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SNHG5 Promotes Breast Cancer Proliferation by Sponging the miR-154-5p/PCNA Axis

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Breast cancer is the most common malignant tumor and the main cause of cancer-associated mortality in females worldwide. Long non-coding RNAs (lncRNAs) have been reported to play vital roles in breast cancer development and progression; however, our understanding of most lncRNAs in breast cancer is still limited. In this study, we demonstrated that small nucleolar RNA host gene 5 (SNHG5) promotes breast cancer cell proliferation both *in vitro* and *in vivo*, and depletion of SNHG5 significantly led to cell-cycle arrest at G1 phase. Accumulating evidence has shown that many lncRNA transcripts could function as competing endogenous RNAs (ceRNAs) by competitively binding common microRNAs (miRNAs). We found that SNHG5 acts as a sponge for miR-154-5p, reducing its ability to repress proliferating cell nuclear antigen (PCNA). SNHG5 promoted breast cancer proliferation and cell-cycle progression by upregulation of PCNA expression. Clinically, we observed an increased SNHG5 expression in breast cancer, whereas miR-154-5p was decreased in breast cancer tissues compared with the adjacent normal breast tissues. Furthermore, the SNHG5 expression was significantly negatively correlated with miR-154-5p expression. Taken together, our data uncover the SNHG5-miR-154-5p-PCNA axis and provide a novel mechanism to explain breast cancer proliferation.

INTRODUCTION

Breast cancer is the most common malignant tumor and the main cause of cancer-associated mortality in females worldwide, with 2.08 million new cases diagnosed and leading to 626,679 deaths in 2018, followed by colorectal and lung cancer.[1] Although great progress has been made in the earlier diagnosis and systemic therapy of patients in recent years, recurrence or distant metastasis of breast cancer has become a barrier to the successful treatment of patients. However, our understanding of the molecular pathogenesis in breast cancer remains largely limited.[2,3] Therefore, a better understanding of the molecular mechanisms is required to facilitate the development of effective therapeutic strategies, as well as more accurate prognostic markers against breast cancer.

Although mammalian genomes can be widely transcribed, only 1.2% of the genome could be encoded for proteins in humans.[4] Among the large RNAs transcripts longer than 200 nt without the open reading frame (ORF) that do not code for proteins are long non-coding RNAs (lncRNAs).[5] Thanks to the development of RNA sequencing and microarray technology, thousands of lncRNAs have been identified. Originally, lncRNAs were considered as the "transcriptional noise" during the expression of the coding genes. Since then researchers found that lncRNA Xist is relative to the inactivation of allele X chromosome and led to the transcriptional silence of chromosome X,[6] and lncRNA HOTAIR (HOX transcript antisense RNA) also can bind with the histone-modifying complex to mediate transcriptional silence.[7] In recent years, increasing evidence supports that lncRNAs are involved in multiple biological processes, including chromatin remodeling, transcriptional regulation, post-transcriptional regulation, and translational regulation.[8,9]

lncRNAs are frequently dysregulated in human cancer and act as either oncogenes or tumor suppressors and critical regulators of carcinogenesis and cancer progression, as well as useful diagnostic and prognostic markers.[10,11] Among them, the aberrant expression of lncRNA small nucleolar RNA host genes (SNHGs) was observed in many types of human cancers, and SNHGs were involved in multiple steps during tumorigenesis and progression.[12,13] SNHG1 promotes tumor growth by regulating gene expression both in *cis* and in *trans*. In *cis*, SNHG1 promotes the transcription of SLC3A2 in colorectal cancer. In *trans*, SNHG1 affects the binding of FIR to FUBP1 and

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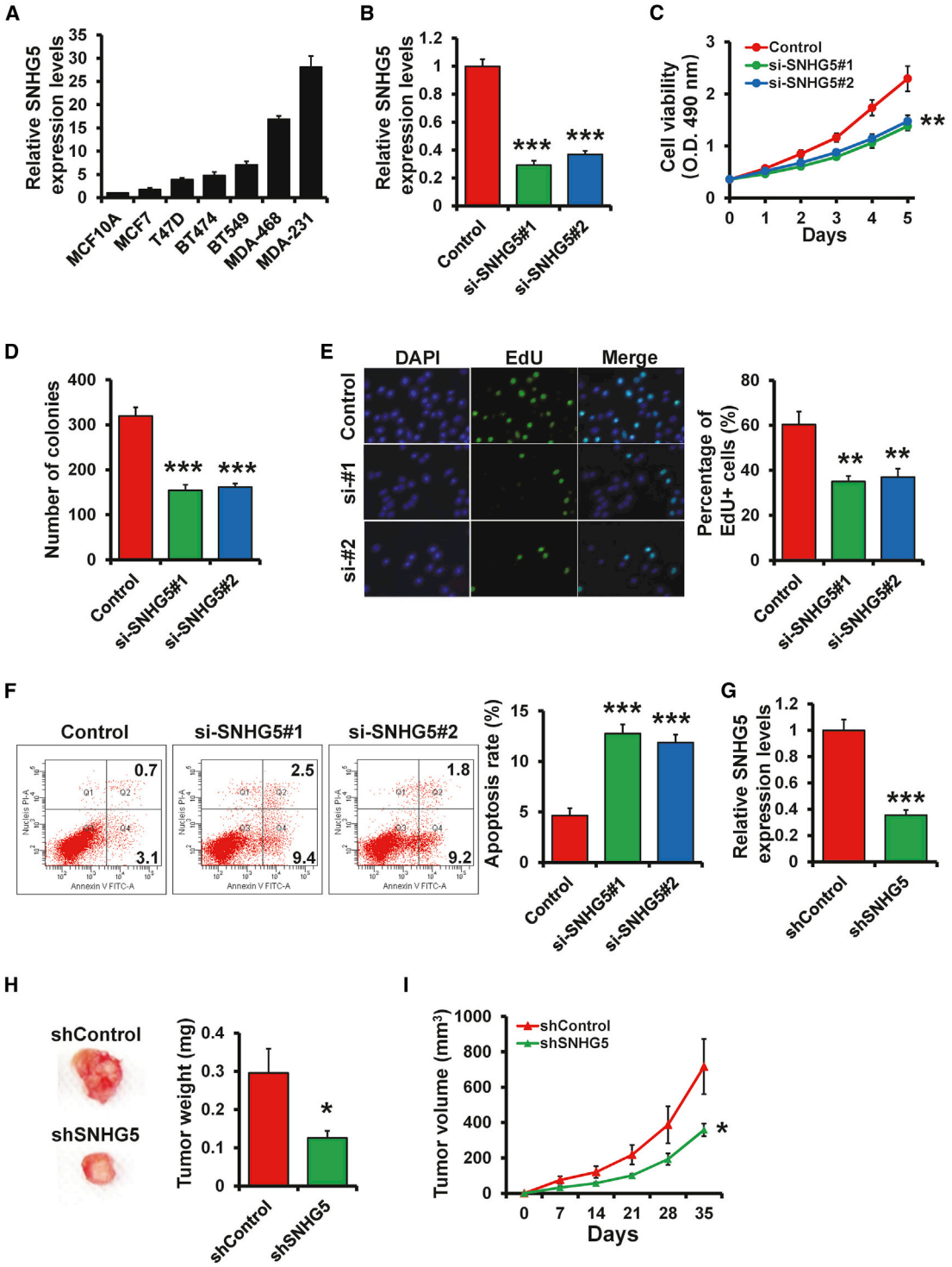

Figure 1. Knockdown of SNHG5 Inhibits Cell Proliferation and Induces Apoptosis in Breast Cancer Both *In Vitro* and *In Vivo*

(A) SNHG5 expression in breast cancer cell lines as determined by qRT-PCR. (B) SNHG5 expression in MDA-MB-231 cells transfected with small interfering RNAs targeting SNHG5 as determined by qRT-PCR. (C–E) Cell growth inhibition was determined by MTT (C), colony formation (D), and EdU (E) assays in cells as in (B).

