exert on proteins: (1) altering interactions between proteins; (2) tethering or sequestering proteins; (3) recruiting proteins to chromatin; (4) forming circRNA-protein-mRNA ternary complexes; and (5) translocating or redistributing proteins.[19] Circ0006646 could bind with nucleolin (NCL) to prevent interaction between NCL and the E3 ligase tripartite motif-containing 21 (TRIM21) leading to promote hepatocellular carcinoma invasion and migration.[20] CircFIRRE stabilizes GLI2 mRNA and subsequent transcription of its target genes MYC, CCNE1, and CCNE2 by interacting with the heterogeneous nuclear ribonucleoprotein C (HNRNPC) protein, ultimately leading to BC progression.[21] CircRHOBTB3 can suppress the aggressiveness of colorectal cancer by binding to human antigen R (HuR) and promote β-Trcp1-mediated ubiquitination of HuR which degrades HuR and reduce the downstream target polypyrimidine tract-binding protein 1 (PTBP1) expression level.[22] Although circRNAs lack the 5' 7-methylguanosine cap and 3' poly (A) tail, which is the key elements for recruiting translation initiation factors for protein synthesis in linear mRNA. They can rely on internal ribosome entry site (IRES) or m6A modification-dependent modifications for translation.[23,24] A few studies have found that circRNAs have the potential to modulate tumor behaviors by encoding proteins.[25] CircCOPA mainly contributes to inhibiting the glioblastoma malignant phenotype through its encoded COPA-99aa and that COPA-99aa increases temozolomide-induced DNA damage by interfering with the dimerization of NONO and SFPQ.[26] A novel protein TRIM1-269aa encoded by circTRIM1 enhanced the interaction between MARCKS and calmodulin, thus promoting the calmodulin-dependent translocation of MARCKS, which further initiated the activation of the PI3K/AKT/mTOR pathway in TNBC.[27]

Accumulating evidence indicates that circRNAs binding with proteins can play an important role in epigenetic modification of downstream mRNAs. As we all know, N6-methyladenosine (m6A) is the most common post-transcriptional RNA modification in mammals, which can regulate RNA splicing, translation, stability.[28–30] As an m6A reader, IGF2BP2 stabilizes m6A-modified RNAs, influencing gene expression in ways that support cancer cell stemness, proliferation, migration, and evasion of programmed cell death.[31] The exploration of m6A modification in tumors has been of particular interest.

Colony-stimulating factor 1 (CSF-1), a cytokine secreted by tumors can recruit macrophages and support tumorigenesis by enhancing angiogenesis and metastases via the secretion of metalloproteinases and inhibiting antitumor immunity by secreting immunosuppressive cytokines.[32] CSF-1 and IGF2BP2 can both promote the malignancy of various tumors. However, the interaction between them is unclear.

In this study, we found a circular RNA named hsa_circ_0007292 (also known as circATP5C1) generated from the exons 3–8 of ATP5C1 gene in BC tissues from GEO DataSets, which might serve as a prognosis-related biomarker in BC. We also discovered that circATP5C1 can facilitate the proliferation potential and influence the process of epithelial-to-mesenchymal transition (EMT) to promote the migration and invasion in TNBC cells. Mechanistically, circATP5C1 accelerates the progression of TNBC by interacting with an m6A reader protein insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP2) leading to improve the stability of the downstream gene Colony Stimulating Factor 1 (CSF-1). Collectively, our findings revealed a novel potential target for the treatment and prognosis prediction in patients with TNBC and discovered IGF2BP2/CSF-1, a pathway regulated by circRNA.

Results

CircATP5C1 was identified in BC

We downloaded GSE182471, a circRNA microarray for five pairs of BC tissue samples and adjacent non-tumor samples obtained from five BC patients on GEO DataSets. Microarray analysis using GEO2R and the differentially expressed genes between BC samples and adjacent non-tumor samples were based on the threshold ($|\log2FoldChange| > 2$ and $p < .05$) (Figure 1a). From the differential genes, we noticed circATP5C1 was upregulated in BC tissues ($\log2FoldChange = 2.43$ and $p < .001$) compared to normal tissues. Based on circBase database, we found circATP5C1 is derived from the exons 3–8 of ATP5C1 gene located on chromosome 10 (p14)

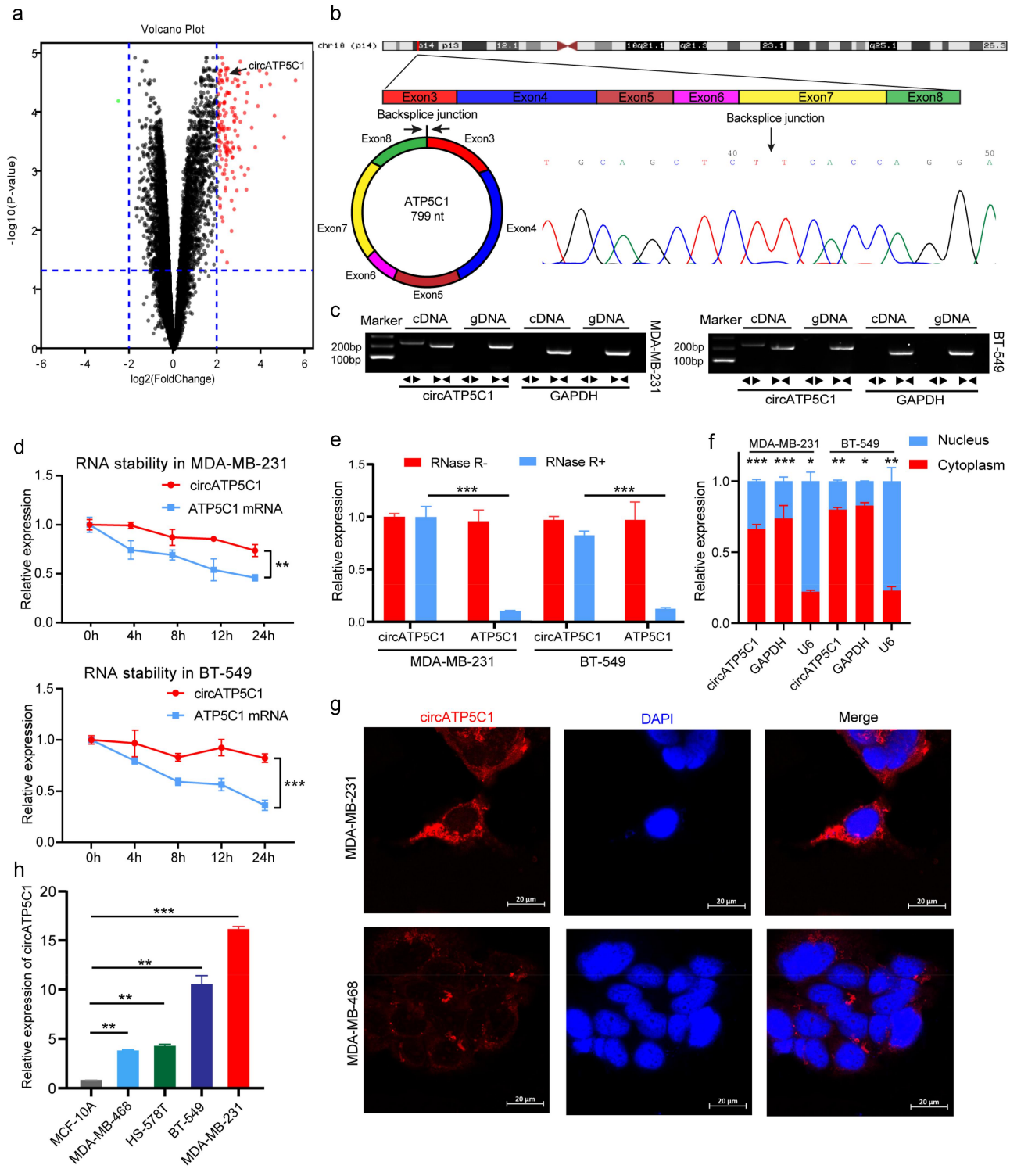

Figure 1. Characteristics of circATP5C1 in breast cancer (BC). (a) volcano plot showed differentially expressed circRNAs in BC tissues compared with adjacent normal tissues. The red represent the upregulated circRNAs based on the threshold (log2FoldChange >2 and $p < .05$). (b) The genomic structure of circATP5C1 and its backsplice junction site was shown in Sanger sequencing. (c) The expression of circATP5C1 in MDA-MB-231 and BT-549 cells was verified by real-time PCR (RT-PCR). (d) RNA stability of circATP5C1 and ATP5C1 was examined by quantitative real-time PCR (rt-qPCR) after treated with actinomycin D (2 μg/ml). (e) Levels of circATP5C1 and ATP5C1 was detected by rt-qPCR after RNase R treatment. (f) Abundance of circATP5C1 in MDA-MB-231 and BT-549 cells was evaluated by rt-qPCR in cytoplasmic and nuclear mRNA fractionation experiment. (g) RNA fluorescence in situ hybridization (FISH) assay showed the subcellular localization of circATP5C1 in breast cancer cells. (h) CircATP5C1 is up-regulated in breast cancer cell lines compared with normal breast epithelial cells MCF-10A. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

