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Original Article

TRF-20-M0NK5Y93 suppresses the metastasis of colon cancer cells by impairing the epithelial-to-mesenchymal transition through targeting Claudin-1

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Abstract: tRNA-derived fragments (tRFs) are derived from corresponding tRNAs and have been shown by several studies to be novel biological markers for tumour diagnosis and therapy. However, until now, the effects of tRFs on the progression of colorectal cancer (CRC) and especially on the epithelial-to-mesenchymal transition (EMT) have remained unknown. Our study aimed to assess CRC-related tRFs and examine the effects of key tRFs on CRC progression and related mechanisms. After hypoxic treatment, tRF sequencing and real-time PCR assays were performed to identify key tRFs. Then, functional tests were designed to verify the effects and evaluate the mechanism after cell transfection under normoxic conditions. A total of 14 tRFs were differentially expressed in the hypoxia and control groups. Based on the results of PCR assay verification and conditional selection, tRF-20-M0NK5Y93 could be a promising target for exploration, as its expression was significantly lower under hypoxic conditions than under control conditions. tRF-20-M0NK5Y93 inhibited CRC cell migration and invasion partly by targeting Claudin-1, an EMT-related molecule. The results of the present study suggest that tRF-20-M0NK5Y93 promotes CRC cell migration and invasion partly by regulating Claudin-1 during EMT.

Keywords: Colorectal cancer, tRNA-derived fragments, epithelial-to-mesenchymal transition, Claudin-1, hypoxia

Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide. It ranks third in incidence rate among malignant tumours and second among the leading causes of cancer-related death [1, 2]. Although therapeutic methods have improved, invasion and metastasis are the main sources of relapse, poor prognosis and the low long-term survival rate of CRC. In addition, approximately one-third of patients with CRC have liver and lung metastases [3]. Tumour metastasis is a series of complex processes involving multifactor participation, multistep development and multiple gene changes [4]. Among these possible mechanisms, epithelial-to-mesenchymal transition (EMT) is a complex process, characterized by the transformation of epithelial cells into mesenchymal cells. The initiation and development of EMT involve complex interactions between transcriptional regulator networks and signalling pathways. The hallmark of EMT is the downregulation of E-cadherin, which is a representative epithelial marker regulated by a panel of EMT transcription factors [5]. To improve the overall survival of CRC, we must identify new biomarkers to develop better clinical interventions and improve patient prognosis.

Recently, tRNA-derived fragments (tRFs) have attracted much attention as a new class of small non-coding RNAs. Under stress conditions such as hypoxia, mature tRNA or pre-tRNA can be specifically cleaved to generate tRFs or tRNA half molecules (tRNA halves, tiRNA). tRFs are produced by precise biogenetic processes, with specific nucleotide composi-

tion, biogenesis and physiological function [6, 7]. tRFs are approximately 18-24 nucleotides in length, and they can be divided into four classes (tRF-5, tRF-3, tRF-1 and i-tRF) depending on their corresponding positions on the tRNA. Previous studies have shown that tRFs are significantly related to malignant cellular proliferation and apoptosis in several cancer types, whereas their significance in the invasion and metastasis of CRC is not understood [7-13]. Notably, one recent study identified a novel class of tRFs suppressing the stability of multiple carcinogenic transcripts to inhibit cancer progression by interacting with the RNA-binding protein YBX1 in breast cancer [10]. In the present study, we explored the specific roles and regulatory mechanisms of particular tRFs in the EMT of CRC. We investigated whether tRFs might play a role in metastasis progression in CRC. We speculated that tRFs could play a role similar to certain specific miRNAs [14]. Because cells are prone to major stress, such as hypoxia, during cancer progression, and hypoxia is a factor for producing tRFs under stress, we hypothesized that induced tRFs play a role in inhibiting CRC progression under hypoxic conditions might. In our study, using high-throughput sequencing and real-time PCR, we identified tRF-20-M0NK5Y93, which was downregulated under hypoxic conditions in CRC cells. Therefore, to simulate the change in tRF-20-M0NK5Y93 under hypoxic conditions, we transfected CRC cells with inhibitors of tRF-20-M0NK5Y93 under normoxic conditions and found that invasion and metastasis abilities were enhanced, and EMT-related indicators changed accordingly; for example, Claudin-1 was upregulated in the transfected cells versus that in the control cells.

Claudin-1, as one of the crucial components in the tight junction protein family, is distributed on the surface of the cell membrane and is associated with tumour metastasis [15, 16]. It has also been demonstrated that Claudin-1 is an EMT-related marker that correlates with the regulation of cellular transformation, MMP activation and metastasis [17]. Claudin-1 plays different roles in different cancer cells, promoting or suppressing cancer progression. For instance, a previous study on gastric cancer showed that Claudin-1 was highly expressed and responsible for tumour invasion and metastasis: correspondingly treatment with a Claudin-1 inhibitor reversed cell proliferation and metastasis in vitro and in vivo [18, 19]. In CRC, Claudin-1 plays a crucial role in the regulation of cellular transformation and is potentially associated with invasion and metastasis [20, 21]. Thus, the effects of Claudin-1 on EMT in CRC have not been fully elucidated in human CRC cells. Our study verified that Claudin-1 could promote the EMT in vitro.