(legend continued on next page)

increases MYC transcription in lung cancer.[14] SNHG3 enhances the malignant progress of glioma through silencing KLF2 and p21.[15] SNHG6 regulates the expression of ZEB1 by competitively binding miR-101-3p and interacting with UPF1 in hepatocellular carcinoma.[16] SNHG16 enhances the proliferation of osteosarcoma by upregulating ZEB1 expression by acting as an endogenous sponge of miR-205.[17] SNHG5, also named U50HG, is 524 bp in length and is one of the well-defined cytoplasmic lncRNAs.[18] SNHG5 is first mentioned in B cell lymphoma and was identified to locate at the chromosomal translocator breakpoint.[19] SNHG5 has also been identified as a potential oncogene in several types of cancer development and progression, including colorectal cancer,[18] gastric cancer,[20] and hepatocellular carcinoma.[21] However, the role and the mechanism of SNHG5 in breast cancer has not been elaborated.

Here, we investigate the role of SNHG5 in breast cancer progression. Depletion of SNHG5 suppresses breast cancer proliferation and leads to cell-cycle arrest at the G1 phase by sponging miR-154-5p. Furthermore, proliferating cell nuclear antigen (PCNA) is a target of miR-154-5p. Our data uncover the SNHG5-miR-154-5p-PCNA axis and provide a novel mechanism to explain breast cancer proliferation.

RESULTS

Knockdown of SNHG5 Inhibits Proliferation and Induces Apoptosis of Breast Cancer Cells

SNHG5 spliced into two main isoforms: SNHG5-1 (1,032 nt; Figure S1A) and SNHG5-2 (430 nt; Figure S1B). We observed that SNHG5-2 is the most abundant isoform expressed in breast cancer (Figure S1C), and we refer to it as SNHG5 in the following experiments. To investigate the potential biological function of SNHG5 in breast cancer progression, we determined the expression of SNHG5 in breast cancer cell lines (MCF7, T47D, BT474, BT549, MDA-MB-468, and MDA-MB-231) and a normal breast epithelial cell line (MCF10A) by qRT-PCR. The expression of SNHG5 was upregulated in all breast cancer cell lines as compared with MCF10A cells (Figure 1A). Next, we investigated the influence of SNHG5 on cell proliferation by introducing two specific small interfering RNAs (siRNAs) targeting SNHG5 into the MDA-MB-231 cell line, which has a higher endogenous SNHG5 expression level (Figure 1B). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and colony formation assays indicated that knockdown of SNHG5 decreased breast cancer cell proliferation (Figures 1C and 1D). 5-ethynyl-2'-deoxyuridine (EdU) assay further revealed that knockdown of SNHG5 markedly decreased the number of cells in the S phase (Figure 1E). Furthermore, we observed that the number of apoptotic cells was significantly higher in SNHG5-depleted MDA-MB-231 cells compared with that of control cells (Figure 1F). We next established the stable SNHG5-depleted MDA-MB-231 cells, as well as the control cells (Figure 1G). Next, 231-shSNHG5 or 231-shControl cells were injected into the mammary fat pads of female BALB/c nude mice, and tumor growth was quantified. As shown in Figures 1H and 1I, tumor volumes and weights were significantly decreased in mice injected with 231-shSNHG5 cells compared with those in mice injected with 231-shcontrol cells. Similar results were observed in MCF10A cells (Figure S2). These data suggested that knockdown of SNHG5 inhibits cell proliferation and induces apoptosis in breast cancer.

Overexpression of SNHG5 Promotes Tumor Growth Both *In Vitro* and *In Vivo*

Next, we assessed whether SNHG5 overexpression in breast cancer cells can influence tumor growth. We established the stable SNHG5-overexpressed T47D cells through the transfection of pcDNA3.1-SNHG5 expression plasmid, as well as the control cells (Figure 2A). The MTT and colony formation assays indicated that overexpression of SNHG5 promoted cell proliferation in T47D cells (Figures 2B and 2C). EdU assay further revealed that overexpression of SNHG5 markedly increased the number of EdU-positive cells (Figure 2D). Next, T47D-SNHG5 or T47D-control cells were injected into the mammary fat pads of female BALB/c nude mice, and tumor growth was quantified. As shown in Figures 2E and 2F, tumor volumes and weights were significantly increased in mice injected with T47D-SNHG5 cells compared with those in mice injected with T47D-control cells. The expression of Ki-67 was also increased in tumors from T47D-SNHG5 mice compared with that in tumors from T47D-control mice, as evidenced by immunohistochemical staining (Figure 2G). Collectively, these results indicate that overexpression of SNHG5 promotes tumor growth in breast cancer cells both *in vitro* and *in vivo*.

Depletion of SNHG5 Leads to Cell-Cycle Arrest at G1 Phases

The deregulation of the cell cycle is one of the hallmarks of cancer. To investigate the role of SNHG5 in the regulation of cell-cycle progression, we analyzed the cell-cycle distribution in 231-siSNHG5 and control cells by flow cytometry analysis. We observed a decreased number of S phase cells in the 231-siSNHG5 cells compared with that of control cells (Figure 3A). To further assess the role of SNHG5 in cell-cycle progression, we performed a series of cell synchronization experiments using the 231-siSNHG5 and control cells to analyze the cell-cycle transitions from G1 to S phases and S to G2/M phases. First, we synchronized 231-siSNHG5 and 231-Control cells at the G1/S transition double-thymidine block, and the cells were collected at the indicated times. Flow cytometry analysis showed that 231-siSNHG5 and 231-Control cells progressed similarly from S to G2/M phases (Figure 3B). The cell-cycle regulatory protein Cyclin B1 presented similar temporal expression (Figure 3C).

Furthermore, cancer cells were synchronized using a thymidine-nocodazole block to arrest the cells at the mitotic pre-metaphase. Flow cytometry analysis showed that the 231-siSNHG5 cells progressed

(F) Apoptosis analysis of cells as in (B). (G) SNHG5 expression in stable SNHG5-depleted MDA-MB-231 and control cells as determined by qRT-PCR. (H) Representative photos and weights of tumors formed by cells as in (G). (I) Tumor volume of xenograft mice injected with cells as in (G) at the indicated times. The data were presented as the mean ± SD obtained from at least three independent experiments. Significance was determined by Student's t test; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus negative control (NC).