with a length of 799nt. We used divergent primers to amplify the backsplice junction site of circATP5C1 and detected its products by Sanger sequencing (Figure 1b). CircATP5C1 could only be amplified by divergent primers instead of convergent primers. We could only get amplification products of circATP5C1 when cDNA but not gDNA was used as the template (Figure 1c). After treatment with Actinomycin D in MDA-MB-231 and BT-549 cells, RT-qPCR analysis

demonstrated that circATP5C1 was more stable than ATP5C1 mRNA (Figure 1d). Additionally, circATP5C1 was resistant to RNase R exonuclease treatment relative to linear ATP5C1 gene which confirmed that circATP5C1 has a closed loop structure (Figure 1e). Subsequently, we detected the subcellular location of circATP5C1 by nuclear/cytoplasmic fractionation assay (Figure 1f) and fluorescence in situ hybridization (FISH) assay (Figure 1g). These results showed that circATP5C1 is predominantly localized in cytoplasm.

Next, we evaluated the relative expression of circATP5C1 by RT-qPCR in four different TNBC cell lines and normal breast epithelial cells MCF-10A. The results exhibited that the expression of circATP5C1 is relatively high in MDA-MB -231 and BT-549 cells but low in MDA-MB-468 and HS-578T cells. Besides, the expression of circATP5C1 is obviously higher in breast cancer cell lines compared with normal breast epithelial cells MCF-10A (Figure 1h). These findings indicate that circATP5C1 is a stable circRNA transcript and has a certain level of expression in TNBC cells.

The high expression of circATP5C1 indicates poor prognosis of patients with TNBC

To investigate the relationship between circATP5C1 expression and prognosis in TNBC patients, we initially conducted FISH assay on tissue microarray to assess the expression levels of circATP5C1 in 127 BC tissues. Out of 127 BC tissues, there are 25 TNBC tissues (Figure 2a). We explored the association between features of BC patients and the expression of circATP5C1. The results showed that patients with higher T stage, lymph node metastasis exhibited higher expression of circATP5C1 (Figure 2b, Table S2). Combined with the follow-up data of the BC patients, we used Kaplan – Meier analysis and Cox regression model to find out the relationship between circATP5C1 expression and prognosis in BC patients. It revealed that BC patients with higher expression of circATP5C1 had a shorter overall survival and disease-free survival (Figure 2d). We also found that high circATP5C1 expression level was an independent prognostic factor of BC patients (Table S3–4). In addition, we screened out 25 TNBC patients from the total BC patients and analyzed the corresponding indicators. We noticed that those 25 patients with higher T stage or lymph node metastasis also exhibited higher expression of circATP5C1 (Figure 2c). Among the 25 TNBC patients, those with higher circATP5C1 expression also had shorter overall and disease-free survival (Figure 2e).

The findings indicate that high level of circATP5C1 in TNBC indicates poor prognosis and may be associated with progression in TNBC, thus promoting us to further explore the biological function of circATP5C1 in TNBC.

CircATP5C1 promotes TNBC cells progression

To find out the role of circATP5C1 in TNBC, we constructed and transfected circATP5C1 overexpression plasmid in MDA-MB-468 and HS-578T cells for the expression level of the target circRNA is relatively low in those two cell lines and examined the transfection efficiency. The results showed that the circATP5C1 overexpression plasmid did not change the mRNA level of the ATP5C1 gene which indicated that ATP5C1 cannot be affected by circATP5C1 (Fig. SA). Next, in vitro studies including CCK8 assay (Figure 3a) and colony formation assay (Figure 3c) showed that the proliferation ability of TNBC cells was significantly improved by circATP5C1 overexpression. Besides, the results of transwell (Figure 3e) and wound healing (Figure 3g) assays manifested that circATP5C1 overexpression facilitated the migration and invasion of TNBC cells. Furthermore, we designed specific siRNA targeting the backsplice junction region of circATP5C1 and transfected si-circATP5C1 into MDA-MB -231 and BT-549 cells for the level of circATP5C1 expression is comparatively high in the cell lines mentioned above (Fig. SB). We examined the knockdown efficiency by RT-qPCR. The results showed that the level of circATP5C1 expression decreased significantly by si-circATP5C1, however the level of ATP5C1 mRNA expression was not changed (Fig. SC). From the results of the CCK8 assay (Figure 3b) and colony formation assay (Figure 3d) we found that knock down of circATP5C1 effectively suppressed the proliferation abilities of TNBC cells. Besides, the migration and invasion ability of the treated cells were examined by transwell (Figure 3f) and wound healing (Figure 3h) assays. It revealed that knockdown of circATP5C1 significantly inhibit the migration and invasion ability of the TNBC cells. Consistent with in vitro experiment, xenograft experiments showed that the proliferation of MDA-MB-231 cells and Ki-67 levels were suppressed by circATP5C1 knockdown (Figure 3i–l).

The results of the in vitro and in vivo experiments added new evidence to the notion that circATP5C1 may stimulate the pathogenesis and progression of TNBC.

CSF-1 is a downstream target gene of circATP5C1

To know the downstream-signaling pathways and target genes regulated by circATP5C1, we conducted RNA-seq analyses on MDA-MB-231 cells (si-NC vs si-circATP5C1). Four hundred and forty-two genes were changed after knockdown of circATP5C1, of which 108 genes were up-regulated and 334 genes showed obviously down-regulated in si-circATP5C1 cells compared with si-NC cells ($|\log_2\text{Fold Change}|>1$, $p < .05$) (Figure 4a). Gene ontology biological processes (GO_BP) (Figure 4b) and KEGG (Figure 4c) pathways enrichment analysis revealed that circATP5C1 was involved in regulation of plasma membrane and cytokine–cytokine receptor interaction. Among the genes likely to be regulated by circATP5C1, we selected some genes encoding cytokines or cytokine receptors that were most significantly altered according to RNA-seq, to be validated by RT-qPCR (Figure 4d). Among the representative genes, we noticed that colony stimulating factor 1 (CSF-1) was one of the most significantly changed genes. CircATP5C1 knockdown reduced the expression of CSF-1, while circATP5C1 overexpression promoted the expression of CSF-1 (Figure 4d).

Colony stimulating factor 1 also known as macrophage colony stimulating factor (M-CSF), is a secreted cytokine that can differentiate hematopoietic stem cells into macrophages or other related cell types.[33] For BC patients, the expression of both CSF-1 and its receptor in BC cells is significantly related with poor

