Pathology - Research and Practice 215 (2019) 152526

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

The effects of miR-429 on cell migration and invasion by targeting Slug in esophageal squamous cell carcinoma

Mingzhu Zong[a,b], Yan Liu[a], Kai Zhang[c], Yi J[c], Longbang Chen[a,c,*]

[a] Jinling Hosp Dept of Medical Oncology, Nanjing Med Univ, Nanjing, 210002, PR China
[b] The Affiliated Huaian No.1 People, s Hospital of Nanjing Medical University, PR China
[c] Jinling Hosp Dept of Medical Oncology, Nanjing Univ, Sch Med, Nanjing, 210002, PR China

ARTICLE INFO

Keywords:
microRNAs
miR-429
Slug
EMT
ESCC

ABSTRACT

Increasing evidence indicates that microRNAs may play important roles in tumor development and may take part in different processes in different cancers. miR-429 is known as a cancer suppressor or oncogene that is dysregulated in different malignancies, including esophageal squamous cell carcinoma (ESCC). However, the effect of miR-429 in ESCC has not been fully explored. The purpose of this study was to investigate the functions of miR-429 in ESCC. qRT-PCR assays were performed to detect miR-429 expression in ESCC tissues and cell lines. To assess the effects of miR-429 on ESCC cells, wound healing and transwell assays were used. Luciferase reporter and western blot assays were employed to determine whether Slug is a major target of miR-429.Our results showed that the expression levels of miR-429 in ESCC tissues and cells were lower than in normal esophageal epithelial tissues and cells. Furthermore, overexpression of endogenous miR-429 inhibited the migration and invasion of ESCC cell lines. In addition, Luciferase reporter and western blot assays provided evidence that miR-429 can bind to the 3′ untranslated regions of Slug to regulate its expression and that of downstream epithelial-to-mesenchymal transition (EMT) markers. We found that Slug serves as a major target of miR-429. miR-429 plays a vital role in ESCC progression and represents a new therapeutic target for ESCC.

1. Introduction

Esophageal cancer (EC) is the sixth most common cause of cancer death, with a high global mortality rate [1]. It is the fourth most common cause of cancer death in developing countries. China had an estimated 477,900 new diagnoses and 375,000 deaths in 2015 [2]. Based on its pathological features, esophageal cancer is mainly divided into esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EA), of which ESCC accounts for more than 90%. The main treatment methods of esophageal cancer are surgery, chemotherapy, radiotherapy, and combined treatments. Targeted therapies such as trastuzumab plus chemotherapy are recommended as first-line therapies for patients with advanced or metastatic adenocarcinoma who are HER2-positive [3]. Despite great advances in treatment, the 5-year survival rate for esophageal cancer remains below 15% [4]. Recurrence and metastasis are the fundamental causes affecting the long-term survival. Therefore, it is of great significance and clinical value to understand the molecular mechanisms of the occurrence, development, and metastasis of esophageal cancer cells and to screen for new, specific, and sensitive targets to prevent and treat esophageal cancer.

Tumor invasion and metastasis is a complex process in which epithelial-to-mesenchymal transition (EMT) has been reported to play an important role [5–7]. EMT is the differentiation of epithelial cells into mesenchymal cells under specific physiological and pathological conditions [8]. When EMT occurs in tumor cells, the skeleton of the cells recombines, and the epithelial cell phenotype is deleted [9]. Moreover, the expression of proteins that enhance intercellular adhesion, such as E-cadherin, is reduced, and molecular markers of mesenchymal cells such as vimentin and N-cadherin are increased [10]. As a result, the adhesion between cells is reduced or lost, and intercellular connections become loose. In addition, the polarity is lost, and the ability to migrate, avoid apoptosis, and degrade the extracellular matrix is obtained, which is an important development for the invasion of surrounding tissues and metastasis of distant organs [11].

Slug is a member of the transcription factor Snail family. It encodes a zinc finger protein that plays an important role in embryonic development and is one of the most important transcription factors in the process of activating EMT [12,13]. Slug is significantly upregulated in a variety of malignant tumors, and tumors in which Slug is upregulated are prone to distant metastases and are correlated with poor prognosis

* Corresponding author at: Jinling Hosp Dept of Medical Oncology, Nanjing Med Univ, Nanjing, 210002, PR China.
E-mail address: doctorclb@163.com (L. Chen).

https://doi.org/10.1016/j.prp.2019.152526
Received 2 May 2019; Received in revised form 27 June 2019; Accepted 5 July 2019

[14,15]. E-cadherin is one of the most important markers of epithelial cell adhesion plaques. Slug has a highly conserved C2H2 zinc finger domain, which can bind directly to a target gene sequence promoter in the nucleus to directly regulate its transcription [16]. It has been reported that Slug can inhibit E-cadherin gene transcription and expression by combining with the E-box on its gene promoter, which is a key factor in EMT [17].

As an initiator of tumor metastasis, EMT is regulated by microRNAs (miRNAs). MiRNAs are a class of noncoding small RNAs about 18—25 nucleotides in length [18,19] that interact with the 3′-untranslated region (UTR) of target mRNAs, causing the expression of the target to decrease or experience accelerated degradation, reducing protein translation. miRNAs participate in many physiological and pathological processes, such as cell differentiation, embryonic development, organ formation, and tumor progression [20]. Abnormal miRNA expression exists in many types of tumor tissues and cells, and the expression profiles of miRNA at different stages of the same tumor are also different. According to its regulated target genes, miRNAs play a role in inhibiting or promoting tumor growth. miR-200 is the main regulatory molecule of EMT and helps to maintain the epithelial phenotype, playing an important role in the occurrence of EMT and tumor progression [21,22]. Due to its location in different chromosomes, miR-200 can be divided into two gene clusters: miR-200b, miR-200a, miR-429 and miR-200c, miR-141. It is predicted that members of the same family may have similar target gene profiles and may participate in the same or related physiological processes [23,24]. miR-429 is a member of the miRNA-200 family, which is implicated in EMT. miR-429 has been reported to be dysregulated in NSCLC, colorectal cancer, nasopharyngeal cancer, and bladder cancer and is associated with poor outcomes, suggesting that it may play a role in tumorigenesis and progression in different types of tumors [25–28]. Wang et al. reported that miR-429 expression in esophageal cancer is downregulated, and the expression level is closely related to lymph node metastasis [29]. However, few studies have been conducted on the potential molecular mechanisms of miR-429 in regulating the invasion and metastasis of esophageal cancer.

In this study, miR-429 was found to be downregulated in ESCC, and it negatively regulated cell migration and invasion of ESCC cells by directly targeting Slug. In addition, downregulation of miR-429 induced EMT.

2. Materials and methods

2.1. Cell culture and tissue samples

Human EC cells ECA109, TE-1, TE-2, TE-3, TE-8, TE-12, and TE-13 and the normal human esophageal epithelial cell line HEEC were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. All cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA) at 37 °C with 5% CO_2.

Pairs of ESCC tissues and their paracancerous tissues(n = 23) were collected from Jinling Hospital (Jiangsu, China). No radiotherapy and chemotherapy were performed before operation, and the pathological diagnosis was clear. The study protocol was reviewed and approved by the Ethics Committee and Institutional Review Committee of Jinling Hospital and obtained the written informed consent of each patient participating in the study.