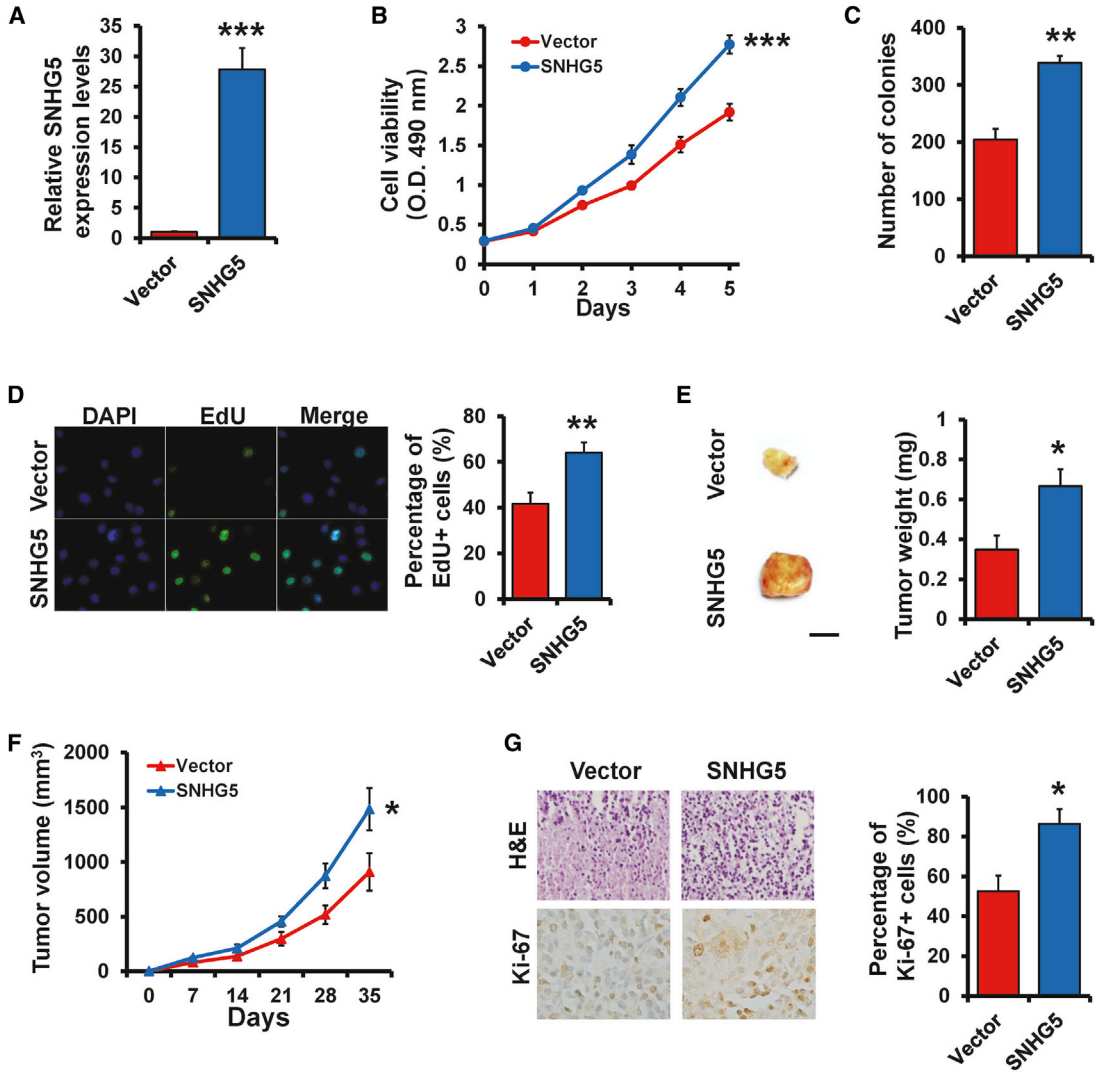

Figure 2. Overexpression of SNHG5 Promotes Breast Cancer Proliferation Both *In Vitro* and *In Vivo*

(A) The SNHG5 expression in stably transfected T47D with a SNHG5 expression vector or empty vector as determined by qRT-PCR. (B–D) Cell growth inhibition was determined by MTT (B), colony formation (C), and EdU (D) assays in cells as in (A). (E) Representative photos and weights of tumors formed by cells as in (A). (F) Tumor volume of xenograft mice injected with cells as in (A) at the indicated times. (G) H&E staining or Ki-67 expression in primary tumors harvested from mice bearing the indicated xenograft tumors. The data were presented as the mean ± SD obtained from at least three independent experiments. Significance was determined by Student's t test; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus empty vector.

slower from mitotic pre-metaphase to S phase, particularly arresting at G1 phase (Figure 3D), and Cyclin D1 expression was decreased in the 231-siSNHG5 cells compared with the 231-Control cells (Figure 3E). The EdU assay showed that the number of EdU-positive cells was decreased in the 231-siSNHG5 cells compared with that of control cells at 12 h after release (Figure 3F). Together, these results indicated that depletion of SNHG5 leads to cell-cycle arrest at G1 phase.

SNHG5 Functions as a Sponge for miR-154-5p

To further investigate the potential mechanism by which SNHG5 contributed to the malignant phenotypes of breast cancer cells, we first examined the subcellular localization of SNHG5. We observed that SNHG5 was mainly expressed in the cytoplasm by using fluorescence *in situ* hybridization (FISH) (Figure 4A) and subcellular fractionation by qRT-PCR (Figure 4B). Many cytoplasmic lncRNAs have been reported to act as competing endogenous RNAs (ceRNAs) by competitively binding microRNAs (miRNAs). We found that miR-154-5p was predicted to have a high probability of binding to SNHG5 by using starBase v3.0 (http://starbase.sysu.edu.cn/; Figure 4C). To further identify whether miR-154-5p could bind to the SNHG5, we constructed wild-type (SNHG5-WT) and miR-154-5p binding site mutant type (SNHG5-mut) SNHG5 luciferase reporters.

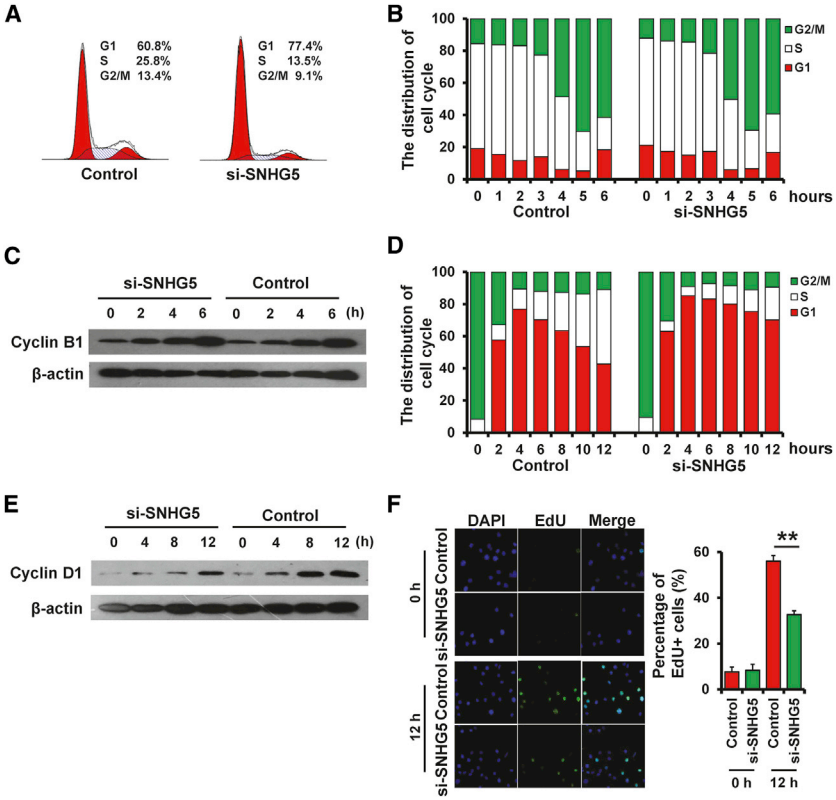

Figure 3. Knockdown of SNHG5 Leads to Cell-Cycle Arrest at the G1 Phase

(A) The cell-cycle distribution of 231-siSNHG5 and 231-siControl cells by flow cytometry analysis. (B) The cell-cycle distribution of 231-siSNHG5 and 231-siControl cells by flow cytometry analysis. These cells synchronized at the G1/S transition by a double-thymidine block and release for the indicated times. (C) The expression of Cyclin B1 in cells treated in (B) was detected by western blotting. (D) The cell-cycle distribution of 231-siSNHG5 and 231-siControl cells by flow cytometry analysis. The cells were synchronized at mitotic pre-metaphase by a thymidine-nocodazole block and were released for the indicated times. (E) The expression of Cyclin D1 in cells treated in (D) was detected by western blotting. (F) EdU analysis of cells as in (D) after released for 12 h. The data were presented as the mean ± SD obtained from at least three independent experiments. Significance was determined by Student's t test; **p < 0.01 versus NC.