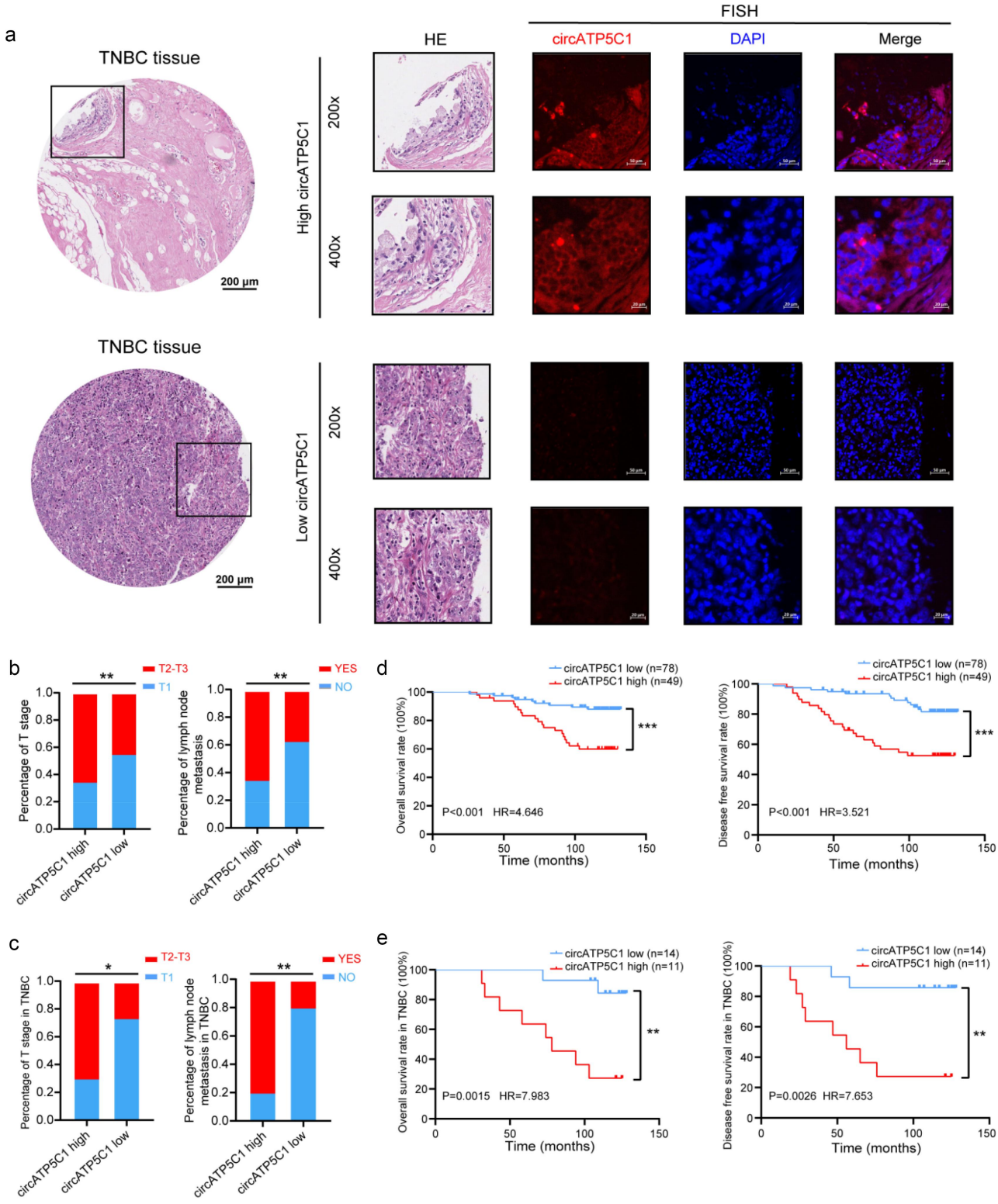

Figure 2. Higher expression level of circATP5C1 in breast cancer tissue is correlated with poor prognosis. (a) the representation pictures of circATP5C1 expression in 25 triple-negative breast cancer (TNBC) tissues in RNA FISH assay by tissue microarray. (b, c) the percentage of T stage and lymph node metastasis in BC tissues (b) and TNBC tissues (c). (d, e) Kaplan-Meier analysis showed an association between circATP5C1 expression and overall survival as well as disease-free survival in breast cancer patients ($n = 127$, D) or triple-negative breast cancer (TNBC) patients ($n = 25$, E). *$p < .05$, **$p < .01$.

prognosis.[34] ELISA assays in TNBC cell culture supernatants indicated that circATP5C1 knockdown suppressed the secretion of CSF-1, while circATP5C1 overexpression enhanced the secretion of CSF-1 (Figure 4E). Recent studies showed that CSF-1/CSF-1 receptor (CSF-1 R) signaling could improve invasiveness and had an impact on epithelial-to-mesenchymal transition (EMT) in BC cells.[35] Thus, we performed western blot assay to quantify protein levels of epithelial marker E-cadherin with mesenchymal markers N-cadherin and vimentin in TNBC cell lines. The results revealed that after circATP5C1 overexpressed,

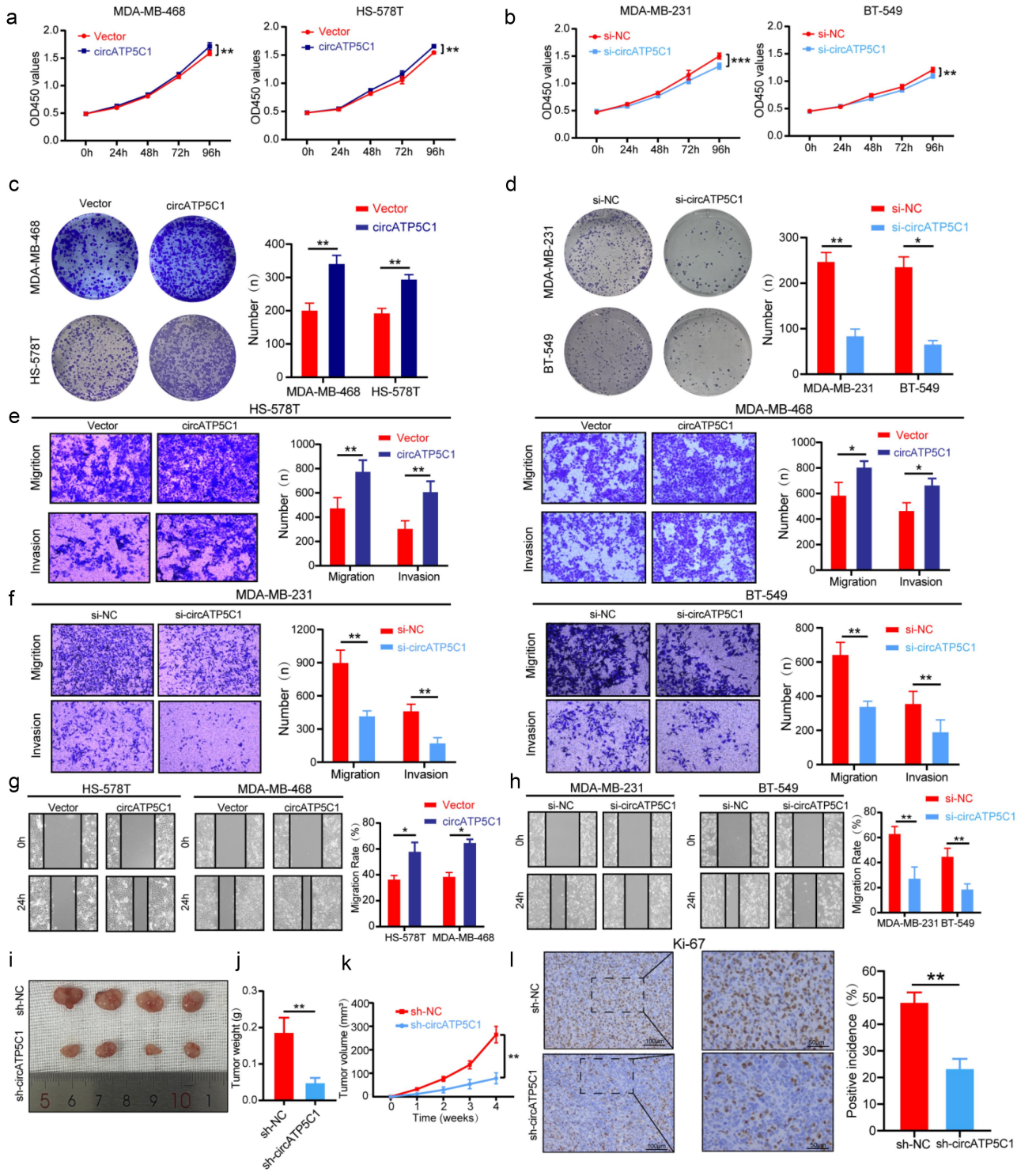

Figure 3. CircATP5C1 accelerates progression of TNBC cells. (a, b) CCK8 assays showed the proliferation ability of MDA-MB-468 and HS-578T cells with circATP5C1 overexpression (a) as well as MDA-MB-231 and BT-549 cells transfected with si-circATP5C1 (b). (c, d) colony formation assays showed the potential of proliferation of MDA-MB-468 and HS-578T cells treated with circATP5C1 overexpression plasmid (c) along with MDA-MB-231 and BT-549 cells after down-regulation of circATP5C1 (d). (e, f) transwell migration and matrigel invasion assays showed the migration and invasion abilities of MDA-MB-468 and HS-578T cells transfected with circATP5C1 plasmid (e) as well as MDA-MB-231 and BT-549 cells with circATP5C1 knockdown (f). (g, h) the migration ability tested by wound healing experiments in MDA-MB-468 and HS-578T cells transfected with circATP5C1 plasmid (g) as well as MDA-MB-231 and BT-549 cells after treated with si-circATP5C1 (h). (i–l) representative tumor images (i), tumor weights (j), growth curves (k), and ki-67 immunohistochemistry (IHC) staining (l) of subcutaneous tumors in the xenograft model of MDA-MB-231 cells treated with sh-nc or sh-circATP5C1 ($n = 4$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

levels of mesenchymal markers including N-cadherin and vimentin rose, the expression of E-cadherin had a reduction. On the contrary, knockdown of circATP5C1 downregulated the expression of N-cadherin and vimentin but elevated the expression level of E-cadherin (Figure 4f). Previous studies demonstrated the expression of CSF-1 in BC cells and revealed the association of CSF-1/CSF-1 R signaling in the invasiveness, proliferation and apoptosis resistance of BC cells.[36–38] Next, we