2.2. Quantitative RT-PCR of miRNAs

Total RNA was extracted from cells and tissues using Trizol reagent (Invitrogen, USA), according to the manufacturer's instructions. Total RNA was reverse transcribed to cDNA using an SYBR® PrimeScript™ RT-PCR Kit (TaKaRa, Japan). The expression level of miR-429 and Slug was determined using the SYBR® PrimeScript™ RT-PCR Kit in conjunction with an ABI 7500 Fast Thermal Cycler (Applied Biosystems, USA). U6 small nuclear RNA was used as an endogenous normalized reference. The experiment was repeated three times, and data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

2.3. Transfection with miRNA mimics or siRNAs

TE-2 and TE-13 cells were selected for further functional study. Oligonucleotides miR-429 mimic and miR-NC used in this study were prepared by Realgene Biotechnology (Nanjing, China). siRNA/Slug and siRNA/control (siRNA-NC) were synthesized by Shanghai Gene-Pharma Co. Ltd. The cells were seeded into 6-well plates with 2×10^5 cells/2 ml/well. Transfection was performed by Lipofectamine 2000 (Invitrogen, USA) according to the instructions.

2.4. Proliferation assays

Cell viability was analyzed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Transfected cells were seeded into 96-well plates (5.0×10^3 cells per well) and cultured for 24–72 h followed by the addition of MTT solution (5 mg/mL) and then further incubated for 4 h at 37 °C. After removing the medium, 100 μL of dimethyl sulfoxide (DMSO) was added into each well. The transfected cells were shaken at a low speed for 10 min, then the absorbance value of each well was determined at 490 nm by an automatic enzyme marker. Three independent experiments were conducted.

2.5. Luciferase reporter assay

The 3′-UTR sequence of Slug was predicted to interact with miR-429 by two established miRNA-target prediction programs (TargetScan, miRanda). The luciferase reporter assay was used to assess the effects of miR-429 on the 3′-UTR of Slug mRNAs by the Dual-Glo Luciferase Assay System (Promega, USA). The pLUC firefly luciferase vectors contained the wild-type and mutant Slug 3′-UTR sequence, respectively. Cells were co-transfected with hsa-miR-429/NC miRNA and the wild-type/mutant 3′-UTR of Slug using Lipofectamine 2000 (Invitrogen, CA, USA). After 48 h, the Dual-Luciferase Reporter Assay System (Promega, USA) was employed to analyze the luciferase activity according to the manufacturer's instructions. The experiment was repeated in triplicate.

2.6. Wound-healing assay

For wound-healing assays, transfected TE-2 and TE-13 cells and control-transfected cells were seeded onto 6-well plates with 2×10^5 cells/well. The cells were cultured to a subconfluent state. After 24 h of starvation in serum-free medium, disinfected pipette tips were used to scratch through the confluent monolayer. After washing three times with PBS, cells were incubated in RPMI-1640 containing 10% FBS. Their migrations were quantified after 48 h under a microscope. All wound-healing assays were conducted in triplicate.

2.7. Transwell migration and invasion assay

The Transwell migration and invasion assay was used to evaluate the metastasis and invasion of transfected cells. For the Transwell migration assay, the above transfected cells were added to the Transwell upper chambers without Matrigel coating at a concentration of 1×10^5/ml. For the Matrigel-coated Transwell invasion assay, Matrigel-coated transwell chambers were used to assess cell invasion according to the manufacturer's instructions (Corning, MA, USA). Precoated Matrigel and the above transfected cells were added to the upper chambers and incubated in serum-free RPMI 1640 in 8-μm pore size Transwell plates (Corning). Every experiment was performed in triplicate. After 48 h incubation at 37 °C in 5% CO_2, the cells in the upper chamber were carefully cleaned with a cotton swab. The lower chamber

was fixed with methanol for 15 min, stained with 1% crystal violet for 5 min, and cells were counted under a microscope.

2.8. Western blotting

Total protein was extracted from cultured cells using RIPA lysis buffer. Protein concentrations were assessed by a BCA protein assay kit (Beyotime, China). Extracted proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes, and incubated with 5% skimmed milk for 1 h. Then the membranes were incubated with antibodies specific to E-cadherin, β-catenin, N-cadherin, Snail, or Slug (Abcam, UK) overnight at 4 °C. GAPDH was used as an endogenous reference. After washing with TBST three times, the corresponding secondary antibodies were added. The membranes were incubated at room temperature for 1 h and then washed with TBST three times. The ECL Luminescence Kit was used for darkroom coloration. Image-J software was used to quantitatively analyze the gray values of electrophoretic stripes in scanning images. Each experiment was repeated three times.

2.9. Nude mice xenografts

miR-429 mimics and miR-NC cells in logarithmic growth stages were collected, digested with trypsin, washed with PBS three times, and suspended in serum-free medium. The cell concentration was adjusted to be 5×10^6/ml. Twenty female nude BALB/c-nu/nu mice aged 4—6 weeks (Jinling Hospital, Nanjing, China) were randomly divided into two groups. The two transfected cell lines were inoculated under the skin of the right side of the posterior flank (0.1 ml each). The growth of tumors was closely observed. The length and short diameter of the tumors were measured and recorded with a Vernier caliper every other day after tumor formation. The mice were killed, and the subcutaneous tumors were excised and measured 5 weeks later. Primary tumors were analyzed by western blotting. The experimental protocol was approved by the ethics committee of Jiangsu Province Medical Association. Tumor volume was determined as $mm^3 = A \times B^2/2$, in which A was the length, and B was the short diameter of the tumor. The growth curve of subcutaneous tumors was plotted according to tumor volume.

2.10. Statistical analysis

SPSS 18.0 software was used for statistical analysis of the results, and each experiment was repeated three times. Student's *t* test was used to compare the mean values of the two samples. All data were expressed as the mean ± standard deviation (SD). The test level value was 0.05, and the difference was statistically significant when $P < 0.05$.

3. Results

3.1. miR-429 is downregulated in ESCC cells and tissues and is correlated with proliferation

The results of quantitative real-time PCR (qRT-PCR) showed that miR-429 was aberrantly downregulated in human esophageal squamous cell lines ECA109, TE-1, TE-2, TE-3, TE-8, TE-12, and TE-13 compared with the normal human esophageal epithelial cell line, HEEC (Fig. 1A, $P < 0.01$). Since the expression levels of miR-429 in TE-2 and TE-13 cells were highly reduced, subsequent experiments were conducted with these lines. To clarify the role of miR-429 in esophageal cancer proliferation, miR-429 mimics or miR-NC were used in TE-2 and TE-13 cells for functional verification. After transfection with miR-429 mimics, the expression of miR-429 in TE-2 and TE-13 cells was significantly increased (Fig. 1B and D). MTT was used to detect cell proliferation, and the cell proliferation capacity of the cells transfected with miR-429 mimics was significantly lower than that of cells transfected with miR-NC (Fig. 1C and E). The results of qRT-PCR showed that the expression level of miR-429 in ESCC tissues was significantly lower than that in paracancerous tissues (Fig. 1F).

3.2. miR-429 regulates migration, invasion, and EMT in EC cells

To detect the effect of miR-429 on migration and invasion of EC cells, transwell assays were used to observe the effect of miR-429 on migration and invasion of TE-2 and TE-13 cells. We found that cells transfected with miR-429 mimics showed significantly reduced cell migration compared to the miR-NC groups (Fig. 2A, $P < 0.01$). Wound-healing assay results showed that after the overexpression of miR-429 in the esophageal cancer cell lines TE-2 and TE-13, the healing rate of scratches was significantly lower than that of NC-transfected cells at 48 h, and the difference was statistically significant (Fig. 2B, $P < 0.01$). EMT is closely related to tumor cell invasion and metastasis. To study the role of miR-429 in EMT, we examined EMT-related molecular markers. Western blotting results revealed that miR-429 overexpression in both TE-2 and TE-13 upregulated the expression of epithelial markers (E-cadherin and β-catenin) and downregulated the expression of mesenchymal markers (N-cadherin and Snail) (Fig. 2C, $P < 0.01$). This finding suggested that miR-429 may be involved in the regulation of EMT-related molecular marker expression in EC cells.