As shown in Figure 4D, co-transfection of the SNHG5-WT with miR-154-5p mimics, but not the SNHG5-mut, significantly reduced the luciferase activity in MDA-MB-231 cells. In addition, an RNA immunoprecipitation (RIP) assay indicated that SNHG5 and miR-154-5p were significantly enriched in AGO-containing micro-ribonucleoprotein complexes, suggesting that the AGO2 bound to SNHG5 and miR-154-5p directly in breast cancer cells (Figure 4E). The expression of miR-154-5p was significantly increased in SNHG5-depleted MDA-MB-231 cells (Figure 4F), whereas the expression of miR-154-5p was decreased in T47D-SNHG5 cells compared with those of control cells by qRT-PCR (Figure 4G). miR-154 functions as a tumor suppressor in several types of cancers;[22–24] however, the role of miR-154-5p in breast cancer is limited. To further confirm whether SNHG5 promotes breast cancer proliferation by regulating miR-154-5p, we transfected miR-154-5p mimic into T47D-SNHG5 cells (Figure 4H). As expected, miR-154-5p overexpression in T47D-SNHG5 cells decreased cell viability (Figure 4I), the number of colonies (Figure 4J), and the percentage of cells in the S phase (Figure 4K). Taken together, our results indicated that SNHG5 promotes breast cancer proliferation partly dependent on sponging miR-154-5p.

PCNA Is a Target of miR-154-5p

To elucidate the biological mechanisms underlying the role of miR-154-5p in breast cancer proliferation, we investigated the potential targets of miR-154-5p by using starBase v3.0. We found eight candidate genes by using multiple target-predicting programs (Figure 5A). PCNA, an important proliferation biomarker in many types of cancer and immunohistochemical staining of PCNA, has been used extensively in breast cancer diagnosis and prognosis.[25] Thus, we identified PCNA as a putative miR-154-5p target. To further confirm this regulation, PCNA 3′ UTR and its mutant containing the putative miR-154-5p binding sties were cloned into the downstream luciferase ORF (Figure 5B). As compared with that in control cells, the luciferase activity was significantly decreased in miR-154-5p-transfected MDA-MB-231 cells with inhibition rates of 40% (Figure 5C). This effect was abolished in mutated PCNA 3′ UTR, in which the binding site for miR-154-5p was inactivated by site-directed mutagenesis (Figure 5C). Furthermore, the expression of PCNA was decreased in miR-190-overexpressing MDA-MB-231 cells and was increased in miR-154-5p-depleted T47D cells compared with that in control cells, as determined by qRT-PCR (Figures 5D and 5E) and western blotting (Figure 5F). Together, these results indicated that PCNA is a direct target of miR-154-5p.

SNHG5 Promotes Breast Cancer Proliferation by Regulating PCNA Expression

To further demonstrate that SNHG5 promotes breast cancer by regulating PCNA expression, we transfected small interfering RNAs targeting PCNA into T47D-SNHG5 and vector control cells (Figure 6A). Whereas SNHG5 overexpression led to an increased PCNA expression, transient transfection of small interfering RNAs targeting PCNA repressed the expression of PCNA (Figure 6B). Meanwhile, T47D-SNHG5 cells exhibited an increased Cyclin D1 expression and a decreased p16 expression, whereas knockdown of PCNA expression could rescue the expression (Figure 6B). Depletion of PCNA abolished the effect of SNHG5 on cell proliferation in T47D cells (Figures 6C–6E). We observed a decreased number of S phase cells in the PCNA-depleted T47D-SNHG5 cells compared with that of control cells (Figure 6F). Immunohistochemical staining