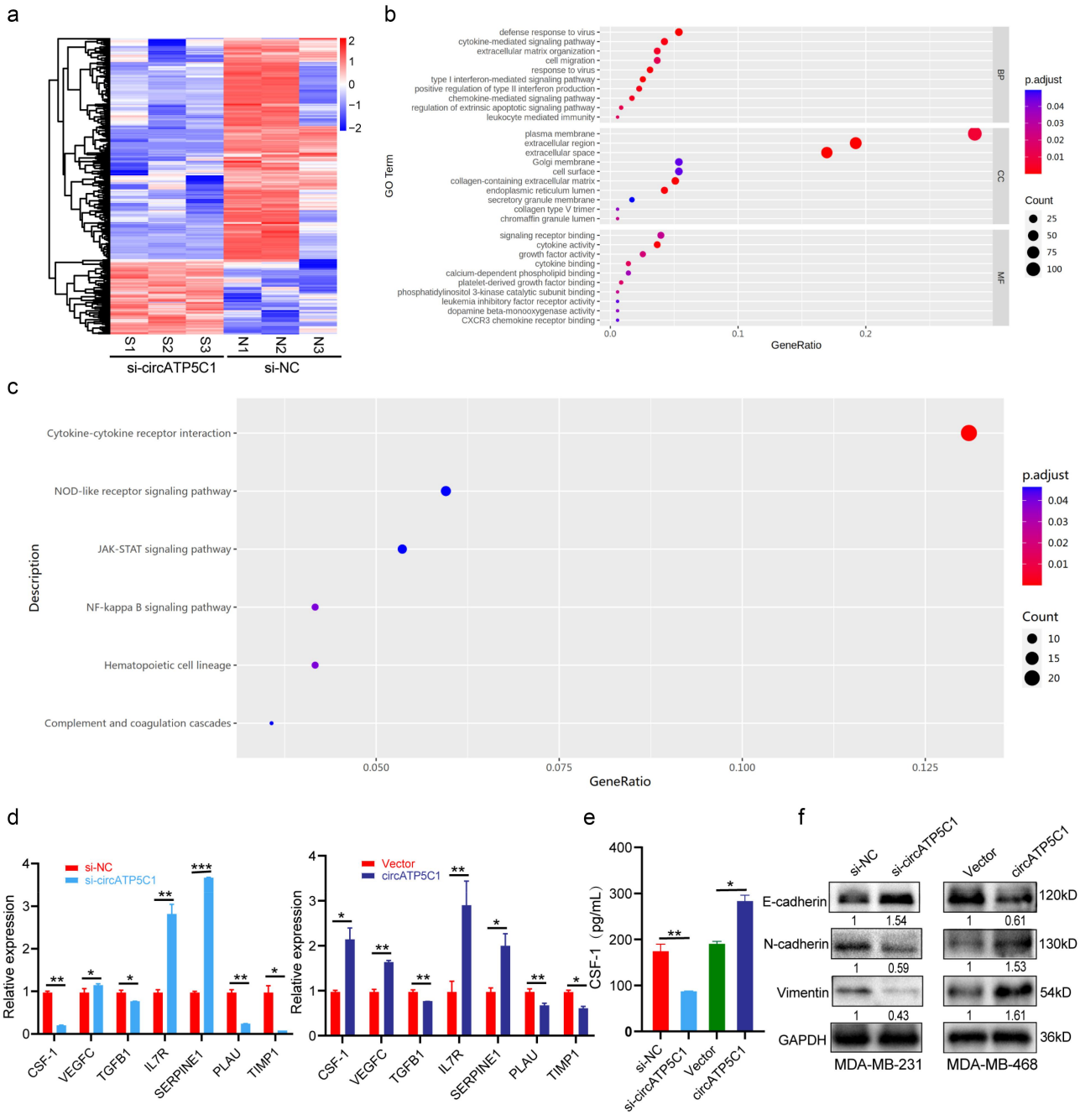

Figure 4. CSF-1 is a functional downstream gene regulated by circATP5C1. (a) Heatmap showed 484 differentially expressed genes between si-nc and si-circATP5C1 groups with the criteria of up-regulation or down-regulation fold change value > 2 and the *p* value < 0.05. (b, c) enrichment analysis for representative GO_BP (b) and KEGG pathways (c) in circATP5C1 target genes. (d) The mRNA levels tested by rt-qPCR of representative genes in MDA-MB-231 cells transfected with si-circATP5C1 (left panel) and in MDA-MB-468 cells after circATP5C1 was up-regulated (right panel). (e) Enzyme-linked-immunosorbent serologic assay (ELISA) measured the secretion levels of colony stimulating factor 1 (CSF-1) in the supernatants of MDA-MB-231 with circATP5C1 knockdown and MDA-MB-468 with circATP5C1 overexpression. (f) Western blot assays examined the protein levels of E-cadherin, N-cadherin and vimentin in MDA-MB-231 cells with circATP5C1 knockdown and MDA-MB-468 cells with circATP5C1 overexpression. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

performed rescue experiments to explore whether the biological functions of circATP5C1 on TNBC cells were caused by the secretion of CSF-1. CCK8 (Figure 5b) and colony formation assays (Figure 5b) indicated that the proliferation of the TNBC cells inhibited by si-circATP5C1 partly reversed by rhCSF-1. Besides, addition of rhCSF-1 partly reversed the suppression of migration and invasion potential in TNBC cells transfected with si-circATP5C1 (Figure 5c,d). Moreover, rhCSF-1 significantly rescued the expression of E-cadherin, N-cadherin and vimentin induced by si-circATP5C1 (Figure 5e). In summary, the results revealed that the effect on pathogenesis and progression of TNBC inhibited by circATP5C1 knockdown partly due to the reduced secretion of CSF-1.

CircATP5C1 influences the stability of CSF-1 mRNA by interacting with IGF2BP2

Subsequently, we commenced exploring the mechanism about how circATP5C1 regulates the secretion of CSF-1. Predictions on websites showed that there are few binding sites of miRNA