3.3. miR-429 inhibits tumor formation in vivo and the relationship between miR-429 and Slug expression in tissue specimens

The results of functional experiments *in vitro* indicated that upregulation of miR-429 significantly inhibited the migration and invasion of TE-2 and TE-13 cells. Therefore, we further investigated whether miR-429 has similar tumor suppressive function *in vivo*. To study the mechanism of tumor proliferation, a subcutaneous xenograft model was established in nude mice. As shown in Fig. 3A—C, the subcutaneous xenograft tumor model established by esophageal cancer cells transfected with miR-429 mimics had weaker tumor proliferation than the miR-NC group. In general, our results showed that miR-429 suppresses the proliferation of EC. The overexpression of miR-429 also affected the expression of EMT-related molecules *in vivo*, which was consistent with the results of the experiments *in vitro* (Fig. 3D, $P < 0.01$ compared to the control group). In the above study, we detected the expression of miR-429 in esophageal carcinoma, and found that the expression of miR-429 was down-regulated in esophageal carcinoma. The mRNA expression level of Slug gene in 23 cases of ESCC tissues and its paracancerous tissues was detected by qRT-PCR. It was found that the mRNA expression level of Slug gene was significantly higher than that in paracancerous tissues ($P < 0.01$), and the expression level of miR-429 was negatively correlated with the expression level of Slug gene (Figure 3 F and G).

3.4. Slug is a direct target of miR-429 in EC cells

Using bioinformatics programs such as TargetScan, we predicted that Slug was the target gene of miR-429. Luciferase reporter gene analysis showed that miR-429 overexpression significantly inhibited the activity of wild-type luciferase (Fig. 4A). Western blotting results showed that the expression level of Slug protein was significantly lower after transfection of miR-429 into TE-2 and TE-13 cells compared to NC-transfected cells (Fig. 4B). This suggested that miR-429 negatively regulates Slug expression. Western blotting was used to detect the influence of si-Slug on Slug expression. The results showed that the expression of Slug in the si-Slug group was downregulated compared with the control group (Fig. 4C). We examined the effects of Slug on cell migration and invasion using transwell migration and invasion and wound-healing assays. Knockdown of Slug suppressed the migration and invasion of the cells transfected with si-Slug (Fig. 4D). Wound-healing assays revealed that the silencing of Slug suppressed wound healing (Fig. 4E). *In vitro* experiments therefore confirmed that the

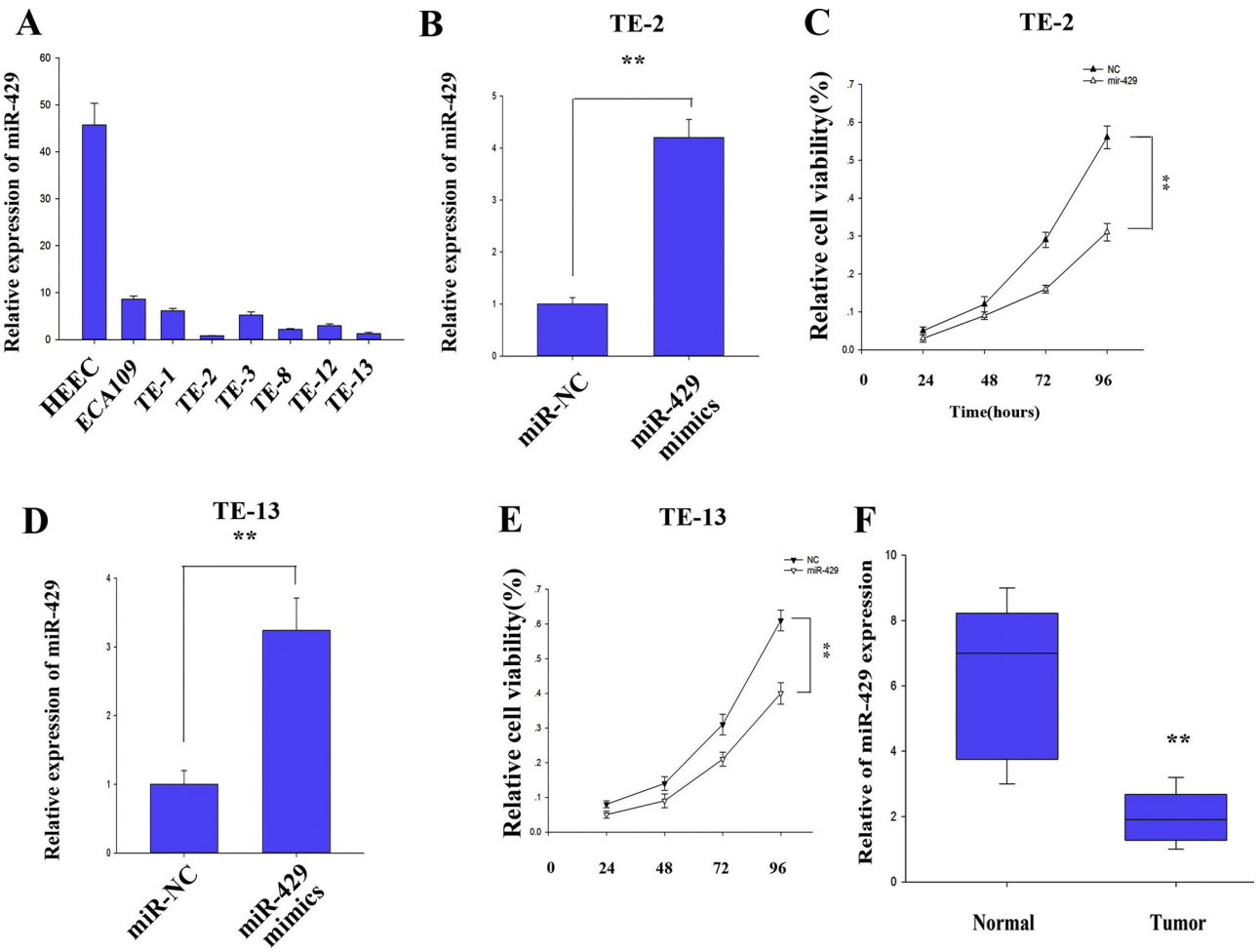

Fig. 1. miR-429 expresses in ESCC cells and tissues and regulates ESCC cell proliferation. (A) Relative miR-429 expression in normal esophageal epithelial cells and EC cell lines was examined by qPCR. (B) Expression levels of miR-429 in TE-2 cells after transfection with miR-NC and miR-429 as detected by qPCR. (C) MTT assays were used to analyze the effect of miR-429 on TE-2 cells. (D) Expression levels of miR-429 in TE-13 cells after transfection with miR-NC and miR-429 as detected by qPCR. (E) MTT assays were used to analyze the effect of miR-429 on TE-13 cells. (F) The expression level of miR-429 in ESCC tissues and paracancerous tissues was detected by qRT-PCR (Fig. 1F).$^{**}P < 0.01$ compared to the control group.

knockdown of Slug had a biological effect similar to the overexpression of miR-429. We further studied whether knockdown of Slug also plays a regulatory role in the EMT process of esophageal cancer cells. Western blot results showed that in Slug-knockdown cells, compared with control cells, the expression levels of epithelial markers (E-cadherin and β-catenin) were significantly increased ($P < 0.01$), while the expression levels of mesenchymal markers (N-cadherin and Snail) were significantly decreased (Fig. 4F, $P < 0.01$). These results suggest that knockdown of the Slug can simulate the effect of miR-429 overexpression on EMT and inhibit the migration and invasion of EC cells *in vitro*.