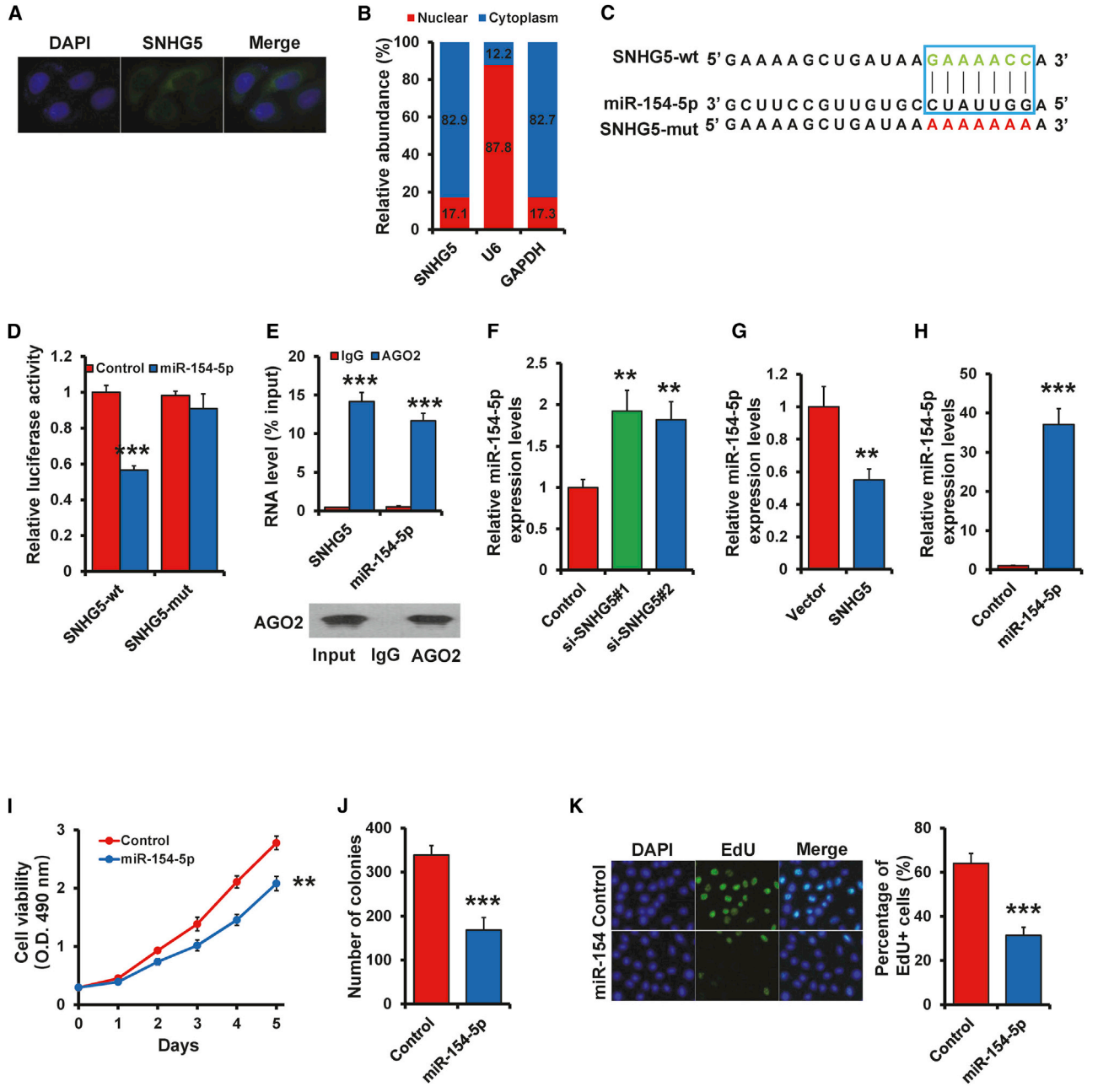

Figure 4. SNHG5 Acts as a Sponge for miR-154-5p

(A) Representative fluorescence *in situ* hybridization indicated subcellular location of SNHG5 in MDA-MB-231 cells (green). Nuclei were stained by DAPI (blue). (B) Relative SNHG5 expression levels in nuclear and cytoplasmic fractions of MDA-MB-231 cells. (C) The predicted binding of miR-154-5p with SNHG5 3′ UTR. (D) Dual-luciferase reporter assay was performed to validate the interaction between miR-154-5p and SNHG5. (E) An RNA immunoprecipitation analysis of endogenous AGO2 binding to RNA in MDA-MB-231 cells; IgG was used as the control. SNHG5 and miR-154-5p levels were determined by qRT-PCR and presented as fold enrichment in AGO2 relative to input. (F) miR-154-5p expression in SNHG5-depleted MDA-MB-231 cells as determined by qRT-PCR. (G) miR-154-5p expression in stably transfected T47D with a SNHG5 expression vector or empty vector as determined by qRT-PCR. (H) miR-154-5p expression in T47D-SNHG5 cells transfected with miR-154-5p mimics as determined by qRT-PCR. (I–K) Cell growth inhibition was determined by MTT (I), colony formation (J), and EdU (K) assays in cells as in (H). The data were presented as the mean ± SD obtained from at least three independent experiments. Significance was determined by Student's t test; ***$p < 0.001$, **$p < 0.01$ versus NC or empty vector. Mut, contains 7-base mutation at the miR-154-5p target seed region.

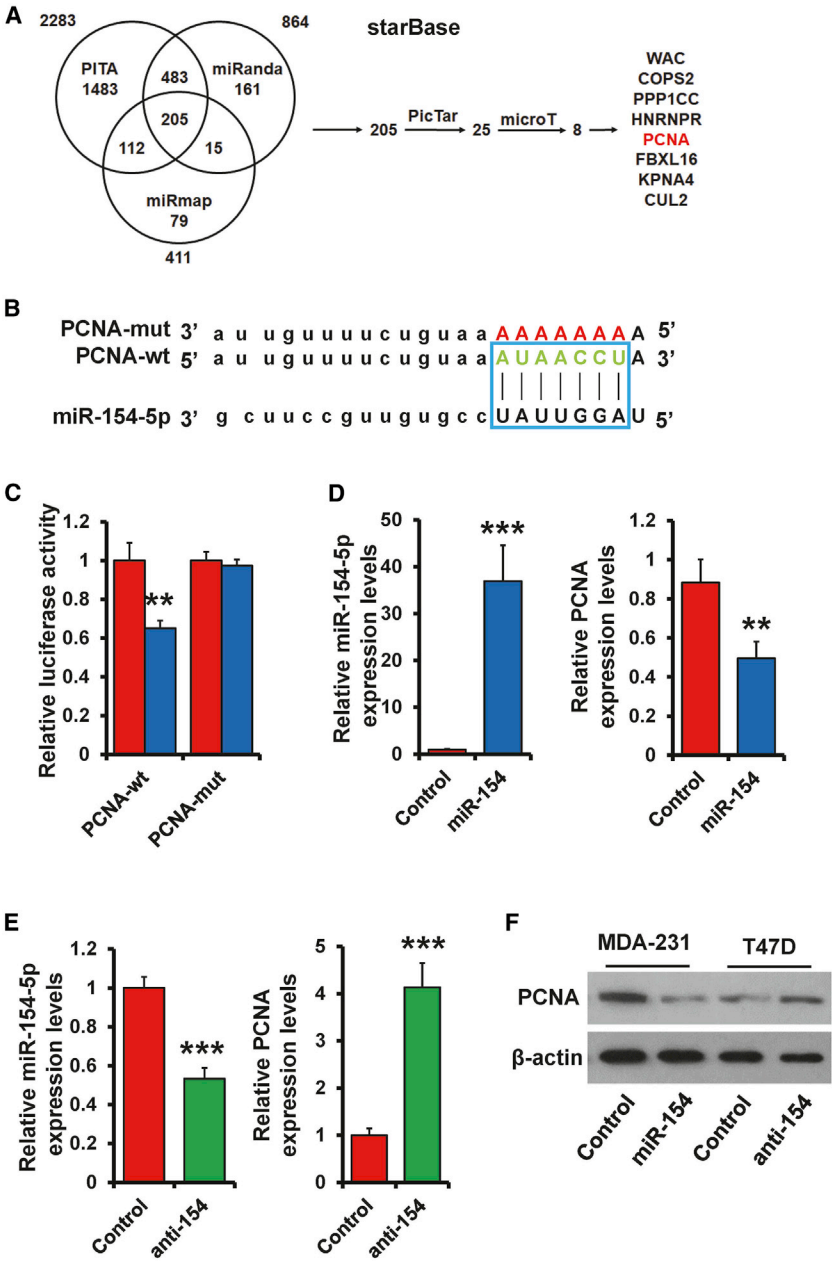

Figure 5. PCNA Is a Direct Target of miR-154-5p

(A) The potential target genes of miR-154-5p analyzed by starBase v3.0. (B) The predicted binding of miR-154-5p with PCNA 3′ UTR. (C) Dual-luciferase reporter assay was performed to validate the miR-154-5p target, PCNA. (D) The expression of miR-154-5p and PCNA in miR-154-5p-overexpressed MDA-MB-231 and control cells determined by qRT-PCR. (E) The expression of miR-154-5p and PCNA in miR-154-5p-depleted T47D and control cells determined by qRT-PCR. (F) The expression of miR-154-5p and PCNA in indicated cells as determined by western blotting. The data were presented as the mean ± SD obtained from at least three independent experiments. Significance was determined by Student's t test; ***$p < 0.001$, **$p < 0.01$ versus NC. Mut, contains 6-base mutation at the miR-154-5p target seed region.

confirmed the upregulation of PCNA in tumors from T47D-SNHG5 mice compared with tumors from T47D-control mice (Figure 6G). Taken together, these results indicated that SNHG5 promotes breast cancer proliferation by regulating PCNA expression.

SNHG5 Predicts Poor Prognosis and Inversely Correlates with miR-154-5p in Clinical Samples

We finally examined the clinical relevance of SNHG5 expression in breast cancer progression. We analyzed the expression of SNHG5 in 30 samples of primary breast cancer tissues and paired adjacent normal breast tissues by qRT-PCR. We observed upregulation of SNHG5 in most of the cases (Figure 7A), whereas the expression of miR-154-5p was downregulated in breast cancer (Figure 7B). More importantly, we compared the overall survival (OS) of breast cancer patients with different levels of SHNG5 expression by Kaplan-Meier (KM)-plotter and found that patients with high SNHG5 expression had a significantly poor overall survival compared with those with low SNHG5 expression (Figure 7C). In addition, the patients with high miR-154-5p expression had a favorable outcome compared with those with low miR-154-5p expression (Figure 7D). Moreover, the expression of miR-154-5p exhibited a significant relationship with SNHG5 expression (Figure 7E). Collectively, our data support the conclusion that SNHG5 promotes breast cancer proliferation by sponging the miR-154-5p.