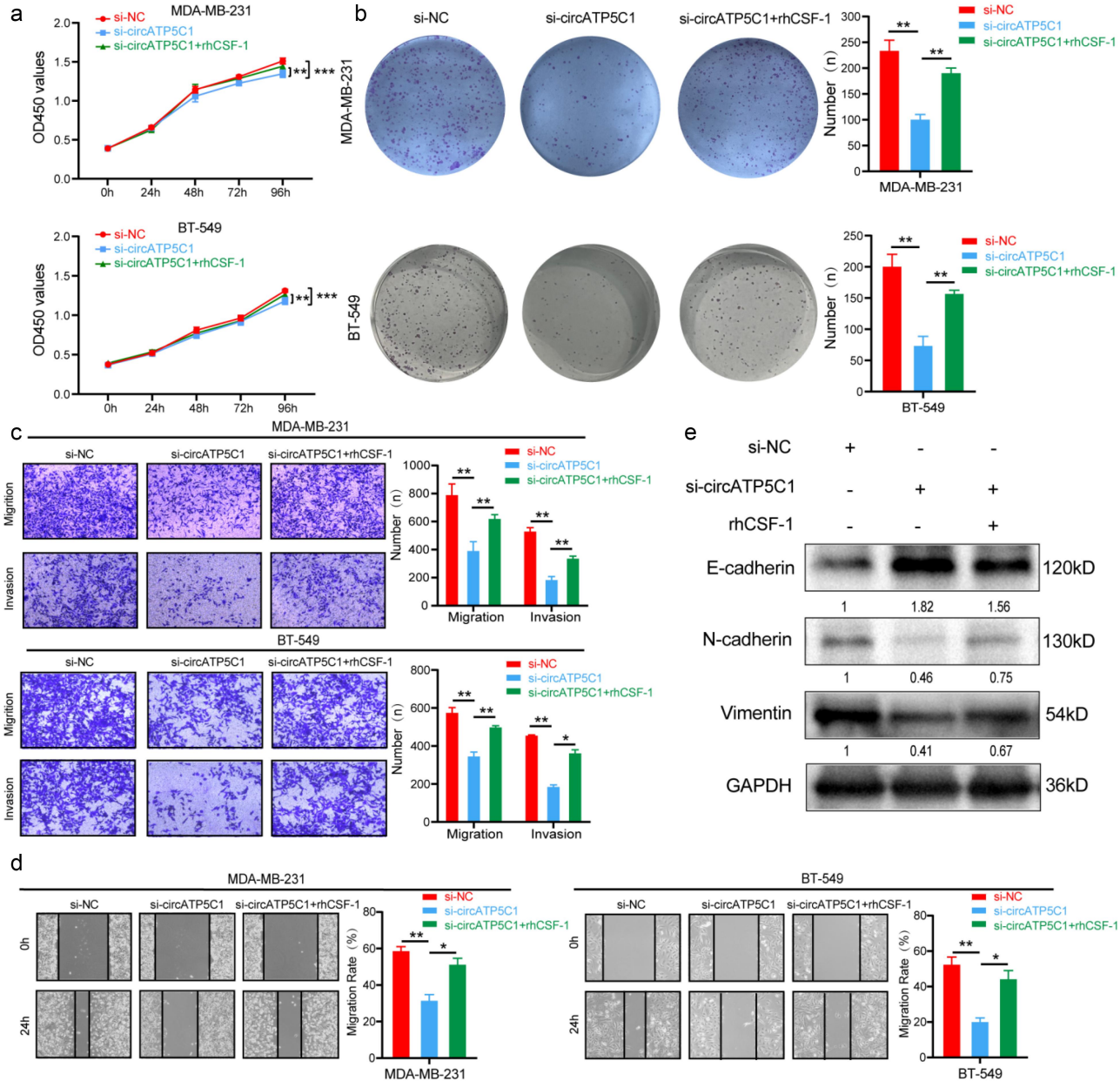

Figure 5. CSF-1 reverses the impairment of proliferation, migration and invasion in TNBC cells caused by si-circATP5C1. (a, b) the addition of recombinant human colony stimulating factor 1 (rhCSF-1) partly rescued the restraint of cell proliferation in MDA-MB-231 and BT-549 cells via circATP5C1 knockdown in CCK8 assays (a) and colony formation assays (b). (c, d) the effects of si-circATP5C1 on migration and invasion potential were partially reversed by adding to rhCSF-1 in MDA-MB-231 and BT-549 cells. (e) Protein levels showed that si-circATP5C1-induced changes in E-cadherin, N-cadherin and vimentin in MDA-MB-231 cells were partially reversed by the addition of rhCSF-1. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

targets of circATP5C1. So, we suspected that circATP5C1 may not act as a miRNA sponge in TNBC progression. To explore whether circATP5C1 played a role by interacting with proteins, we performed RNA pull-down assay. SDS-PAGE was used to separate the proteins from RNA pull-down assay. After silver staining (Figure 6a), we resected the bands and analyzed them by mass spectrometry. From the results, we found a major differential band precipitated in lysates was identified to be the IGF2BP2 protein and we validated it via western blot (Figure 6b,c). Next, we conducted RNA FISH immunofluorescence analysis and it confirmed the colocalization of endogenously expressed circATP5C1 and IGF2BP2 in the cytoplasm of TNBC (Figure 6d). RNA immunoprecipitation (RIP) assays also showed that circATP5C1 could bind with IGF2BP2 protein (Figure 6e). Additionally, we validated that IGF2BP2 protein could have an interaction with CSF-1 mRNA by performing RIP assay. Moreover, the alteration of the expression of circATP5C1 correspondingly effected the interaction between IGF2BP2 and CSF-1 (Figure 6f). To further elucidate whether the interaction between circATP5C1 and IGF2BP2 protein had an impact on the expression of CSF-1, we conducted rescue experiments. As we expected, knockdown of IGF2BP2 reduced the mRNA and protein levels of CSF-1 in TNBC cells. We also found that the rise of CSF-1 caused by circATP5C1 overexpression can be partly reversed by knockdown of IGF2BP2 (Figure 6g).

From the previous studies, IGF2BP2 has been regarded as an m6A reader regulating mRNA stability and binds to the

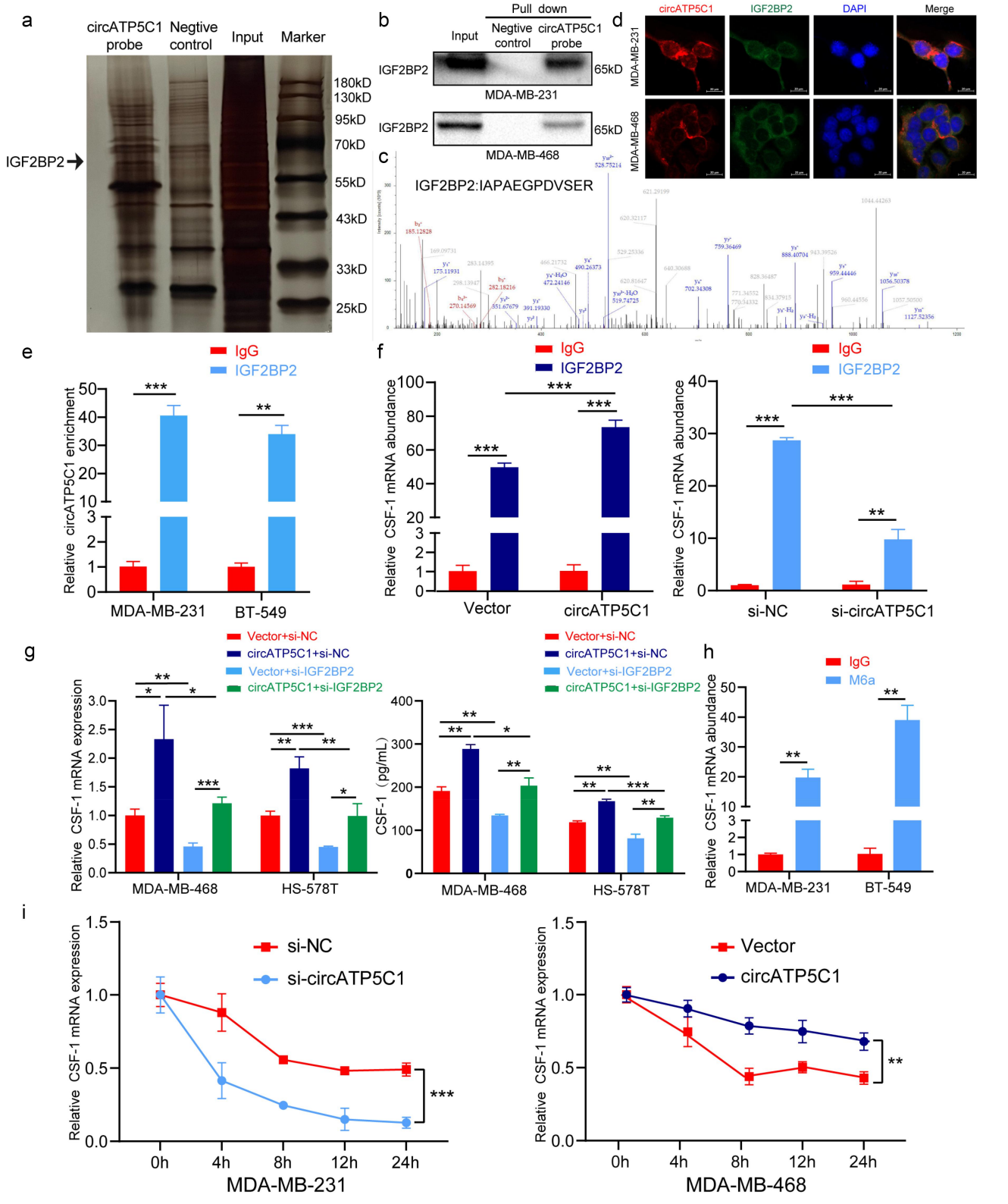

Figure 6. circATP5C1 increases the stability of CSF-1 mRNA through interaction with insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP2). (a) RNA pull-down assay and silver staining were conducted to visualize the possible binding proteins in circATP5C1 probe. (b) RNA pull-down-western blot showed IGF2BP2 could bind with circATP5C1. (c) The specific peptide map for IGF2BP2 identified by mass spectrometry. (d) RNA fish-immunofluorescence in MDA-MB-231 and MDA-MB-468 cells exhibited the colocalization of circATP5C1 with IGF2BP2. (e) RNA immunoprecipitation (RIP) assays validate binding of circATP5C1 to IGF2BP2 protein. (f) RIP assays identified the interaction of IGF2BP2 protein with CSF-1 mRNA. (g) Knockdown of IGF2BP2 rescued the mRNA and protein levels of CSF-1 caused by circATP5C1 overexpression in MDA-MB-468 and HS-578T cells. (h) MeRIP assays validated CSF-1 mRNA contains m6A methylation. (i) Stability of CSF-1 mRNA was detected by rt-qPCR after actinomycin D treatment in transfected MDA-MB-231 and MDA-MB-468 cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