At present, the understanding of tRF-mediated cancer progression is still nascent. There are few studies on tRFs related to cancer invasion and metastasis, especially in CRC. In the present study, we identified tRF-20-M0NK5Y93, whose expression is downregulated in CRC cells under hypoxic conditions compared with normoxic conditions. We subsequently knocked down tRF-20-M0NK5Y93 in RKO and SW480 cell lines, which promoted the invasion and migration of these cells. Furthermore, Claudin-1 was verified as a direct target of tRF-20-M0NK5Y93. These results indicate that tRF-20-M0NK5Y93 might act as a tumour suppressor in CRC progression and is expected to be a novel potential therapeutic target.

Materials and methods

Cell culture

The human CRC cell lines (RKO, SW480) were obtained from the American Type Culture Collection (ATCC). Both cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Life Technologies, USA) supplemented with 10% FBS (fetal bovine serum, Gibco, Life Technologies, USA) containing 1% penicillin-streptomycin (Invitrogen, Carlsbad, USA). All cells were incubated in a CO_2 constant temperature incubator (37°C, 21% O_2, 5% CO_2) (Thermo Fisher Scientific, Rockford, IL, USA) for normal oxygen culture or in a hypoxic incubator (37°C, 1% O_2, 5% CO_2) for hypoxic culture for 24 h.

Cell transfections

RKO and SW480 cells were passaged. When the cells reached 70-80% confluence, the cells were ready for transfection. tRF-20-M0NK5Y93 inhibitor (5'-ACCCACAAUCCCCAG-

Table 1. Primer sequences for qRT-PCR

Gene	Primer Sequence (5'-3')
TRF-20-M0NK5Y93	F: ACTGGATACGACA
	R: GGAGCTGGGG
U6	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT

CUCCG-3') and negative control (NC-inhibitor, 5'-UUCUCCGAACGAGUCACGUTT-3') were purchased from GenePharma (Shanghai, China). The siRNA targeting Claudin-1 and NC-siRNA for transfection were prepared by GenePharma. Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc.) was employed to perform transient transfection of the plasmid into RKO and SW480 cells according to the manufacturer's instructions. Cells were harvested for further experiments at 24 h after transfection. The sequences for the siRNA Claudin1 were as follows: siRNA-1 sense, 5'-GCAAAGUCUUUGACUCCUUTT-3', siRNA-1 antisense, 5'-AAGGAGUCAAAGACUUUGCTT-3'; siRNA-2 sense, 5'-CCACAGCAUGGUAUGGCAATT-3', siRNA-2 antisense, 5'-UUGCCAUACCAUGCUGUGGTT-3'; siRNA-3 sense, 5'-GGUGCCCUACUUUGCUGUUTT-3', siRNA-3 antisense, 5'-AACAGCAAAGUAGGGCACCTT-3'. NC-siRNA sense: UUCUCCGAACGAGUCACGUTT, NC-siRNA antisense: ACGUGACUCGUUCGGAGAATT.

Western blot analysis

CRC cells were washed with PBS and then chilled on ice in RIPA buffer (Sigma-Aldrich, Shanghai, China) containing proteinase inhibitors. Then, sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was used to perform vertical electrophoresis. The gel was loaded with twenty micrograms of protein separated by SDS-PAGE. Then, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The nitrocellulose membrane was blocked in a solution containing 5% nonfat dry milk powder, shaken slowly on a shaker, and sealed for two hours. After that, the blocked PVDF membrane was removed, washed three times, and transferred to be incubated in the diluted primary antibody solution at 4°C overnight. The primary antibodies included rabbit anti-Claudin-1: ab13255, anti-matrix metalloprotein-2 (MMP-2): ab40994, anti-matrix metalloprotein-7 (MMP-7): ab71031, anti-Fibronectin: 15613, anti-HIF-1a: 36169, anti-Twist1: ab-49254, anti-Twist2: 66544, anti-E-Cadherin: sc-8426, anti-N-Cadherin: 13116, anti-Vimentin: 5741, anti-Slug: 9585, anti-ZEB1: 3396 and anti-Snail: ab3879. Then, the membranes were transferred into 1x PBST, to which goat anti-rabbit IgG secondary antibody (ab7074; abcam, USA) was added and incubated on a shaker for two hours. The nitrocellulose membranes were placed in the mixed liquid from the ECL kit (Bio-Rad, Cal, USA) and then exposed with the chemical photosensitive mode on the exposure plate of the instrument. The images were exported and analysed for optical density using ImageJ software (USA).

Quantitative real-time PCR (qRT-PCR)

The cells (transfected cell lines including RKO and SW480) were collected in a 1.5-ml RNase-free Eppendorf (EP) tube, and 500 μl of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added, mixed well and incubated for 5 min to allow the cells to fully lyse. The tRFs were reverse-transcribed using murine leukaemia virus (MMLV) (Promega, Madison, WI, USA). The forward primers and reverse primers of tRFs were designed and synthesized (**Table 1**). qRT-PCR amplifications were performed using SYBR Green qPCR Master Mix (ABI, 4367659) and an ABI 7300 real-time PCR detection system.