DISCUSSION

In the present study, we identified that lncRNA SNHG5 acts as an oncogene in breast cancer and validated that SNHG5 is increased in clinical breast cancer tissues and correlates with survival in patients with breast cancer. Depletion of SNHG5 suppresses breast cancer proliferation and leads to cell-cycle arrest at G1 phase. Furthermore, PCNA is a target of miR-154-5p, and overexpression of SNHG5 induces the expression of PCNA by sponging miR-154-5p. Therefore, our results revealed a SNHG5-miR-154-5p-PCNA axis in regulation of the cell cycle in breast cancer proliferation and demonstrated that SNHG5 functions as an oncogenic lncRNA in breast cancer.

Dysregulation of lncRNAs is involved in almost every cellular process during cancer development and progression, including proliferation, apoptosis, migration, invasion, angiogenesis, and metastasis.[26] SNHG5 has been identified to be an oncogene in several types of cancer,

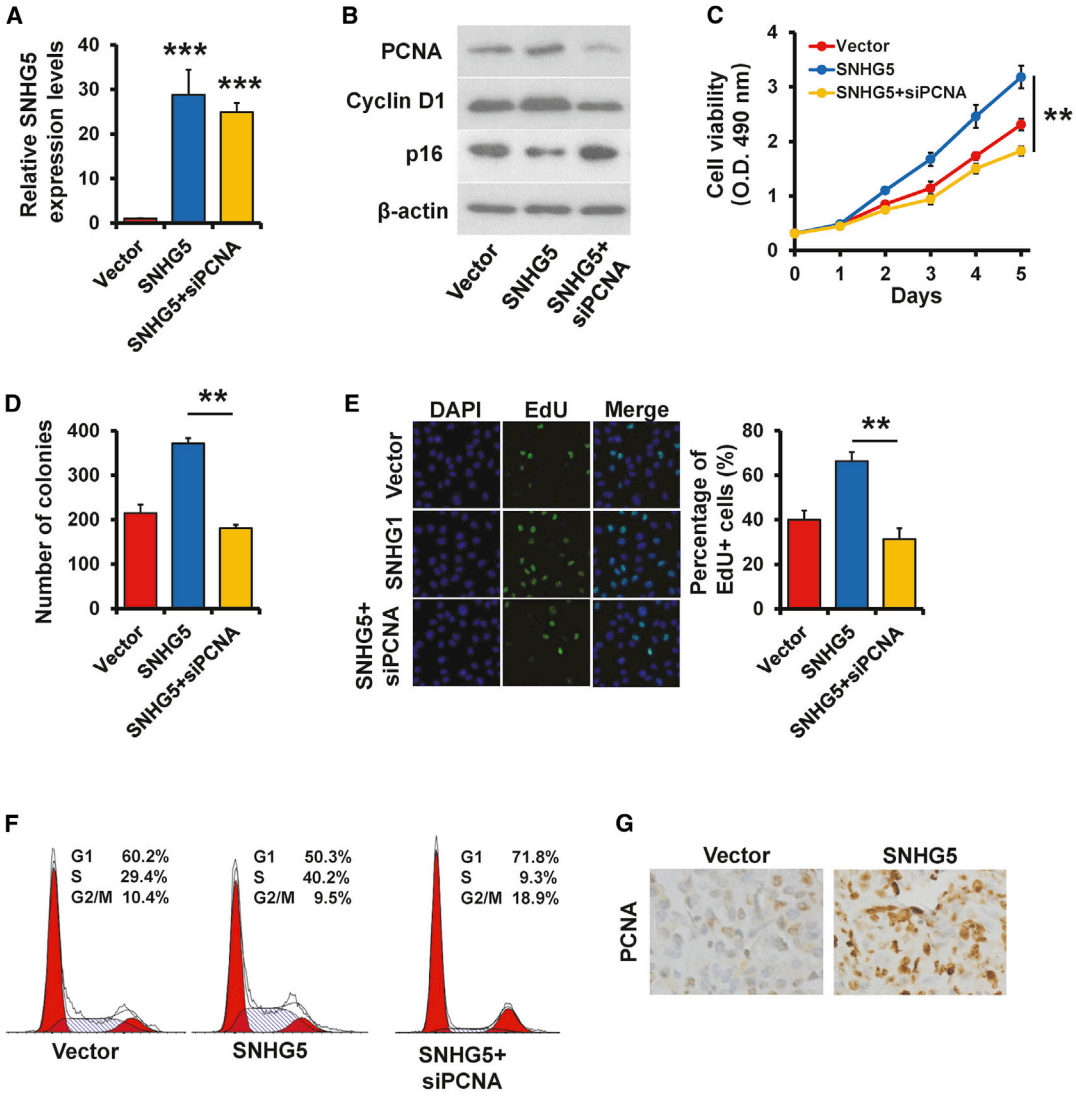

Figure 6. SNHG5 Promotes Breast Cancer Proliferation by Regulating PCNA

(A) SNHG5 expression in indicated T47D cells as determined by qRT-PCR. (B) The expression of PCNA, Cyclin D1, and p16 in indicated T47D cells as determined by western blotting. (C–E) Cell growth inhibition was determined by MTT (C), colony formation (D), and EdU (E) assays in indicated T47D cells. (F) The cell-cycle distribution of cells as in (A) by flow cytometry analysis. (G) The expression of PCNA in T47D-SNHG5 and T47D-vector xenograft tumors was examined by immunohistochemical staining. The data were presented as the mean ± SD obtained from at least three independent experiments. Significance was determined by Student's t test; ***$p < 0.001$, **$p < 0.01$ versus empty vector.

including colorectal cancer,[18] hepatocellular carcinoma,[21] osteosarcoma,[27] melanoma,[28] and gastric cancer.[20] Although it was found that the expression of SNHG5 is upregulated in MDA-MB-231 cells compared with that in MCF7 cells, the role of SNHG5 in breast cancer is still limited.[29] Uncontrolled cell proliferation is a crucial characteristic of human cancer, and numerous lncRNAs were involved in the regulation of cell proliferation during tumorigenesis. Consistent with previous studies in other cancers, we provided evidence that SNHG5 is upregulated in breast cancer tissues and correlates with poor survival in patients with breast cancer. Furthermore, we observed that SNHG5 promotes breast cancer proliferation both in *in vitro* and *in vivo*. Dysregulated cell cycle is a biological feature of uncontrolled cell proliferation in malignant tumors.[30] The cell-cycle progression is dependent on the activity of complexes of cyclin-dependent kinase (CDK) with respective cyclin partners.[31] The activation of CDK4/6-Cyclin D1 and CDK1-Cyclin B1 is the key event in G1 to S and G2 to M transition, respectively.[32,33] We found that depletion of SNHG5 increases the percentage of cells at G1 phase. Furthermore, SNHG5 depletion did not affect the expression of Cyclin B1, whereas the expression of Cyclin D1 was decreased after cell synchronization. Our study reveals that SNHG5 functions as an oncogene in breast cancer proliferation through acceleration of cell-cycle progression from G1 to S phases.