3'UTRs of target mRNAs.[39,40] From the m6A prediction analysis of CSF-1 on SRAMP (http://www.cuilab.cn/sramp/.),[41] we noticed abundant m6A modification sites in CSF-1 (Fig. SD). We hypothesized that IGF2BP2 exerted its role of m6A reader to facilitate the stability of CSF-1 mRNA. Thus, we conducted methylated RNA immunoprecipitation (MeRIP) assay to see whether CSF-1 contains m6A methylation. The RT-qPCR results revealed that the relative enrichment of m6A in CSF-1 group was significantly higher than IgG control group (Figure 6h). Next, we explored whether circATP5C1 could modulate mRNA stability of its downstream gene CSF-1 due to its interaction with IGF2BP2 protein. We found from the predictive website StarBase (https://rnasysu.com/encori/.)[42] that IGF2BP2 had binding sites with 3'UTR of CSF-1 (Fig. SE). Therefore, we performed Actinomycin D assay to test whether the mRNA stability of CSF-1 was influenced by the expression of circATP5C1. The results indicated that upregulation of circATP5C1 significantly improved the stability of CSF-1 mRNA. Conversely, the decay rates of CSF-1 were accelerated after circATP5C1 knockdown (Figure 6i). Collectively, these data suggested that circATP5C1 can upregulate the expression of CSF-1 by enhancing the stability of its mRNA level via interacting with m6A reader protein IGF2BP2.

Consequently, we performed a series of rescue experiments in vitro and the findings suggested that the malignant progression of TNBC facilitated by circATP5C1 overexpression was reversed by abrogation of IGF2BP2 (Figure 7a–d). Moreover, we also confirmed that the promotion of EMT in TNBC caused by circATP5C1 overexpression were rescued by knockdown of IGF2BP2 (Figure 7e).

The results mentioned above indicate that IGF2BP2-induced CSF-1 secretion probably play a critical role in effects mediated by circATP5C1 on TNBC progression.

Discussion

CircRNAs possess high tissue-specificity, conservatism and stability.[8] Large amount of circRNAs are regarded as oncogenes and are related to the occurrence of various cancers including BC.[43,44] CircRNAs play a role in miRNA sponges have been reported extensively in BC.[15] However, emerging studies showed that circRNA-RNA binding proteins (RBPs) interactions also play a critical role in the progression of BC cells.[45–48] Nowadays, many scientific reports find that m6A is widely involved in tumor occurrence and development through modulating tumor metabolism.[49] Nevertheless, the biological mechanism of circRNA-RBP interactions in the progression of BC especially TNBC via m6A modification is not clear to a great extent. In this article, we revealed a potential role and regulatory mechanism of circATP5C1 in promoting progression of TNBC via IGF2BP2/CSF-1 signaling.

In the beginning we found that TNBC patients with high levels of circATP5C1 expression were inclined to get poor prognoses in overall survival and disease-free survival which showed that circATP5C1 may exert promoting function in malignance of TNBC. Consequently, we performed in vitro experiments to demonstrate that circATP5C1 promoted the proliferation, migration along with invasiveness in TNBC cells.

To further explore the downstream target genes and signaling pathways regulated by circATP5C1, we conducted RNA-seq analyses. After a series of validations, we revealed that CSF-1 is a vital downstream target gene of circATP5C1. CSF-1 can be produced by many stromal cells and its chief role is to be a hematopoietic growth factor contributing to the survival, proliferation, differentiation and motility of cells of the monocyte lineage. However, it has been demonstrated CSF-1 and CSF-1 R not only exist in stromal cells but also in several human malignancies including breast, cervical, ovarian, lung, prostate and kidney cancer.[34] Thus, CSF-1 R can either be stimulated by the secretion of CSF-1 from cancer-associated fibroblasts in a paracrine manner or be agitated in an autocrine manner which means the activating ligand is secreted by cancer cell itself. Among epithelial cancers, the role of CSF-1/CSF-1 R has been most comprehensively explored in BC. CSF-1 has tremendous impact on the invasiveness and proliferation in BC cells[36,37] and plays a part in epithelial-to-mesenchymal transition (EMT) of BC cells.[35] After validating that the expression of CSF-1 can be regulated by circATP5C1, we performed a series of in vitro studies to show that CSF-1 played a vital role in facilitating progression of TNBC cells caused by circATP5C1. We also found that circATP5C1 could alter EMT partly due to the secretion of CSF-1.

CircRNAs exert their cellular functions depending on their locations. To further detect the cellular functions of circATP5C1, we performed FISH assay to have a view on the location of circATP5C1 in TNBC cells. It revealed that most of circATP5C1 lied in cytoplasm. CircRNAs located in the cytoplasm could bind with miRNAs to play a role in competing endogenous RNAs[48,50,51] and can also interact with RBPs.[45,52–54] Some cytoplasmic circRNAs can even translate novel polypeptides or proteins if essential elements are available.[52–54] There is extensive evidence that circRNAs function as miRNA sponges in BC.[55] As for circATP5C1, there are few binding sites of miRNA thus it may take effect in other manners. There are emerging evidence indicating that circRNAs could interact with RBPs to exert biological functions.[7,56] Therefore, we considered circATP5C1 might bind with proteins to govern tumor behaviors. After RNA pull-down assay, we found proteins interacting with circATP5C1 contained an m6A reader protein named IGF2BP2 which can promote the stability of mRNAs.

It is reported that IGF2BP2 is not only connected with diabetes or insulin resistance but also associated with cancers.[57] Additionally, IGF2BP2 was recently suggested as a potential biomarker predicting prognosis in several tumors.[58] It was reported that IGF2BP2 can regulate TGF-β-SMAD signaling family in an m6A-dependent manner to suppress proliferation and promote metastasis of clear cell renal cell carcinoma.[59] IGF2BP2 can also promote acute myeloid leukemia (AML) development and self-renewal of leukemia stem/initiation cells by regulating expression of critical targets (e.g., MYC, GPT2, and SLC1A5) in the glutamine metabolism pathways in an m6A-dependent manner.[60] Additionally, IGF2BP2 can improve the RNA stability of FLT4 through m6A modification, thereby activating the PI3K-Akt signaling pathway, and eventually promoting angiogenesis and metastasis in lung adenocarcinoma.[61] An increasing