Transwell migration and invasion assays

For the Transwell assays, RKO and SW480 cells transfected with plasmids were collected and starved for 24 h in serum-free culture solution. Then, for the invasion experiment, 20 μl of Matrigel mixture (mixed with Matrigel and DMEM 1:1) was pre-plated in the upper layer of the chamber and placed at 37°C for 30 min until the gel solidified. Note that the Matrigel was melted into a liquid state in advance, and it was not required for the migration experiment. After activation of the chamber, the medium used for activation was removed, 800 μl of medium containing 10% FBS, functioning as a chemoattractant, was added to the lower chamber, and 300 μl cell suspensions were added to the upper layer of the chamber. After the cultured cells were incubated at 37°C for 24 hours, the cells were fixed in methanol for 20 min and subjected to crystal violet staining. Images were captured

under a microscope (OLYMPUS CKX53). The number of cells passing through the membrane was measured to assess migration and invasion ability. This experiment was repeated three times.

Cellular proliferation

MTT assays were performed to detect cell proliferation. First, tRF-20-M0NK5Y93 inhibitor and its corresponding NC control were transfected into RKO and SW480 cells for 24 h. The cells were digested and harvested, reseeded in 10% FBS DMEM, and plated in 96-well plates. At 24, 48, 72 and 96 h after incubation, 50 μl of MTT (1 mg/ml) reagent (Sigma) was added to each well and incubated further for 2-4 h. Then, 150 μl of DMSO (Sigma) was added to dissolve the purple crystals, and the absorbance was read at 570 nm.

Dual-luciferase reporter assay

RKO cells were seeded in 24-well plates and cultured for 24 h (80% confluency). The Claudin-1 wild-type or mutant 3'-UTR plasmid and tRF-20-M0NK5Y93 inhibitor or tRF-20-M0NK5Y93 NC plasmid (Promega, USA) were co-transfected into cells for 24 h. Luciferase assays were performed with reference to the specifications of the dual-luciferase reporter assay system (Promega, USA).

Statistical analysis

Data analyses were performed using SPSS 20 software (GraphPad Software Inc., USA). The data are presented as the mean ± standard deviation. Student's t-test was performed to detect the differences in measurement data between the two groups. Statistical significance among multiple groups was assessed by one-way ANOVA. When $P < 0.05$, the difference was considered statistically significant.

Results

Hypoxia-induced separation of tRF-20-M0NK5Y93

Changes in the tumour microenvironment, such as hypoxia, nutrient deficiency, low pH, inflammatory factors, and TGF-β, play a key role in initiating tumour cell metastasis. Studies have confirmed that this microenvironment also causes differences in the expression of tRFs [22]. Studies involving the transfection of interfering fragments and synthetic mimetics have shown that these tRFs inhibit breast cancer cell growth, cell invasion and metastasis [10]. In this study, we used hypoxia-induced culture of colon cancer RKO cells for high-throughput sequencing and post-bioinformatics analyses. The results of high-throughput sequencing are shown in **Figure 1A** and **1B**. PCR identification showed the 14 tRFs with the most significant differences in sequencing expression, and three tRFs were identified in both the sequencing analysis and the differential RNA expression analysis (**Figure 1C**). We found that tRF-20-M0NK5Y93 expression was significantly downregulated under hypoxic conditions compared with normoxic conditions. The change in tRF-20-M0NK5Y93 expression detected by real-time PCR under hypoxic conditions was the same as that identified by high-throughput sequencing (**Figure 1D**). The results of three independent implicated assays were consistent.

tRF-20-M0NK5Y93 modulates invasion and migration but not proliferation in CRC cells

The RKO and SW480 cell lines were utilized for subsequent assays. We transfected tRF-20-M0NK5Y93 inhibitors into RKO and SW480 cells in a normal oxygen environment to simulate the change in tRF-20-M0NK5Y93 secretion under hypoxic conditions. We next aimed to decrease tRF-20-M0NK5Y93 expression in both cell lines in a stable manner. Then, a qRT-PCR assay was performed to detect the relative level of tRF-20-M0NK5Y93. The analysis showed a significant decline in tRF-20-M0NK5Y93 expression in the inhibitor group compared with the NC group (**Figure 2A**). To simulate hypoxic conditions and explore the role of tRF-20-M0NK-5Y93 in CRC cell proliferation, we performed MTT assays on RKO and SW480 cells for loss-of-function studies. The MTT results showed that knockdown of tRF-20-M0NK5Y93 had no effect on the proliferation of either RKO or SW480 cells (**Figure 2B**). To study the role of tRF-20-M0NK5Y93 in CRC cell invasion, we performed Transwell invasion assays to evaluate the invasive capacities of RKO and SW480