3.5. Overexpression of Slug partially abrogates the effects of miR-429 upregulation in EC cells

In vitro functional experiments confirmed that the knockdown of Slug can mimic the biological effects of miR-429 overexpression. However, whether miR-429 mainly regulates the invasion and metastasis of EC cells by inhibiting Slug has not been clarified. Therefore, we constructed a Slug-overexpressing plasmid vector and transfected it into TE-2/miR-429 mimic or TE-13/miR-429 mimic cells. MTT assays showed that the proliferation of TE-2/miR-429 mimic or TE-13/miR-429 mimic cells was reduced compared with TE-2/miR-NC or TE-13/miR-NC cells, but the proliferation of TE-2/miR-429 mimic/Slug or TE-13/miR-429 mimic/Slug cells was significantly upregulated (Fig. 5A). Wound healing experiments showed that the wound width of TE-2/miR-429 mimic or TE-13/miR-429 mimic cells was significantly increased compared with TE-2/miR-NC or TE-13/miR-NC cells. However, the wound width of TE-2/miR-429 mimic/Slug or TE-13/miR-429 mimic/Slug cells was significantly reduced (Fig. 5B). In the transwell assays, we also found that overexpression of Slug partially restored miR-429 mimic-induced migration and invasion in both TE-2 and TE-13 cells (Fig. 5C). Moreover, the EMT factors affected by the miR-429 mimic were also suppressed by transfection with the Slug plasmid (Figure 5DEF). These results showed that restoring Slug expression could reverse the inhibitory effects of miR-429 overexpression on EC cell migration, invasion, metastasis, and EMT processes, suggesting that miR-429 regulates these effects by targeting Slug.

4. Discussion

Esophageal cancer is a common malignancy of the digestive system, and China has a high incidence. Clinically, surgery is mainly used in combination with radiotherapy and chemotherapy. However, tumor metastasis results in a low survival rate [4]. It is thus of great significance to elucidate the mechanisms of the occurrence and development of esophageal cancer for prevention and treatment.

Studies have found that miRNAs directly or indirectly participate in the occurrence and development of tumors by regulating a variety of signaling pathways [18]. miRNA-873 acts as a tumor suppressor in

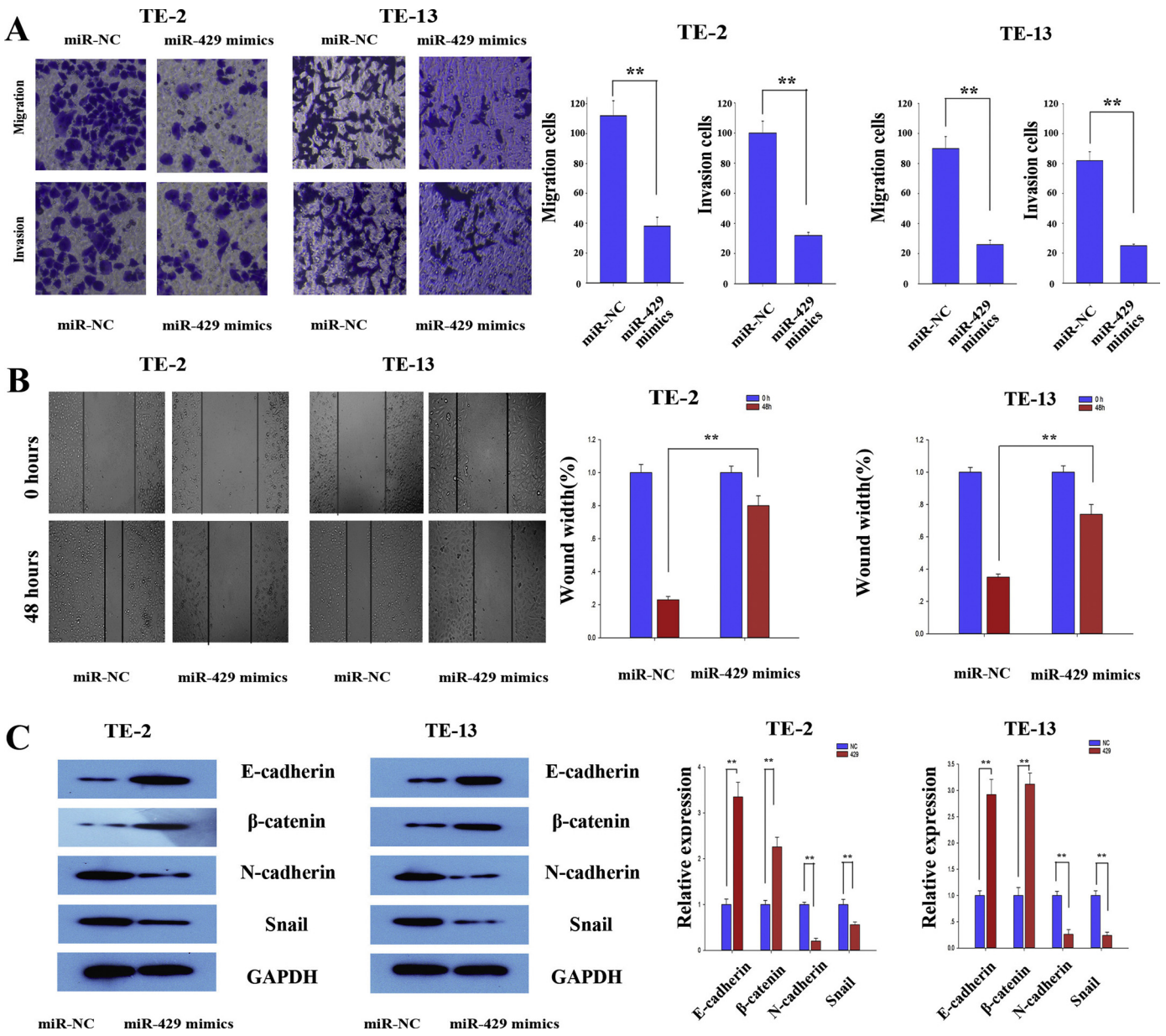

Fig. 2. miR-429 regulates migration, invasion, and EMT in EC cells. (A) Transwell migration and invasion assays were used to evaluate transfected cells. (B) Wound-healing assays were used to evaluate the metastatic ability of transfected cells. (C) Western blots were used to detect the expression levels of EMT-related markers. $^{**}P < 0.01$ compared to the control group.

esophageal cancer by inhibiting differentiated embryonic chondrocyte expressed gene 2 [30]. Downregulation of miR-1 enhances the tumorigenicity and invasiveness of oral squamous cell carcinoma (OSCC) [31]. miR-218-5p is downregulated in invasion front cells and negatively regulates OSCC invasiveness by targeting the CD44-ROCK pathway. Thus, miR-218-5p may serve as a useful therapeutic target for OSCC [32]. The purpose of the present study was to elucidate the relationship between miR-429 and invasion and metastasis of esophageal cancer and its regulatory mechanism.

Studies have shown that miR-429 has abnormal expression levels in various tumors and plays different roles in different tumor types. In gastric cancer, miR-429 acts as a tumor suppressor by targeting FSCN1, suggesting that miR-429 and FSCN1 could both be potential therapeutic targets [33]. In colorectal cancer, miR-429 exerts an oncogenic effect by directly targeting HOXA5, a transcription factor of the HOX family that is involved in cancer's development and progression [34]. In this study, we found that the expression of miR-429 in esophageal cancer cells was lower than that in normal esophageal epithelial cells, and the overexpression of miR-429 resulted in decreased proliferation, invasion, and metastasis of esophageal cancer cells and inhibited tumorigenesis in nude mice *in vivo*. Therefore, we concluded that miR-429 plays a role as a tumor suppressor gene in the occurrence and development of esophageal cancer, though the specific mechanism remained to be studied.