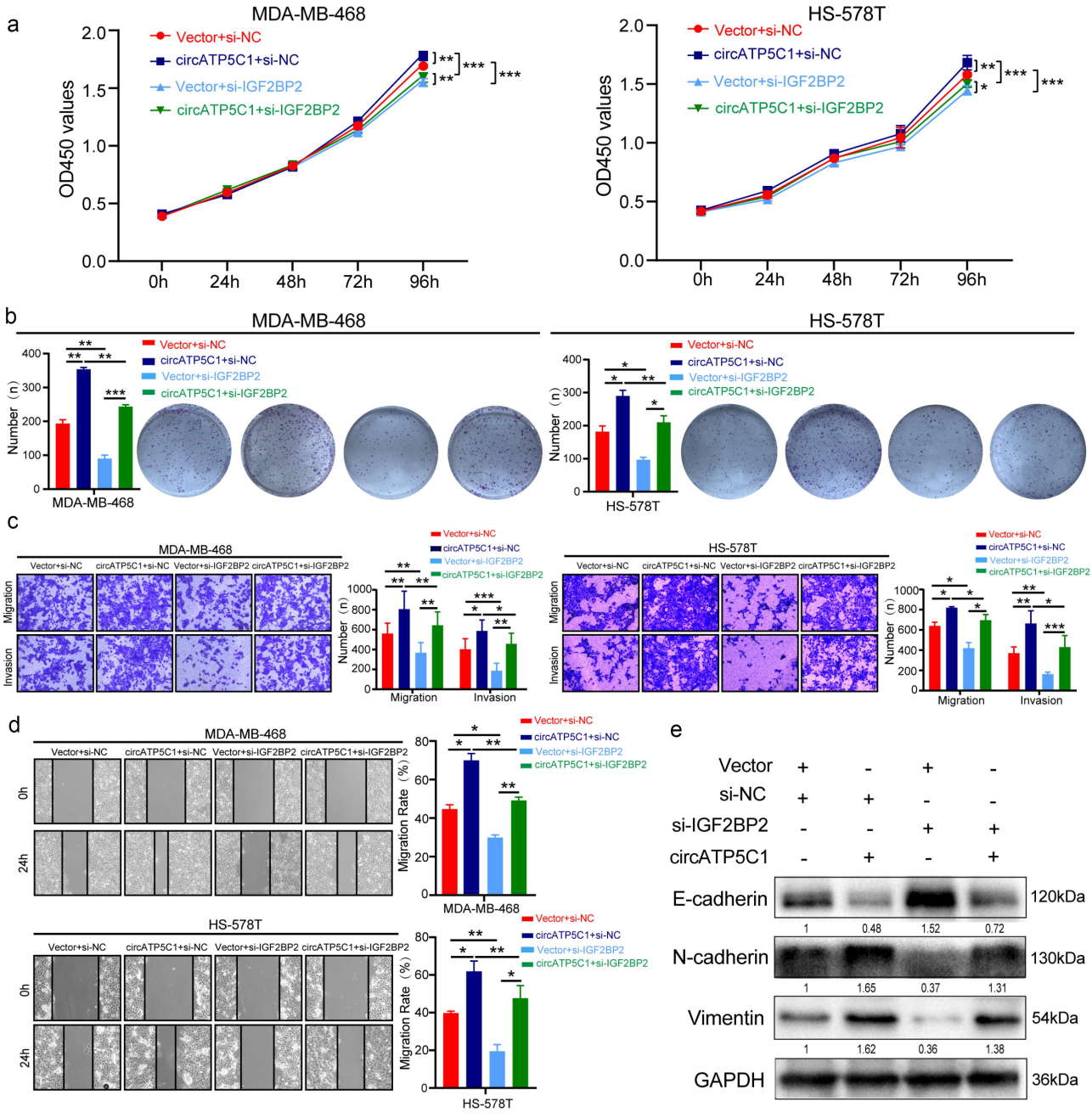

Figure 7. circATP5C1 interacts with IGF2BP2 and enhances progression of TNBC cells in vitro. (a, b) CCK-8 assays (a) and colony formation assays (b) were used to assess the proliferation potential of TNBC cells with circATP5C1 overexpression or IGF2BP2 knockdown. (c, d) the migration and invasion abilities of TNBC cells with circATP5C1 overexpression or IGF2BP2 knockdown were tested by transwell migration and matrigel invasion assays (c) and wound healing experiments (d). (e) Western blot assays showed that si-IGF2BP2 could partially rescue the changes in protein levels of E-cadherin, N-cadherin and vimentin caused by circATP5C1 overexpression in MDA-MB -468 cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

number of evidence indicates IGF2BP2 can be involved in several types of RNAs, including circRNAs, and have an impact on tumor progression. For example, Li et al. showed that circNhibits NSCLC progression by binding to and destabilizing IGF2BP2, thereby activating anti-tumor immunity.[62] CircARHGAP29 increased the stability of lactate dehydrogenase A (LDHA) mRNA by interacting with IGF2BP2, causing enhanced cell proliferation, docetaxel resistance, and glucose metabolism in prostate cancer.[63] By means of binding with IGF2BP2 to stabilize HMGA2 mRNA, circNSUN2 promotes aggressiveness of colorectal carcinoma cell.[64] There exists increasing evidence of circRNAs/IGF2BP2 complex in epithelial carcinomas but few studies were focused on BC especially in TNBC. Herein, we performed RNA pull-down assay, RIP assay as well as RNA FISH-immunofluorescence assay to confirm that circATP5C1 can interact with IGF2BP2. Consistent with the mechanism reported previously, the mRNA stability of the downstream gene of circATP5C1 had a significant elevation by binding with IGF2BP2. Therefore, it was convinced that circATP5C1 could accelerate malignant progression of TNBC by interacting with IGF2BP2 and promotes the secretion of CSF-1. Our study supplied the evidence of circRNAs/

IGF2BP2 complex in facilitating progression of TNBC and it is the first time that the circular RNA circATP5C1 reported in cancer.

It is reported that a potential therapeutic approach is to use specific siRNA to knock down oncogenic circRNAs in tumors.[65] As an oncogene, we hope to see siRNA specifically targeting circATP5C1 can exert therapeutic effects. However, siRNA should target the unique backsplice junction sequence of oncogenic circRNA for it is essential to avoid interfering with the host genes.

For the reasons of limited conditions, this study was unable to detect the presence of circATP5C1 in blood or other body fluids. Therefore, we could not infer whether this molecule has ability to become a rapid and noninvasive diagnostic indicator. Nevertheless, we would like to see combination therapy using circATP5C1 inhibitors with other conventional treatments to bring benefits to patients.

Conclusion

In this article, we identified a new circRNA named circATP5C1. The expression of circATP5C1 was negatively correlated with prognosis in patients with TNBC, indicating it may serve as a novel biomarker for TNBC. After Gain- and loss-of-function experiments, we found circATP5C1 was of vital importance in facilitating malignance in TNBC cells. Mechanistically, circATP5C1 can promote the secretion of CSF-1 via interacting with IGF2BP2 protein. Our study revealed the circATP5C1/IGF2BP2/CSF-1 pathway, which may supply a new perspective for judging prognosis and therapy of TNBC.

Materials and methods

Patients and specimens

The relationship between circATP5C1 expression and prognosis in BC patients was analyzed using HBreD130Su09 tissue microarray purchased from Outdo Biotech Company (Shanghai, China). The clinicopathological characteristics and survival status of the studied BC patients are available from the follow-up data. All the clinical samples were obtained after getting proper consents and ethical approval (License No. SHYJS-CP-1701016) from Ethics Committee of Shanghai Outdo Biotech Company.

Cell culture, drug treatment and transfection

Normal human mammary epithelial cells MCF-10A and four kinds of human TNBC cells including MDA-MB-231, MDA-MB-468, BT-549 and HS-578T were purchased from Procell Life Science&Technology Co., Ltd. (Hubei, China). All cell lines are routinely used for mycoplasma contamination. In addition, all cell lines were authenticated by STR profiling. MCF-10A was cultured in DMEM (GIBCO, USA) with 5% horse serum (HS), 20 ng/mL Epidermal Growth Factor (EGF), 0.5 μg/mL Hydrocortisone, 10 μg/mL Insulin, 1% Non-Essential Amino Acids (NEAA) and 100 U/ml of penicillin, 100 μg/ml streptomycin. MDA-MB-231, HS-578T were cultured with DMEM medium (GIBCO, USA), MDA-MB-468 was cultured with Leibovitz's L-15 medium (GIBCO, USA), BT-549 was cultured with RPMI1640 medium (GIBCO, USA). Four kinds of TNBC cells were all cultured with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml streptomycin. All cells above were maintained at 37°C in a humidified atmosphere of 5% CO2. For drug treatment, MDA-MB-231 was cultured in DMEM, BT-549 was cultured in RPMI1640, without serum for 24 h and then for additional 24 h with medium mentioned above with or without 25 ng/ml recombinant human colony stimulating factor 1 (rhCSF-1) (MedChemExpress, USA). For transfection, siRNAs were purchased from RiboBio Co., Ltd. (Guangzhou, China). The circATP5C1 sequence was cloned into the pCD5-ciR vector and synthesized by Geneseed Biotech Co. (Guangzhou, China) to get the overexpression plasmid. According to the manufacturer's instructions, TNBC cells inoculated in 6-well cell plates were transfected with pCD5-ciR vector by using PolyFect transfection Reagent (Promega, USA). SiRNAs were transfected by HiperFect transfection Reagent (Promega, USA) for 6 h. After that, the complete medium was changed into the medium without transfection. The cells were cultivated for a period of time and then collected.

mRNA sequencing

Total RNA was extracted in triplicate from both the negative control MDA-MB-231 cells and MDA-MB-231 cells with circATP5C1 knockdown. RNA quality measuring and transcriptome analysis were conducted by Annoroad Gene Technology Co., Ltd. (Beijing, China). We identified and selected differentially expressed genes with $|\log2FoldChange| > 1$ and $p < .05$ for subsequent validation.