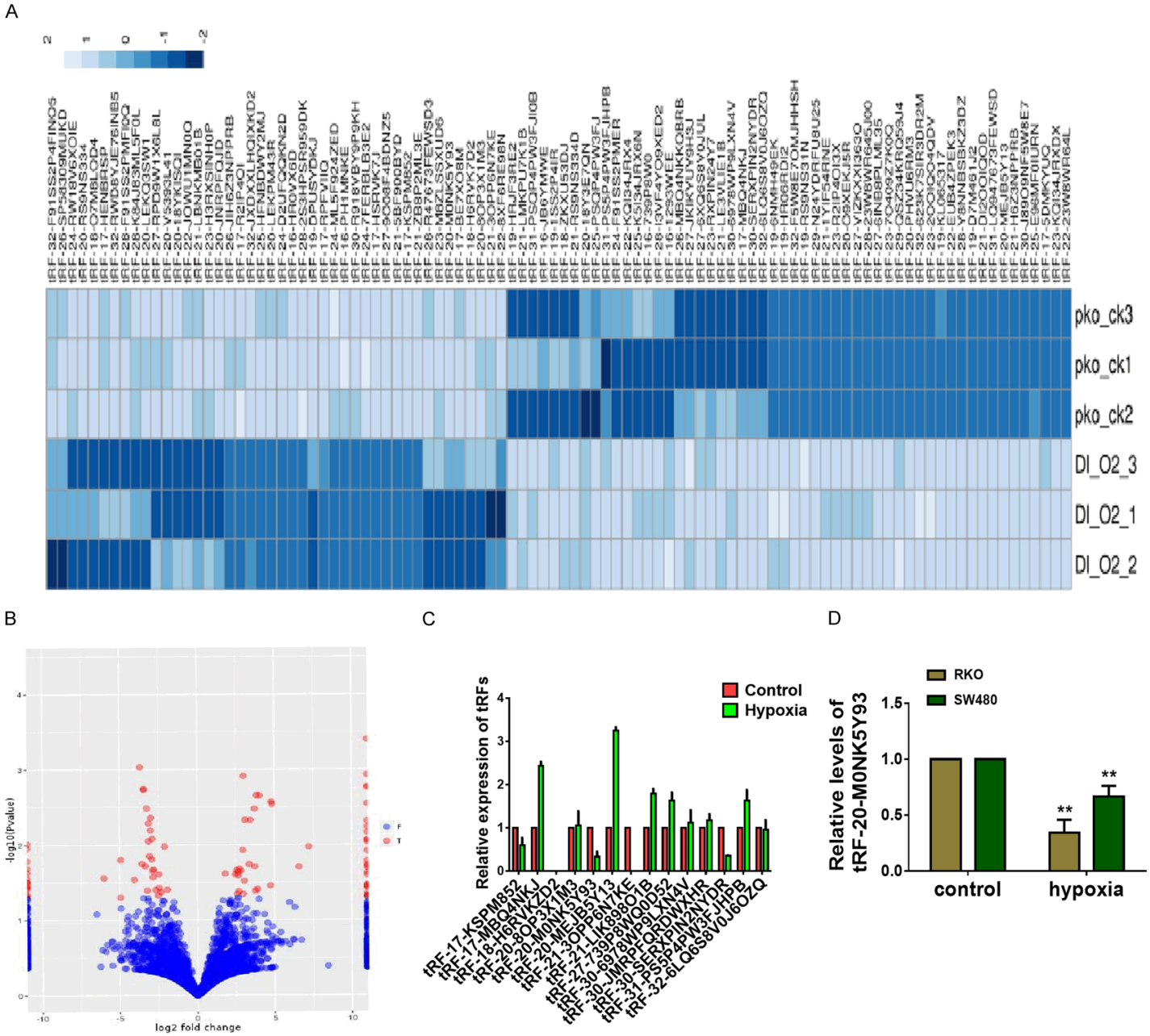

A
pko_ck3
pko_ck1
pko_ck2
Dl_O2_3
Dl_O2_1
Dl_O2_2
B
log2 fold change
-log10(Pvalue)
C
Relative expression of tRFs
Control
Hypoxia
D
Relative levels of tRF-20-M0NK5Y93
RKO
SW480
control
hypoxia

Figure 1. tRF expression pattern analysis in CRC cell lines under hypoxia. A and B. High-throughput sequencing results showed changes in tRFs in the hypoxia-induced culture of colon cancer RKO cells. C. PCR identification for the 14 tRFs that had most significant differences in high-throughput sequencing. D. qRT-PCR assay for the expression levels of tRF-20-M0NK5Y93 under hypoxic conditions in both RKO and SW480 cells. NC, negative control; qRT-PCR, quantitative real-time polymerase chain reaction. **P < 0.01.