EMT was initially discovered to be a critical stage of embryonic development but has been found to play an extremely important role in tumor invasion and metastasis [35–37]. miRNAs, which are key factors in the post-transcriptional regulation of gene expression, play an important role in malignant tumor progression. Studies have shown that miRNAs also play a regulatory role in the development of EMT, which has potential diagnostic and therapeutic implications for tumor metastasis [38,39]. In this study, we found that upregulated miR-429 expression resulted in increased expression of E-cadherin and β-catenin, decreased expression of N-cadherin and Snail, and significantly reduced the number of cells passing through the basement membrane. This

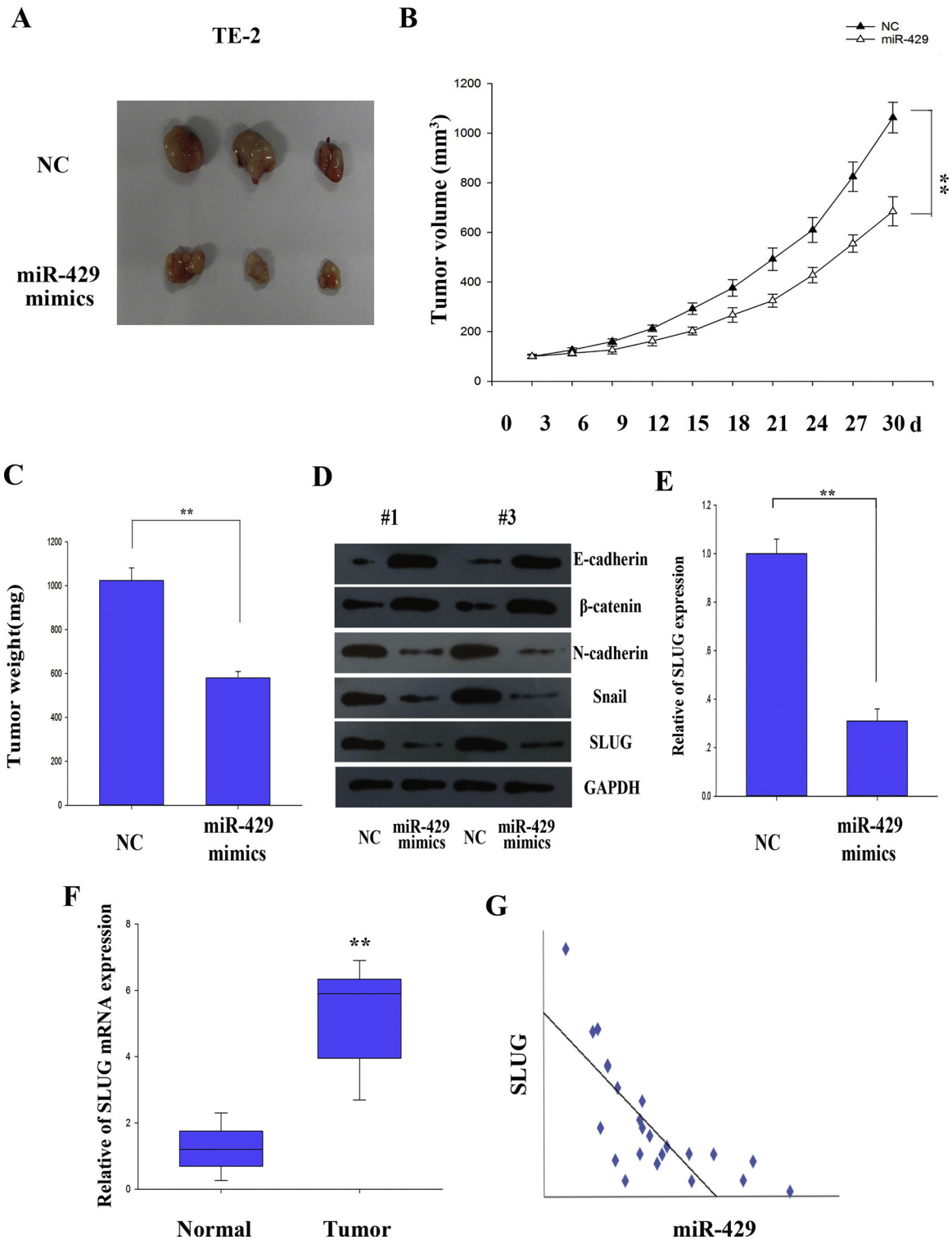

Fig. 3. miR-429 inhibits tumor formation *in vivo*and the relationship between miR-429 and Slug expression in tissue specimens.(A) Overexpression of miR-429 attenuated tumorigenesis. (B) Volumes of xenograft tumors in nude mice were measured every three days. (C) Average weights of xenograft tumors of nude mice were lower in the miR-429 mimic group than in the miR-NC group. (D) The overexpression of miR-429 affected the expression of EMT-related molecules *in vivo*. (F) The mRNA expression level of Slug gene in 23 cases of ESCC tissues and its paracancerous tissues was detected by qRT-PCR (G) Analysis of correlation between Slug and miR-429 expression levels in 23 pairs of ESCC tissue specimens $^{**}P < 0.01$ compared to the control group.

indicated that the number of invasive and metastatic cells was significantly reduced, suggesting that miR-429 regulates EMT.

To further study the mechanism of miR-429 in esophageal cancer, we predicted possible target genes of miR429 through TargetScan and other programs and found that miR-429 potentially targets Slug. Luciferase reporter gene assays and western blotting verified this. The results showed that overexpression of miR-429 significantly inhibited the activity of wild-type luciferase but had no effect on the activity of mutant luciferase, and the overexpression of miR-429 inhibited the expression of Slug. Therefore, we speculate that miR-429 may regulate