Genomic DNA (gDNA)/RNA extraction, RNase R treatment and nuclear-cytoplasmic fractionation

The gDNA from TNBC cells were extracted by the gDNA extraction kit (TIANGEN, China). Total RNA from cells were extracted by the TRIzol reagent (Invitrogen, USA). Total RNA was incubated with 3 U/mg of RNase R in 37°C for 15 min (Epicenter, USA). Nuclear and cytoplasmic RNA in MDA-MB-231 and BT-549 cells was isolated by PARIS™ Kit (Life Technologies, Austin, Texas, USA) according to the manufacturer's protocols.

CDNA synthesis, and quantitative real-time PCR assays (rt-qPCR)/RT-PCR

Reverse transcription was performed to synthesize cDNA by the GoScript Reverse Transcription System (Promega, USA). GoTaq qPCR Master Mix (Promega, USA) and GoTaq Green Master Mix were used to perform the amplification process. PCR amplification primers are listed in Table S1. We used U6 nsRNA as internal controls for circRNAs and GAPDH was used as internal controls for mRNAs. We analyzed the relative expression levels by $2^{-\triangle\triangle}$CT method. Two percent agarose gel electrophoresis was used to detect the RT-PCR products. Safe Green (Monad, China) was used to visualize the DNA fragmentation.

Actinomycin D assay

Cells were seeded in six-well plates when growing up to 60% confluence after 24 h. Cells in plates were treated with 2 μg/mL actinomycin D (AdooQ Bioscience, USA) for 4 h, 8 h, 12 h, 24 h and the control group was treated with DMSO (Solarbio, China). After the RNA was extracted, we detected the RNA stability by RT-qPCR.

Fluorescence in situ hybridization (FISH)

FISH in TNBC cells and tumor tissues was used by the Cy3-labeled circATP5C1 probe targeting the back splice site (Guangzhou Geneseed Biotech Co., Ltd., China). The probe sequence was listed in Table S1. MDA-MB-231 and MDA-MB -468 cells were seeded on the coverslips. After the cells were fixed with 4% paraformaldehyde and prehybried for 1 h, the cells were incubated with circATP5C1 probe overnight at 37°C. The BC tissue microarray slides should be dewaxed in xylene and gradient ethanol hydration at first. Coverslips were sealed with parafilm containing DAPI. The images were captured by confocal microscope (LSM 900, Zeiss, Germany).

Cell proliferation and colony formation assays

TNBC cells were seeded in 96-well plates with the density of 3×10^3 cells/well for overnight. The viability of cells was evaluated with Cell Counting Kit-8 (MedChemExpress, USA) kit at 24, 48, 72, and 96 h. The absorbance at 450 nm was detected. For colony formation assays, TNBC cells were seeded in 6-well plates with the density of 1500 cells per well culturing for nearly ten days. Colonies were fixed with 4% paraformaldehyde and stained with crystal violet.

Wound healing assay

Cells of different treatments were inoculated in 6-well plates. When cells reached 80% confluence, a 10 μl sterile pipette was applied to scratch the cell monolayer vertically. The pictures of scratched areas were obtained with an inverted microscope at 0 h and 24 h post scratch wound formation.

Transwell assays and inverted invasion assays

The transwell assays for migration detection were conducted by the transwell chambers (Corning, USA). As for invasion assay, Matrigel (BD biosciences, USA) was spread in the chamber at 37°C overnight in advance. 3×10^4 cells/chamber were seeded in the upper chamber for 24–48 h. The chambers were fixed with 4% paraformaldehyde and stained by crystal violet, three visual fields were randomly observed and photographed under an inverted microscope (DFC295, Leica, Buffalo Grove, United States).

Immunohistochemistry (IHC)

Tissue sections were dewaxed and then treated with xylene. EDTA was used for antigen retrieval and the sections were then treated with 3% hydrogen peroxide. After incubation with primary antibodies and secondary antibodies, the sections were counterstained with diaminobenzidine (DAB) and hematoxylin.

Enzyme-linked-immunosorbent serologic assay (ELISA)

Cell culture medium of different treatments was analyzed for cytokines production using Human M-CSF ELISA kit (Proteintech, China) according to the manufacturer's instructions.

Western blot

TNBC cells were lysed using RIPA or IP protein lysis buffer and incubated on ice for 1 h. Equal amounts of proteins were loaded into a 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). Then, the membranes were sealed by blocking liquid (Seven, China) for 20 min and incubated with antibodies against E-cadherin (1:3000 dilution; proteintech, China), N-cadherin (1:3000 dilution; proteintech, China), Vimentin (1:8000 dilution; proteintech, China) IGF2BP2 (1:3000 dilution; proteintech, China), and GAPDH (1:10000 dilution; proteintech, China) overnight at 4°C. The bands were illuminated using Electrochemiluminescence (ECL) detection kit (Solarbio, China).

RNA fish-immunofluorescence

TNBC cells seeded on the glass cover slips were fixed at room temperature with 4% paraformaldehyde and prehybried for 1 h. Then, the cells were overlaid with the Cy3-labeled circATP5C1 probe overnight at 37°C. After being blocked by 10% BSA for 30 min at 37°C, the cells were incubated with IGF2BP2 antibody (1:200 dilution; proteintech, China) at room temperature for 1 h and Alexa Fluor™ 488-conjugated secondary antibodies (1:200 dilution; proteintech, China) for 1 h at 37°C. Coverslips were sealed with parafilm containing DAPI. The images were captured with the confocal microscope (LSM 900, Zeiss, Germany).

RNA pull-down and silver staining assays

The RNA pull-down probe, designed to specifically target the junction site of circATP5C1, was synthesized by RiboBio (Guangzhou, China) and listed in Table S1. MDA-MB-231 and BT-549 cells were lysed with lysis buffer and incubated with biotin-labeled circATP5C1 probe. The cell lysates were incubated with streptavidin-coated agarose magnetic beads to pull-down the biotin-labeled RNA complex using Pierce™ Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific, Waltham, USA). Then the agarose magnetic beads were collected and RNA-binding proteins were eluted and identified by western blot. Silver staining was performed by the Fast Silver Stain Kit (Beyotime, Haimen, China) as the protocol described, and the mass spectrometry analysis was then performed.

RNA immunoprecipitation (RIP) and methylated RNA immunoprecipitation (MeRIP) assays

RIP assays were performed by the RIP Kit (Guangzhou Geneseed Biotech Co., Ltd., China). 1×10^7 cells were lysed

by lysis buffer within protease and RNase inhibitors for 10 min. The magnetic beads were incubated with 5 μg antibody against IGF2BP2 (proteintech, China) or anti-m6A antibody (proteintech, China), or control IgG (proteintech, China). Then cell lysates were added and incubated overnight at 4°C. The RIP efficiency was examined by Western blot; the enrichment of circRNAs or mRNAs was detected through RT-qPCR analysis.

In vivo studies

The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Fourth Affiliated Hospital of Hebei Medical University (IACUC Approval No. 20240732). All of the BALB/c nude mice were obtained from Hfk (Beijing, China) Bioscience Co., Ltd. Four-week-old female mice (weighing 18–20 g) were randomly assigned to two groups. After an 7-day acclimatization, mice were injected subcutaneously with 3×10^6 MDA-MB-231 cells infected with sh-NC or sh-circATP5C1 lentiviruses. The width and length of the tumors were measured once a week. The diameter of the subcutaneous graft tumors in the nude mice did not exceed 12 mm. After 4 weeks, mice were collected and euthanasia was performed for necropsy. All the experimental mice were sacrificed by cervical dislocation and tumors were harvested and weighed.

Statistical analysis

Statistical analysis was conducted by GraphPad Prism 8 and SPSS 22.0 software. Data are represented with mean ± S.D (Standard Deviation). The comparisons of two groups were computed using student's t test or ANOVA, Fisher's exact test, chi-square test. Survival curves were made by the Kaplan–Meier method. Differences were considered statistically significant when $P < 0.05$.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

Cuizhi Geng http://orcid.org/0000-0001-5541-5412

Data availability statement

The original contributions presented in this study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author(s).

Ethics approval and consent to participate

The present study was approved by the Fourth Hospital of Hebei Medical University. Animal studies were approved by the Institutional Animal Care and Use Committee of Fourth Hospital of Hebei Medical University. The approval number is IACUC-4th Hos Hebmu −20,240,732.

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