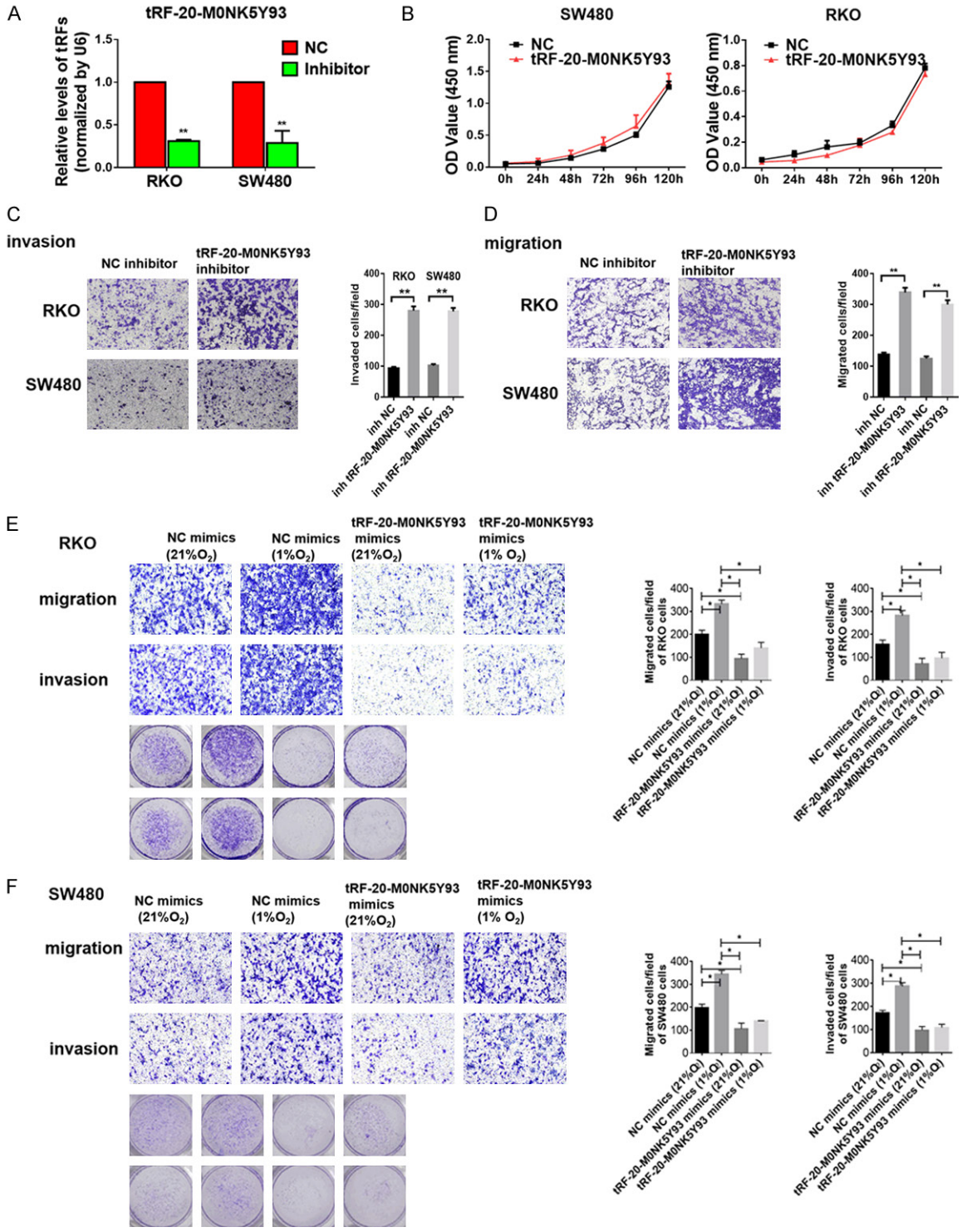


Figure 2. The role of tRF-20-M0NK5Y93 in CRC malignant phenotypes in CRC cells. RKO and SW480 cells were transfected with the tRF-20-M0NK5Y93 inhibitor and inhibitor NC and validated by qPCR assay (A). The MTT proliferation assay was performed to determine cell proliferative potential (B). Transwell assays were performed to

detect the migration and invasion of RKO cells (C) and SW480 cells (D). Transwell assays was used to determine the invasion and migration potential in both RKO cells and SW480 cells under hypoxic conditions or tRF-20-M0NK5Y93 overexpression alone or in combined in RKO cells (E) and SW480 cells (F). MTT, 3-(4,5)-dimethylthiahiazo(-z-yl)-3,5-diphenyltetrazolium bromide; NC, negative control. *P < 0.05, **P < 0.01. The assays were repeated more than three times. (A) RKO cells. (B) SW480 cells.