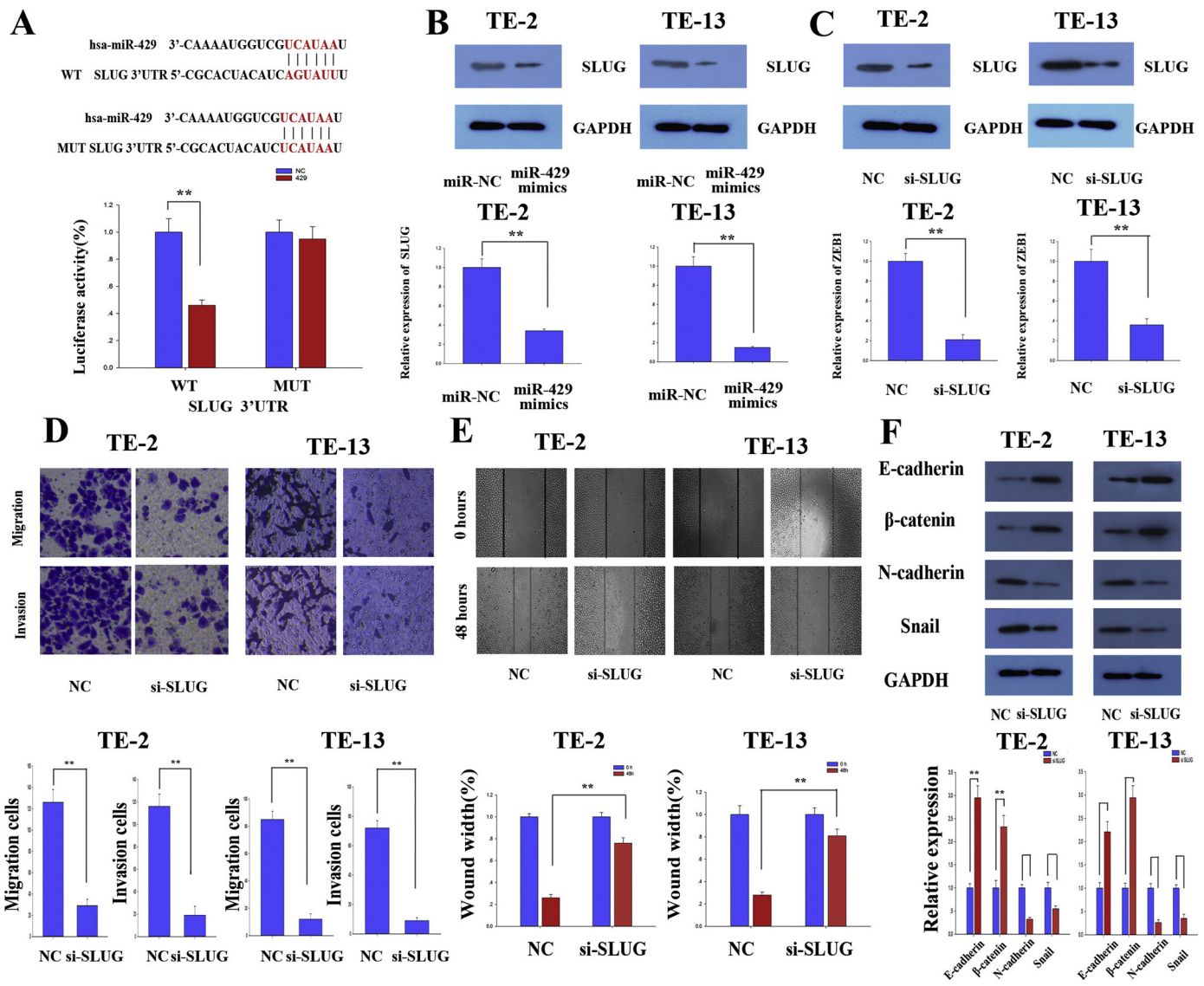

Fig. 4. Slug is a direct target of miR-429 in EC cells. (A) The 3′-UTR sequence of Slug was predicted to interact with miR-429 by two established miRNA-target prediction programs. The dual-luciferase reporter assay showed that luciferase activity was significantly inhibited in esophageal cancer cells when co-transfected with miR-429 mimics and pGL3-Slug vector. (B) Western blotting showed that the expression of Slug was inhibited after transfection with miR-429 mimics in TE-2 and TE-13 cells. (C) The expression levels of Slug in TE-2 and TE-13 cells after transfection with si-Slug were detected by western blot. (D) Knockdown of Slug suppressed the migration and invasion of cells transfected with si-Slug. (E) Wound-healing assays evaluated the metastatic ability of transfected cells. (F) Western blotting was used to detect the expression levels of EMT-related molecular markers in transfected cells. **P < 0.01 compared to the control group.

Slug by binding with its 3′-UTR, though the specific mechanism remains to be further studied.

Slug encodes a zinc finger protein in the transcription factor Snail family and is significantly upregulated in a variety of malignant tumors. Slug is related to the occurrence and angiogenesis of malignant tumors and is also a key transcription factor and marker of EMT [40,41]. Our results showed that interfering with Slug expression could cause a decrease in the proliferation, invasion, and metastasis of esophageal cancer cells, lead to an increase in the expression of E-cadherin and β-catenin and a decrease of N-cadherin and Snail, which is consistent with previous research. To further study the mechanism of miR-429 regulation of Slug expression, we conducted restoration experiments. We found that overexpressing Slug partially reversed the inhibitory effects of miR-429 overexpression on proliferation, migration, invasion, metastasis, and EMT of esophageal cancer cells, suggesting that miR-429 regulates these effects by targeting Slug. Therefore, our results suggest that Slug may be the functional target of miR-429.

In conclusion, the overexpression of miR-429 in esophageal cancer cells downregulated their invasion and metastasis ability, Slug is a target of miR-429, and the high expression of Slug induced the occurrence of EMT and enhanced invasion and metastasis. This study confirms for the first time a direct targeting effect of miR-429 on the EMT-related transcription factor Slug. This indicates that miR-429 plays an important role in the development of esophageal cancer, though the function of Slug as regulated by miR-429 requires further study.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Acknowledgments

This study was supported by the Jiangsu Natural Science Foundation of China (No. BK20161387). We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

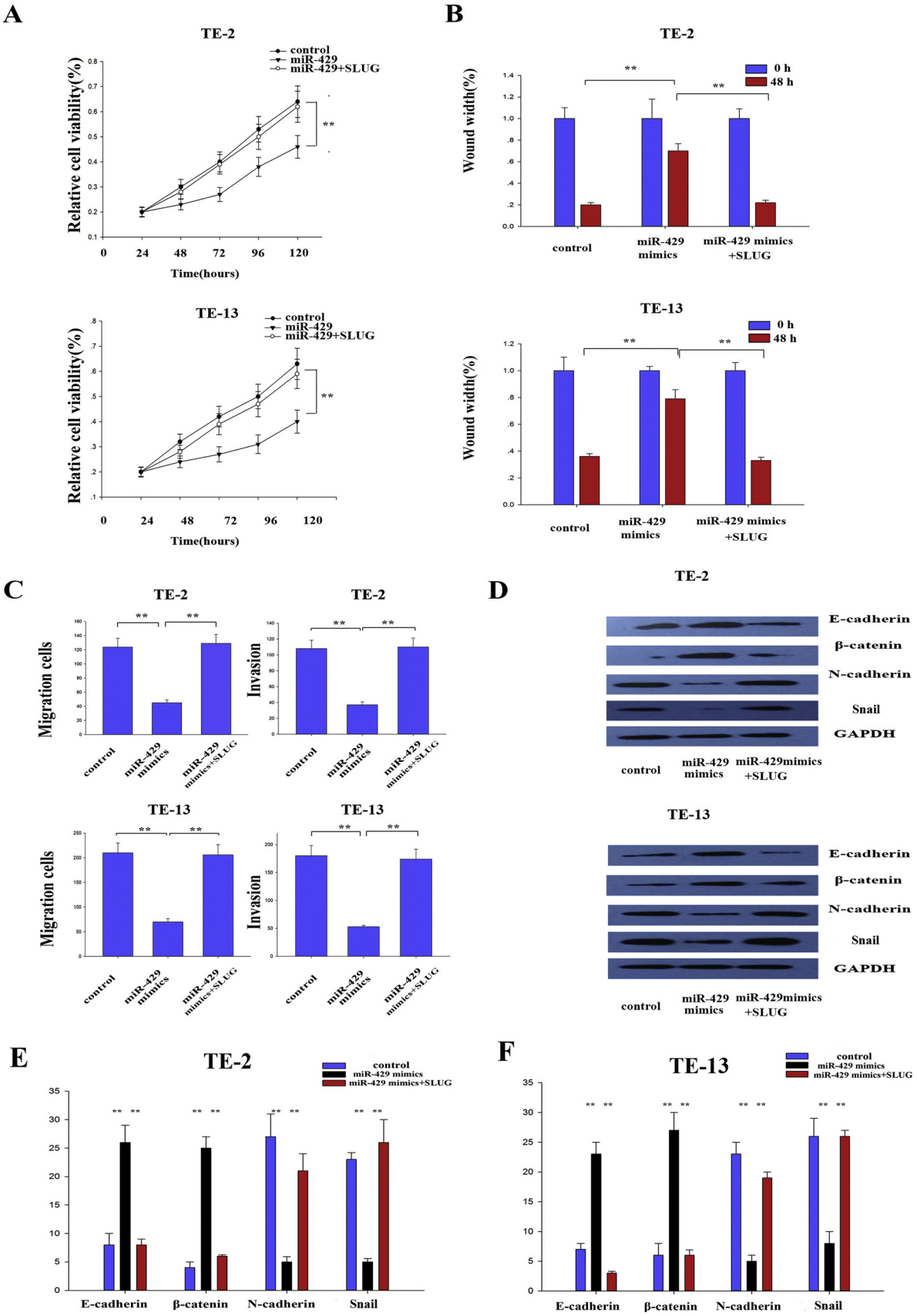

Fig. 5. Overexpression of Slug partially abrogated the effects of miR-429 upregulation on EC cells. (A) Transfection with Slug and miR-429 mimics reversed the growth inhibition by miR-429 mimics. (B) Transfection with Slug and miR-429 mimics increased the metastatic ability inhibited by miR-429 mimics. (C) Transfection with Slug and miR-429 mimics significantly enhanced the average number of migrating and invading cells. (DEF) Overexpression of Slug reversed the changes of EMT-related molecular markers in EC cells induced by miR-429 upregulation.