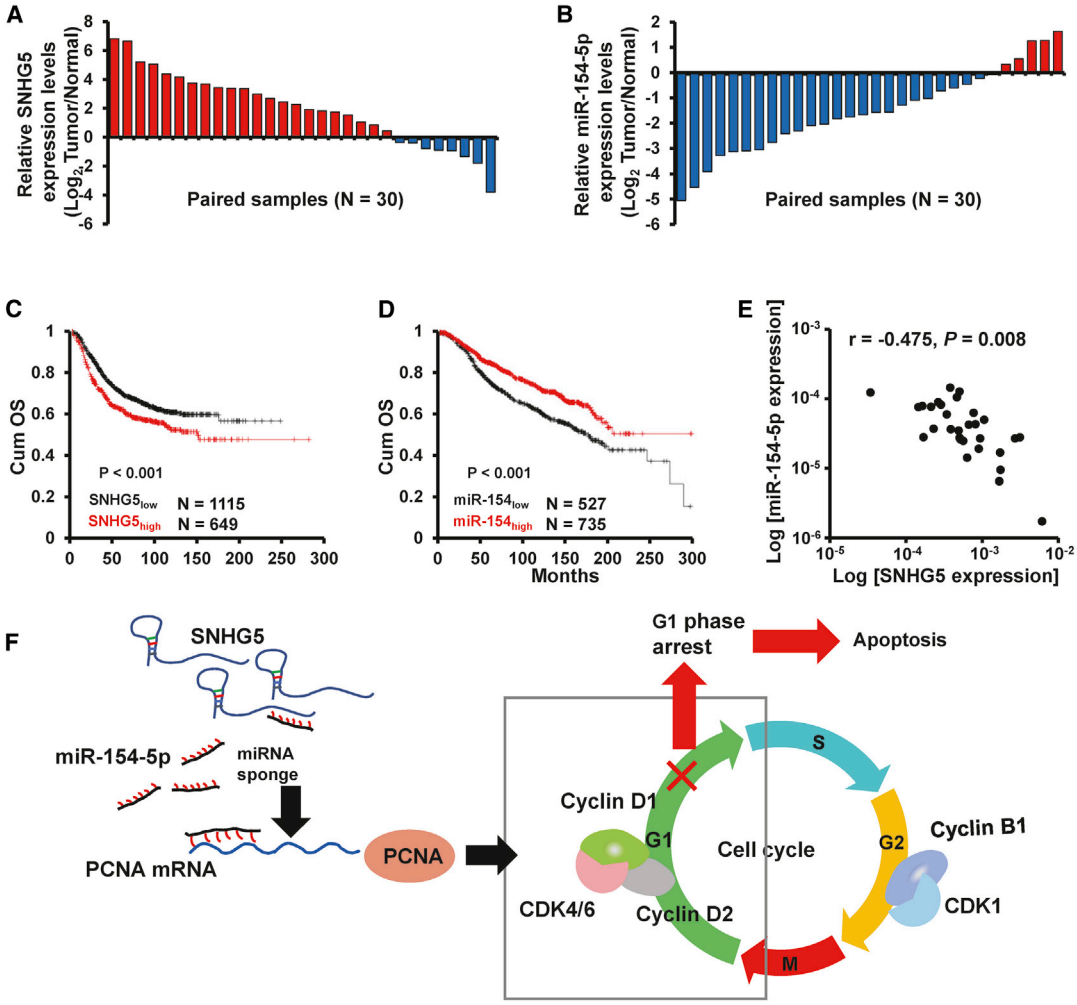

Figure 7. SNHG5 Predicts Poor Prognosis and Inversely Correlates with miR-154-5p in Clinical Samples

(A) SNHG5 expression in primary breast cancer tissues and paired normal breast tissues as determined by qRT-PCR. (B) miR-154-5p expression in primary breast cancer tissues and paired normal breast tissues as determined by qRT-PCR. (C) Kaplan-Meier analysis of the overall survival in patients with different SNHG5 expression levels by KM-plotter. (D) Kaplan-Meier analysis of the overall survival in patients with different miR-154-5p expression levels by KM-plotter. (E) The relationship between SNHG5 and miR-154-5p expression as determined by qRT-PCR. (F) A model for the role of SNHG5 in breast cancer proliferation.

In addition to regulation through direct interaction with the target genes, the hypothesis of ceRNAs, which is supported by numerous studies, has been presented to describe a novel regulatory mechanism of RNA.[20] SNHG5 acts as a sponge for miR-26a and promotes cancer tumorigenesis and progression in hepatocellular carcinoma[21] and osteosarcoma.[26] However, the mechanism of SNHG5 in breast cancer is still unclear. In the current study, we provided evidence of a novel ceRNA regulatory network, where SNHG5 functions as a sponge for miR-154-5p. We found that SNHG5 is a cytoplasmic lncRNA in breast cancer, and this makes it possible for SNHG5 to act as ceRNAs for binding to miRNAs. Increasing evidence shows that miRNAs are frequently dysregulated in breast cancer, and act as either oncogenes or tumor suppressors and critical regulators of carcinogenesis and cancer progression, as well as useful diagnostic and prognostic markers in breast cancer.[34,35] miR-154-5p has been identified to be a tumor suppressor in several types of cancer, including glioblastoma,[36] osteosarcoma,[22] colorectal cancer,[23] prostate cancer,[24,37] and breast cancer.[38] We present evidence that SNHG5 is able to repress miR-154-5p and promote breast cancer proliferation by regulation of miR-154-5p expression. These results provide further supporting evidence for the ceRNA regulatory network.

miRNAs are a class of small non-coding RNAs, which are believed to negatively regulate gene expression by binding to complementary sequences in the 3′ UTRs by translational inhibition and destabilization of target mRNAs.[39] The ceRNA hypothesis clarifies that lncRNAs may exert ceRNA roles by acting as endogenous decoys for miRNAs, which in turn affects the binding of miRNAs on their targets.[40] In our

study, we identified PCNA as a direct target of miR-154-5p. In agreement with SNHG5 being a sponge for miR-154-5p, we proved that PCNA suppression by miR-154-5p could be partially rescued in the presence of SNHG5. PCNA was shown to act as a processivity factor of DNA polymerase δ (polδ), which is required for DNA synthesis during replication.[40] In cell-cycle progression, PCNA interacts with several eukaryotic cell-cycle-related proteins and functions as an oncogene in various types of human cancers.[41–43] Therefore, the SNHG5-miR-154-5p/PCNA axis may function as an important player in breast cancer proliferation.

Conclusions

In summary, we demonstrated that SNHG5 is an oncogene in breast cancer and also a potential independent predictor in patients with breast cancer. Depletion of SNHG5 suppresses breast cancer proliferation and leads to cell-cycle arrest at G1 phase. Furthermore, SNHG5 acts as a sponge for miR-154-5p to weaken the suppressive effect of miR-154-5p on PCNA, thus facilitating cell proliferation. Based on the findings from this study and others, we propose a model that highlights the role of SNHG5 in regulating cell-cycle progression during breast cancer proliferation (Figure 7F). Taken together, our data indicate that the SNHG5-miR-154-5p-PCNA axis functions as an important player in breast cancer proliferation and serves as a promising target for breast cancer therapy.