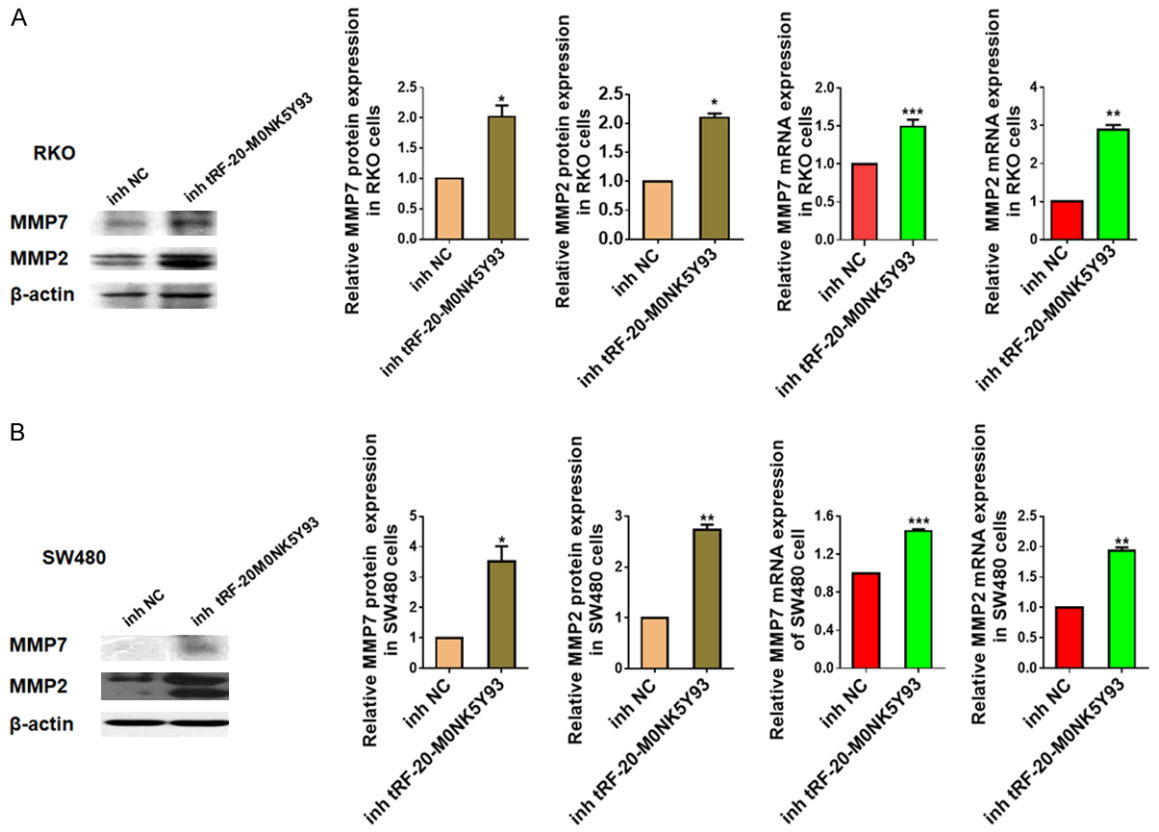


Figure 3. The effects of tRF-20-M0NK5Y93 in regulating the expression of MMPs. Western blot and qRT-PCR assays were designed to detect the protein and mRNA expression of MMP-7 and MMP2 in RKO cells (A) and in SW480 cells (B) transfected with tRF-20-M0NK5Y93 inhibitor or NC. These assays were repeated more than three times. *P < 0.05, ***P < 0.001.

cells transfected with the tRF-20-M0NK5Y93 inhibitor. Transwell assays illustrated that the migration and invasion abilities of RKO and SW480 cells were markedly promoted in the inhibitor group compared with the NC group (**Figure 2C** and **2D**), the cell migration and invasion of both RKO and SW480 cells can be promoted by hypoxic condition, while overexpression of tRF-20-M0NK5Y93 which inhibited the invasion and migration of RKO and SW480 cells effectively impaired the role of hypoxic condition (**Figure 2E** and **2F**). These data suggested that tRF-20-M0NK5Y93 plays a critical role as a tumor suppressor for CRC in the inhibition of CRC cell migration and invasion.

Effect of tRF-20-M0NK5Y93 on the metastasis-related molecules of CRC cells

Some molecules, such as MMPs, have been verified to play a critical role in the metastatic potential of CRC cells. Western blot assays and qRT-PCR assays were performed to detect the protein and mRNA levels of MMPs in CRC cells transfected with tRF-20-M0NK5Y93 inhibitor or anti-tRF-NC. The assay results indicated that knockdown of tRF-20-M0NK5Y93 could enhance both the protein and mRNA levels of MMP-7 and MMP-2 in RKO cells compared with control cells (**Figure 3A**). Consistently, the protein and mRNA levels of MMP7 and MMP-2 in SW480 cells were also significantly

increased (**Figure 3B**). Thus, our findings illustrate that tRF-20-M0NK5Y93 might play a critical role in the inhibition of CRC cell invasion and that the upregulation of MMPs might be the underlying mechanism.

Effects of tRF-20-M0NK5Y93 on the EMT of CRC cells

Subsequently, we explored the effects of tRF-20-M0NK5Y93 on the expression of EMT markers in RKO and SW480 cells by performing Western blot and qRT-PCR assays. The protein levels of Claudin-1, Snail, E-cadherin, Vimentin, Fibronectin showed significant differences (**Figure 4A**), and the mRNA levels of Claudin-1, Snail, HIF1α, Twist1, Twist2, Fibronectin, ZEB1 showed significant differences in RKO cells (**Figure 5A**). The results also showed that Claudin-1 and Fibronectin increased significantly in protein levels (**Figure 4B**), and Claudin-1, Snail, HIF1α, Twist1, E-cadherin, N-cadherin, Vimentin, Fibronectin and ZEB1 showed significant differences in mRNA levels in SW480 cells (**Figure 5B**). HIF-1α, which is known as a hypoxia inducible factor, is also involved in cancer metastasis and induces EMT. Thus, the change of HIF-1α under tRF-20-M0NK5Y93 inhibitor treatment in RKO and SW480 cells was also examined and the data indicated that tRF-20-M0NK5Y93 inhibitor can increase the expression of HIF-1α in both mRNA and protein levels (**Figures 4**, **5A**, **5B**).