References

[1] L.A. Torre, F. Bray, R.L. Siegel, J. Ferlay, J. Lortet-Tieulent, A. Jemal, Global cancer statistics, 2012, CA Cancer J. Clin. 65 (2015) 87–108, https://doi.org/10.3322/caac.21262 PMID:25651787.

[2] W. Chen, R. Zheng, P.D. Baade, S. Zhang, H. Zeng, F. Bray, A. Jemal, He J. Yu XQ, Cancer statistics in China, 2015, CA Cancer J. Clin. 66 (2016) 115–132, https://doi.org/10.3322/caac.21338 PMID:26808342.

[3] Y.J. Bang, E. Van Cutsem, A. Feyereislova, H.C. Chung, L. Shen, A. Sawaki, F. Lordick, A. Ohtsu, Y. Omuro, T. Satoh, G. Aprile, E. Kulikov, J. Hill, M. Lehle, J. Rüschoff, Y.K. Kang, ToGA Trial Investigators. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomized controlled trial, Lancet 9742 (2010) 687–697, https://doi.org/10.1016/S0140-6736(10)61121-X PMID:20728210.

[4] Y. Lin, Y. Totsuka, Y. He, S. Kikuchi, Y. Qiao, J. Ueda, W. Wei, M. Inoue, H. Tanaka, Epidemiology of esophageal cancer in Japan and China, J. Epidemiol. 27 (2013) 233–242 PMID:23629646 PMCID:PMC3709543.

[5] M. Guarino, Epithelial-mesenchymal transition and tumour invasion, Int. J. Biochem. Cell Biol. 39 (2007) 2153–2160, https://doi.org/10.1016/j.biocel.2007.07.011 PMID:17825600.

[6] J.P. Thiery, H. Acloque, R.Y. Huang, M.A. Nieto, Epithelial-mesenchymal transitions in development and disease, Cell 139 (2009) 871–890, https://doi.org/10.1016/j.cell.2009.11.007 PMID:19945376.

[7] S.A. Mani, W. Guo, M.J. Liao, E.N. Eaton, A. Ayyanan, A.Y. Zhou, M. Brooks, F. Reinhard, C.C. Zhang, M. Shipitsin, L.L. Campbell, K. Polyak, C. Brisken, J. Yang, R.A. Weinberg, The epithelial-mesenchymal transition generates cells with properties of stem cells, Cell 133 (2008) 704–715, https://doi.org/10.1016/j.cell.2008.03.027 PMID:18485877 PMCID:PMC2728032.

[8] J. Yang, R.A. Weinberg, Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis, Dev. Cell 14 (2008) 818–829, https://doi.org/10.1016/j.devcel.2008.05.009 PMID:18539112.

[9] R. Kalluri, R.A. Weinberg, The basics of epithelial-mesenchymal transition, J. Clin. Invest. 119 (2009) 1420–1428, https://doi.org/10.1172/JCI39104 PMID:19487818 PMCID:PMC2689101.

[10] W.R. Luo, X.Y. Chen, S.Y. Li, A.B. Wu, K.T. Yao, Neoplastic spindle cells in nasopharyngeal carcinoma show features of epithelial-mesenchymal transition, Histopathology 61 (2012) 113–122, https://doi.org/10.1111/j.1365-2559.2012.04205.x PMID:22486228.

[11] J.P. Thiery, Epithelial-mesenchymal transitions in tumour progression, Nat. Rev. Cancer 2 (2002) 442–454, https://doi.org/10.1038/nrc822 PMID:12189386.

[12] T. Anh Tran, K. Krishnamoorthy, Y.W. Song, S.K. Cho, S.J. Kim, Toxicity of nano molybdenum trioxide toward invasive breast cancer cells, ACS Appl. Mater. Interfaces 6 (2014) 2980–2986, https://doi.org/10.1021/am405586d PMID:24417578.

[13] P. Jethwa, M. Naqvi, R.G. Hardy, N.A. Hotchin, S. Roberts, R. Spychal, C. Tselepis, Overexpression of Slug is associated with malignant progression of esophageal adenocarcinoma, World J. Gastroenterol. 14 (2008) 1044–1052 PMID:18286686 PMCID:PMC2689407.

[14] B. Uygur, W.S. Wu, SLUG promotes prostate cancer cell migration and invasion via CXCR4/CXCL12 axis, Mol. Cancer 10 (2011) 139, https://doi.org/10.1186/1476-4598-10-139 PMID:22074556 PMCID:PMC3226635.

[15] M. Emadi Baygi, Z.S. Soheili, F. Essmann, A. Deezagi, R. Engers, W. Goering, W.A. Schulz, Slug/SNAI2 regulates cell proliferation and invasiveness of metastatic prostate cancer cell lines, Tumour Biol. 31 (2010) 297–307, https://doi.org/10.1007/s13277-010-0037-5 PMID:20506051.

[16] P. Molina-Ortiz, A. Villarejo, M. MacPherson, V. Santos, A. Montes, S. Souchelnytskyi, F. Portillo, A. Cano, Characterization of the SNAG and SLUG domains of Snail2 in the repression of E-cadherin and EMT induction: modulation by serine 4 phosphorylation, PLoS One 7 (2012) e36132, https://doi.org/10.1371/journal.pone.0036132 PMID:22567133 PMCID:PMC3342263.

[17] M. Canel, A. Serrels, M.C. Frame, V.G. Brunton, E-cadherin-integrin crosstalk in cancer invasion and metastasis, J. Cell. Sci. 126 (2013) 393–401, https://doi.org/10.1242/jcs.100115 PMID:23525005.

[18] I. Berindan-Neagoe, C. Monroig Pdel, B. Pasculli, G.A. Calin, MicroRNAome genome: a treasure for cancer diagnosis and therapy, CA Cancer J. Clin. 64 (2014) 311–336, https://doi.org/10.3322/caac.21244 PMID:25104502 PMCID:PMC4461198.

[19] D.P. Bartel, MicroRNAs: target recognition and regulatory functions, Cell 136 (2009) 215–233, https://doi.org/10.1016/j.cell.2009.01.002 PMID:19167326 PMCID:PMC3794896.

[20] V. Ambros, The functions of animal microRNAs, Nature 431 (2004) 350–355, https://doi.org/10.1038/nature02871 PMID:15372042.

[21] E.L. Paterson, J. Kazenwadel, A.G. Bert, Y. Khew-Goodall, A. Ruszkiewicz, G.J. Goodall, Down-regulation of the miRNA-200 family at the invasive front of colorectal cancers with degraded basement membrane indicates EMT is involved in cancer progression, Neoplasia 15 (2013) 180–191 PMID:23441132 PMCID:PMC3579320.