MATERIALS AND METHODS

Cell Culture and Clinical Samples

MCF10A, MCF7, T47D, BT474, BT549, MDA-MB-468, and MDAMB-231 cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured as previously described.[44]

Breast cancer specimens were obtained from Tianjin Medical University Cancer Institute and Hospital (TMUCIH). Thirty cases of primary breast cancer tissue and paired adjacent normal breast tissue specimens were included in this study. All tumor samples were from patients with a newly diagnosed breast cancer who had received no therapy before sample collection. After mastectomy surgery, the primary cancer tissues and the adjacent normal tissues were immediately snap-frozen in liquid nitrogen and stored at −80°C until use. This study was approved by the Institutional Review Board of the Tianjin Medical University Cancer Institute and Hospital, and written consent was obtained from all participants.

Antibodies, Reagents, Plasmids, miRNA, and Small Interfering RNA

The antibodies, reagents, plasmids, miRNAs, and small interfering RNAs used in this study are listed in the Supplemental Materials and Methods.

Cell Synchronization

To obtain synchronized cell populations in S phase, MDA-MB-231 cells were blocked for 18 h in complete medium containing 2 mM thymidine, released for 8 h by washing out the thymidine, and then blocked again with 2 mM thymidine for 18 h to arrest at the beginning of S phase. To obtain synchronized cell populations in mitosis, MDA-MB-231 cells were blocked at the G1/S border with 2 mM thymidine for 18 h, followed by a 4-h release, and then blocked with 100 ng/mL nocodazole for 12 h to arrest the cells in mitosis.

Transfection and Generation of Stable SNHG5-Overexpressed Cell Line

For transient transfection, miRNA or small interfering RNAs were transfected into different cell lines using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA), and plasmids were transfected using TransFast Transfection Reagent (Promega) according to the manufacturer's recommendations. To generate stable SNHG5 overexpression cells, T47D cells were cultured in a 12-well plate and were transfected with 1 μg of either pcDNA3.1-SNHG5 or vector plasmid using TransFast Transfection Reagent. The cells were then cultured in the medium containing 800 μg/mL G418 (Sigma-Aldrich, St. Louis, MO, USA) for 3 weeks. Monoclonal resistant cells were obtained using limiting dilution assay and maintained in medium supplemented with 400 μg/mL G418.

Proliferation Assays

MTT, plate colony formation, and EdU assays were used to evaluate cell proliferation ability. Experiments were carried out as described in the Supplemental Materials and Methods.

RNA Fluorescence *In Situ* Hybridization

Fluorescence *in situ* hybridization assay was performed using the Fluorescent *in situ* Hybridization Kit (RiboBio) according to the manufacturer's recommendations. Fluorescence-conjugated SNHG5 probes were purchased from RiboBio (China). Cells were first fixed in 4% formaldehyde for 15 min at room temperature. Then the cells were permeabilized in PBS containing 0.5% Triton X-100 at 4°C for 30 min and pre-hybridized at 55°C for 30 min in pre-hybridization buffer (Life Technologies, Grand Island, NY, USA). After that, probes were added in the hybridization solution and incubated with the cells at 37°C overnight in a humidified chamber. The next day, the cells were counterstained with DAPI and imaged.

Flow Cytometry Analysis

Cells were fixed in 70% ethanol at 4°C overnight and washed with PBS. Then the cells were resuspended in 0.1% Triton X-100/PBS, concomitantly treated with RNaseA (Sigma-Aldrich), and stained with 50 μg/mL propidium iodide (PI) for 30 min. Cell cycle was analyzed using FACSCanto II (BD Biosciences, San Diego, CA, USA).

Cell apoptosis was assessed using an Annexin V Staining Kit (BD Biosciences, San Diego, CA, USA). In brief, cells were harvested, washed with cold PBS and resuspended in binding buffer, and incubated with Annexin V and PI for 15 min at room temperature. Sorting was performed, and cells were analyzed using BD FACSCanto II.

Western Blotting

Cells were lysed in protein lysis buffer (20 mM Tris-HCl [pH 7.4], 5 mM EDTA, 1% Triton X-100, 150 mM NaCl, and 1% DTT)

containing a protease inhibitor cocktail tablet (Roche Molecular Biochemicals, Indianapolis, IN, USA). Proteins were resolved by SDS-PAGE, transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA), and incubated with primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody. The blots were visualized with enzyme chemiluminescence (ECL) reagent (Millipore).

RNA Extraction and qRT-PCR

Total RNA of cultured cells, surgically resected fresh breast tissues, and formalin-fixed paraffin-embedded clinical specimens was extracted using the mirVana PARIS kit (Life Technologies) according to the manufacturer's recommendations. qPCR was performed to detect mRNA expression using GoTaq qPCR Master Mix (Promega). TaqMan qRT-PCR was performed to detect mature miRNA expression using the TaqMan miRNA reverse transcription kit, has-RNU6B (U6, ABI Assay ID: 001093), and miR-154-5p (ABI Assay ID: 000477) according to the manufacturer's protocol (Life Technologies). The sequences of PCR primers are listed in the Supplemental Materials and Methods.

Luciferase Reporter Assay

Luciferase assays were carried out using a dual-luciferase assay kit according to the manufacturer's recommendations, as previously described.[20] PCNA-WT/mut or SNHG5-WT/mut was co-transfected with miR-154-5p mimics or mimic control into MDA-MB-231 cells by using TransFast Transfection Reagent. The cells were collected 48 h after transfection, and the luciferase activities were measured using the Dual Luciferase Reporter System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

RNA Immunoprecipitation Assay

An RNA immunoprecipitation experiment was conducted using the EZ-Magna RIP Kit (Millipore, USA) following the manufacturer's protocol. MDA-MB-231 cells were lysed in an RNA immunoprecipitation lysis buffer, followed by incubation of 100 μL of whole-cell extract with an RNA immunoprecipitation buffer containing A/G magnetic beads conjugated with human anti-AGO2 antibody (Millipore). Normal mouse immunoglobulin G (IgG) (Millipore) was used as a negative control. Samples were incubated with Proteinase K by shaking in order to digest the protein, followed by isolation of immunoprecipitated RNA. qRT-PCR was performed to analyze the presence of the binding targets.

Xenograft

The stable SNHG5-overexpressed T47D cells or SNHG5-depleted MCF10A cells, as well as the control cells (3×10^6 cells) together with 100 μg of Matrigel (BD Biosciences), were inoculated into the mammary fat pads of 5-week-old female SCID mice (five mice per group). Tumor growth was recorded twice a week with a caliper-like instrument. Tumor volume was calculated according to the formula: volume = (width^2 × length)/2. The mice were sacrificed at 5 weeks considering animal welfare, and the final volume and weight of tumor tissues were determined. All *in vivo* experiments were reviewed and approved by the Animal Ethics Committee of Tianjin Medical University Cancer Institute and Hospital and were performed according to the guidelines for the welfare and use of animals in cancer research and national law.

Statistical Analysis

Data are presented as mean ± SD. The Student's t test (two-tailed) was used to determine the differences between the experimental and control groups. The level of significance was set to $p < 0.05$. All calculations were performed with the SPSS for Windows statistical software package (SPSS, Chicago, IL, USA).

Availability of Data and Materials

All data generated or analyzed during this study are included in this article and the Supplemental Information.

Ethics Approval and Consent to Participate

This study was approved by the Institutional Review Board of Tianjin Medical University Cancer Institute and Hospital, and written consent was obtained from all participants.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.05.013.

AUTHOR CONTRIBUTIONS

Y.Y. and X.-C.C. designed the study; J.-R.C., Z.-H.Y., and B.-W.L. performed the experiments; J.-R.C. and D.Z. performed the statistical analysis; J.G. participated in the clinical specimen's detection; and J.-R.C., Y.Y., and X.-C.C. wrote and revised the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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