TRF-20-M0NK5Y93 targets claudin-1 in CRC cells

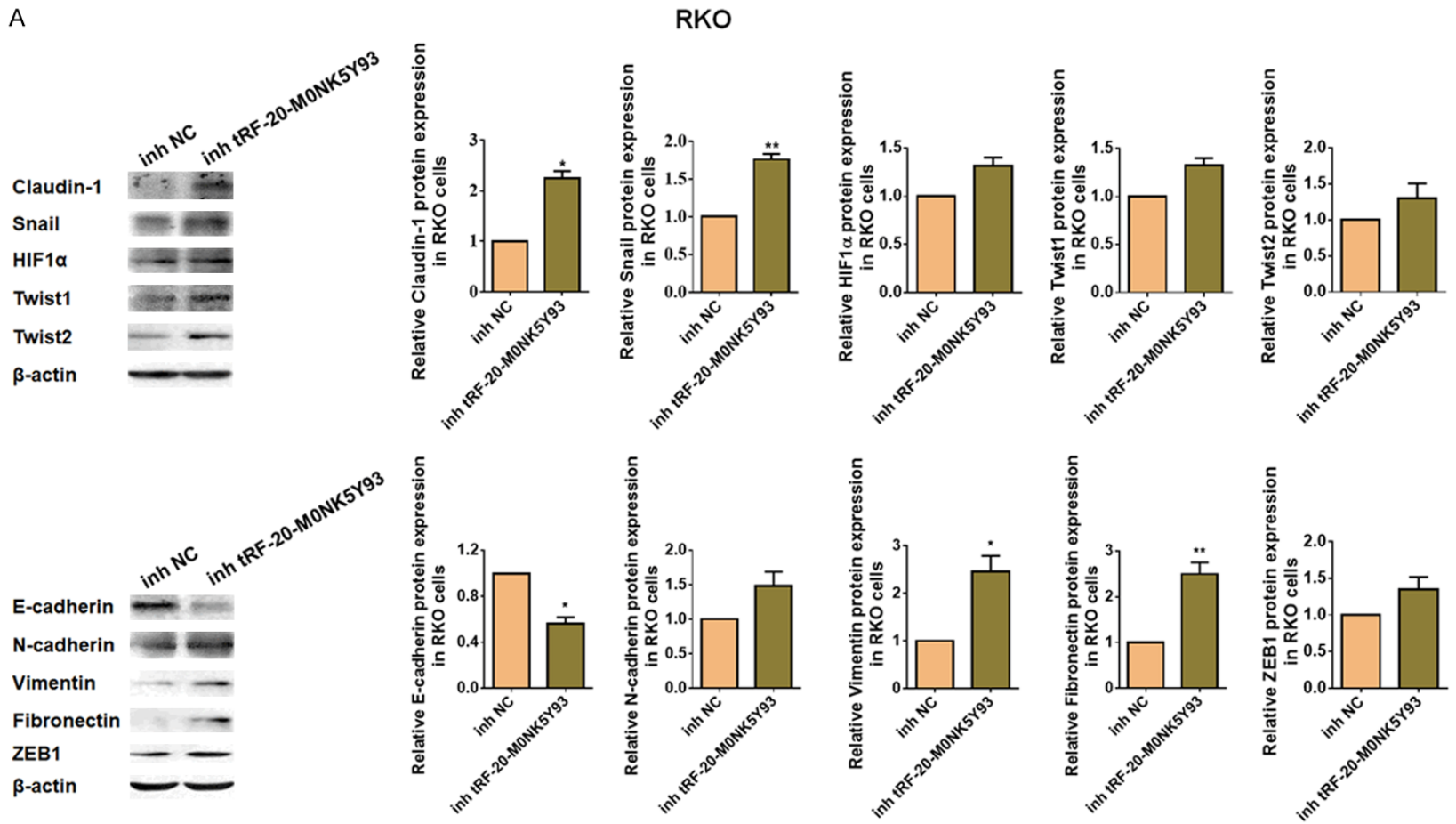
Next, we explored the downstream target molecules with which tRF-20-M0NK5Y93 interacts in the EMT process of CRC cells. Based on informatics analysis of the Santa Cruz database with TargetScan, we found a potential tRF-20-M0NK5Y93-binding site in the Claudin-1 3'-UTR. Next, we constructed two types of plasmids that contained a luciferase reporter gene and wild-type (Wt) or mutant (Mut) Claudin-1 3'-UTR (**Figure 5C**). Then, each plasmid was co-transfected with either tRF-NC or tRF-20-M0NK5Y93 mimics into RKO cells. The findings showed that tRF-20-M0NK5Y93 overexpression decreased Claudin-1 luciferase activity in RKO cells transfected with Claudin-1 3'-UTR-WT plasmid compared with those transfected with the control plasmid. However, the effects were abolished when RKO cells were transfected with the Claudin-1 3'-UTR-Mut plasmid (**Figure 5D**), which suggested that tRF-20-M0NK5Y93 directly and negatively regulates the expression of Claudin-1. Western blot and qRT-PCR assays illustrated the regulatory effects of tRF-20-M0NK5Y93 on Claudin-1 in CRC cells; inhibition of tRF-20-M0NK5Y93 increased the protein and mRNA expression of Claudin-1 (**Figures 4A** and **5A**). In conclusion, all of the above results revealed a direct interaction between tRF-20-M0NK5Y93 and Claudin-1 in CRC cells.

TRF-20-M0NK5Y93 suppressed the EMT of CRC cells through its inhibition of Claudin-1

To further validate the role of tRF-20-M0NK5Y93 in the invasion and migration of CRC and to explore its detailed mechanism, we transfected tRF-20-M0NK5Y93 and si-NC into RKO cells. The Transwell assay results revealed that compared with those in the control group, the invasion and metastasis of CRC cells in the experimental group were significantly increased (**Figure 6B**).

To confirm the role of Claudin-1 in EMT in CRC cells, we transfected RKO cells with si-NC or si-Claudin-1. The knockdown effect of Claudin-1 was detected by Western blot assay. The results showed that the expression of Claudin-1 was significantly decreased at the protein level in RKO cells transfected with si-Claudin-1 (**Figure 6A**). Transwell assay results revealed that the downregulation of Claudin-1 suppressed the migration and invasion of RKO cells (**Figure 6B**). In addition, downregulation of Claudin-1 could also partially decreased the expression of EMT-related molecules (**Figures 6C**, **7**). These data confirmed that the downregulation of Claudin-1 inhibited the migration and invasion of CRC cells.