[22] M.J. Schliekelman, D.L. Gibbons, V.M. Faca, C.J. Creighton, Z.H. Rizvi, Q. Zhang, C.H. Wong, H. Wang, C. Ungewiss, Y.H. Ahn, D.H. Shin, J.M. Kurie, S.M. Hanash, Targets of the tumor suppressor miR-200 in regulation of the epithelial-mesenchymal transition in cancer, Cancer Res. 71 (2011) 7670–7682, https://doi.org/10.1158/0008-5472.CAN-11-0964 PMID:21987723 PMCID:PMC3419137.

[23] S.M. Park, A.B. Gaur, E. Lengyel, M.E. Peter, The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2, Genes Dev. 22 (2008) 894–907, https://doi.org/10.1101/gad.1640608 PubMed PMID: 18381893; PubMed Central PMCID: PMC2279201.

[24] P.A. Gregory, A.G. Bert, E.L. Paterson, S.C. Barry, A. Tsykin, G. Farshid, M.A. Vadas, Y. Khew-Goodall, G.J. Goodall, The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1, Nat. Cell Biol. 10 (2008) 593–601, https://doi.org/10.1038/ncb1722 PubMed PMID: 18376396.

[25] W. Zhu, J. He, D. Chen, B. Zhang, L. Xu, H. Ma, X. Liu, Y. Zhang, H. Le, Expression of miR-29c, miR-93, and miR-429 as potential biomarkers for detection of early stage non-small lung cancer, PLoS One 9 (2014) e87780, https://doi.org/10.1371/journal.pone.0087780 PMID:24523873 PMCID:PMC3921142.

[26] Y. Sun, S. Shen, X. Liu, H. Tang, Z. Wang, Z. Yu, X. Li, M. Wu, MiR-429 inhibits cells growth and invasion and regulates EMT-related marker genes by targeting Onecut2 in colorectal carcinoma, Mol. Cell. Biochem. 390 (2014) 19–30, https://doi.org/10.1007/s11010-013-1950-x PMID: 24402783 PMCID:PMC3972435.

[27] F. Wang, C. Jiang, Q. Sun, F. Yan, L. Wang, Z. Fu, T. Liu, F. Hu, Downregulation of miR-429 and inhibition of cell migration and invasion in nasopharyngeal carcinoma, Mol. Med. Rep. 13 (2016) 3236–3242, https://doi.org/10.3892/mmr.2016.4940 PMID:26936585.

[28] C.L. Wu, J.Y. Ho, S.C. Chou, D.S. Yu, MiR-429 reverses epithelial-mesenchymal transition by restoring E-cadherin expression in bladder cancer, Oncotarget 7 (2016) 26593–26603, https://doi.org/10.18632/oncotarget.8557 PMID:27058893 PMCID:PMC5042001.

[29] Y. Wang, M. Li, W. Zang, Y. Ma, N. Wang, P. Li, T. Wang, G. Zhao, MiR-429 up-regulation induces apoptosis and suppresses invasion by targeting Bcl-2 and SP-1 in esophageal carcinoma, Cell. Oncol. (Dordr.) 36 (2013) 385–394, https://doi.org/10.1007/s13402-013-0144-6 PMID: 23999873.

[30] Y. Liang, P. Zhang, S. Li, H. Li, S. Song, B. Lu, MicroRNA-873 acts as a tumor suppressor in esophageal cancer by inhibiting differentiated embryonic chondrocyte expressed gene 2, Biomed. Pharmacother. 105 (2018) 582–589, https://doi.org/10.1016/j.biopha.2018.05.152 PMID:29890466.

[31] C.Y. Peng, Y.W. Liao, M.Y. Lu, C.H. Yu, C.C. Yu, M.Y. Chou, Downregulation of miR-1 enhances tumorigenicity and invasiveness in oral squamous cell carcinomas, J. Formos. Med. Assoc. 116 (2017) 782–789, https://doi.org/10.1016/j.jfma.2016.12.003 PMID:28089494.

[32] X. Li, J. He, M. Shao, B. Cui, F. Peng, J. Li, Y. Ran, D. Jin, J. Kong, J. Chang, L. Duan, X. Yang, Y. Luo, Y. Lu, B. Lin, T. Liu, Downregulation of miR-218-5p promotes invasion of oral squamous cell carcinoma cells via activation of CD44-ROCK signaling, Biomed. Pharmacother. 106 (2018) 646–654, https://doi.org/10.1016/j.biopha.2018.06.151 PMID:29990854.

[33] M. Zhang, B.B. Dong, M. Lu, M.J. Zheng, H. Chen, J.Z. Ding, A.M. Xu, Y.H. Xu, miR-429 functions as a tumor suppressor by targeting FSCN1 in gastric cancer cells, Onco-Targets Ther 9 (2016) 1123–1133, https://doi.org/10.2147/OTT.S91879 PMID:27042104 PMCID:PMC4780435.

[34] Y. Han, Q. Zhao, J. Zhou, R. Shi, miR-429 mediates tumor growth and metastasis in colorectal cancer, Am. J. Cancer Res. (2017) 7 218-7233. PMID:28337372 PMCID:PMC5336497.

[35] M.A. Nieto, Epithelial plasticity: a common theme in embryonic and cancer cells, Science 342 (2013) 1234850, https://doi.org/10.1126/science.1234850 PMID:24202173.

[36] L. Chen, W. Jian, L. Lu, L. Zheng, Z. Yu, D. Zhou, Elevated expression of E-cadherin in primary breast cancer and its corresponding metastatic lymph node, Int. J. Clin. Exp. Med. 8 (2015) 11752–11758 PMID:26380015 PMCID:PMC4565398.

[37] F. Trillsch, S. Kuerti, C. Eulenburg, E. Burandt, L. Woelber, K. Prieske, K. Eylmann, L. Oliveira-Ferrer, K. Milde-Langosch, S. Mahner, E-Cadherin fragments as potential mediators for peritoneal metastasis in advanced epithelial ovarian cancer, Br. J. Cancer 114 (2016) 213–220, https://doi.org/10.1038/bjc.2015.436 PMID:26757261 PMCID:PMC4815811.

[38] L. Li, M.Y. Shao, S.C. Zou, Z.F. Xiao, Z.C. Chen, MiR-101-3p inhibits EMT to attenuate proliferation and metastasis in glioblastoma by targeting TRIM44, J. Neurooncol. 141 (2019) 19–30, https://doi.org/10.1007/s11060-018-2973-7 PMID:30539341.

[39] C. Zhang, Y. Liang, M.H. Ma, K.Z. Wu, C.D. Zhang, D.Q. Dai, Downregulation of microRNA-376a in gastric Cancer and association with poor prognosis, Cell. Physiol. Biochem. 51 (2018) 2010–2018, https://doi.org/10.1159/000495820 PMID:30522118.

[40] W. Zhang, J. Sun, J. Chen, C. Xu, L. Zhang, Downregulation of miR-95 in gastric cancer promotes EMT via regulation of Slug, thereby promoting migration and invasion, Oncol. Rep. 41 (2019) 1395–1403, https://doi.org/10.3892/or.2018.6911 PMID:30535480.

[41] J. Wu, Y. Zhang, R. Cheng, W. Gong, T. Ding, Q. Zhai, Y. Wang, B. Meng, B. Sun, Expression of EMT regulators TWIST, SLUG, and SNAIL in follicular thyroid tumours may relate to widely invasive, poorly differentiated, and distant metastasis, Histopathology 74 (2019) 780–791, https://doi.org/10.1111/his.13778 PMID:30368884.