Based on the results above, we then cotransfected CRC cells with a tRF-20-M0NK5Y93 inhibitor and the si-Claudin-1 to confirm our hypothesis that tRF-20-M0NK5Y93 suppresses EMT in CRC cells through its inhibition of Claudin-1. The transfection assay results showed that compared with transfection of the tRF-20-M0NK5Y93 inhibitor and si-NC into CRC cells, transfection with both tRF-20-M0NK5Y93 inhibitor and si-Claudin-1 dramatically decreased RKO cell metastasis, similar to


A
RKO
inh NC
inh tRF-20-M0NK5Y93
Claudin-1
Snail
HIF1α
Twist1
Twist2
β-actin
E-cadherin
N-cadherin
Vimentin
Fibronectin
ZEB1
Relative Claudin-1 protein expression in RKO cells
Relative Snail protein expression in RKO cells
Relative HIF1α protein expression in RKO cells
Relative Twist1 protein expression in RKO cells
Relative Twist2 protein expression in RKO cells
Relative E-cadherin protein expression in RKO cells
Relative N-cadherin protein expression in RKO cells
Relative Vimentin protein expression in RKO cells
Relative Fibronectin protein expression in RKO cells
Relative ZEB1 protein expression in RKO cells

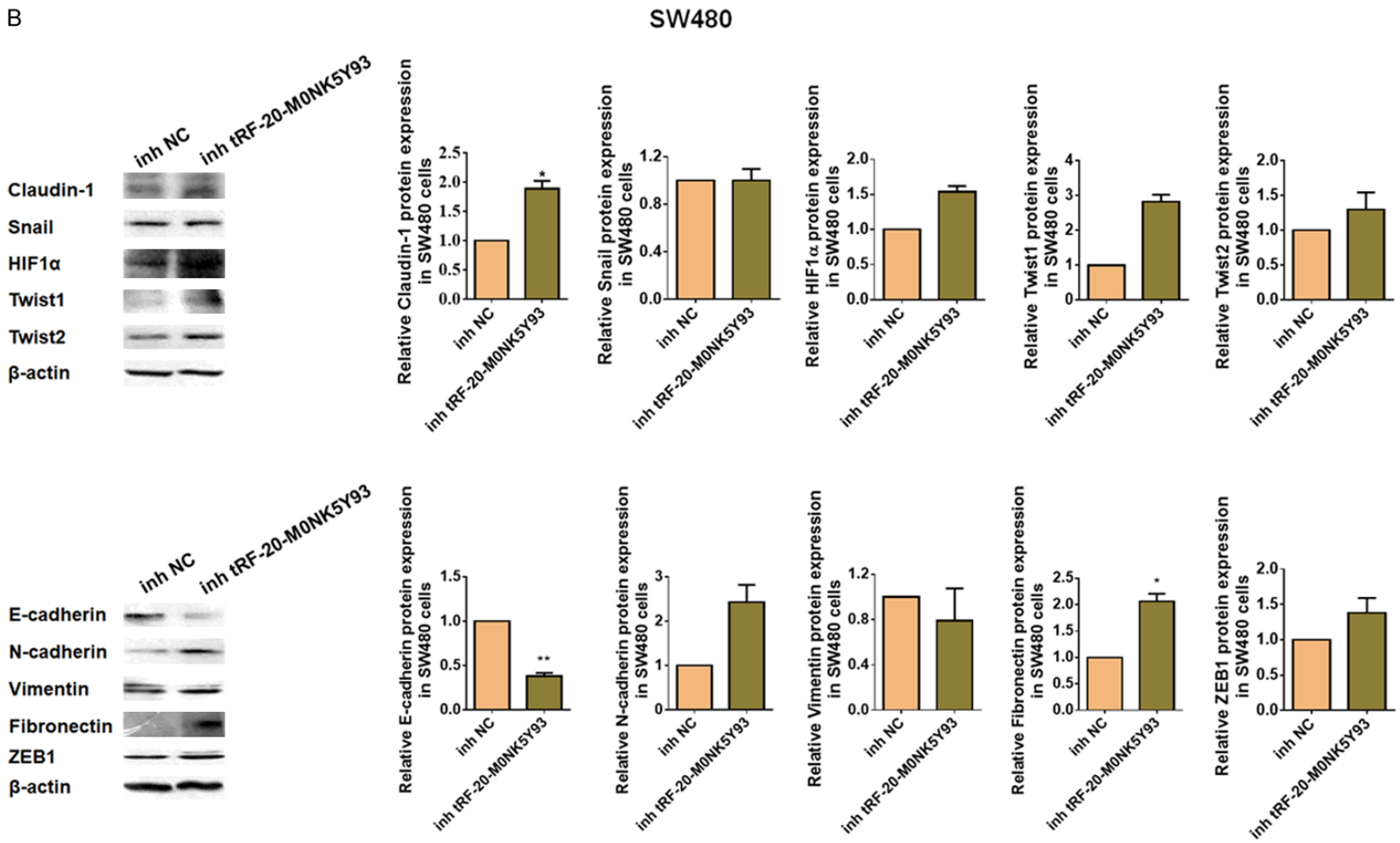
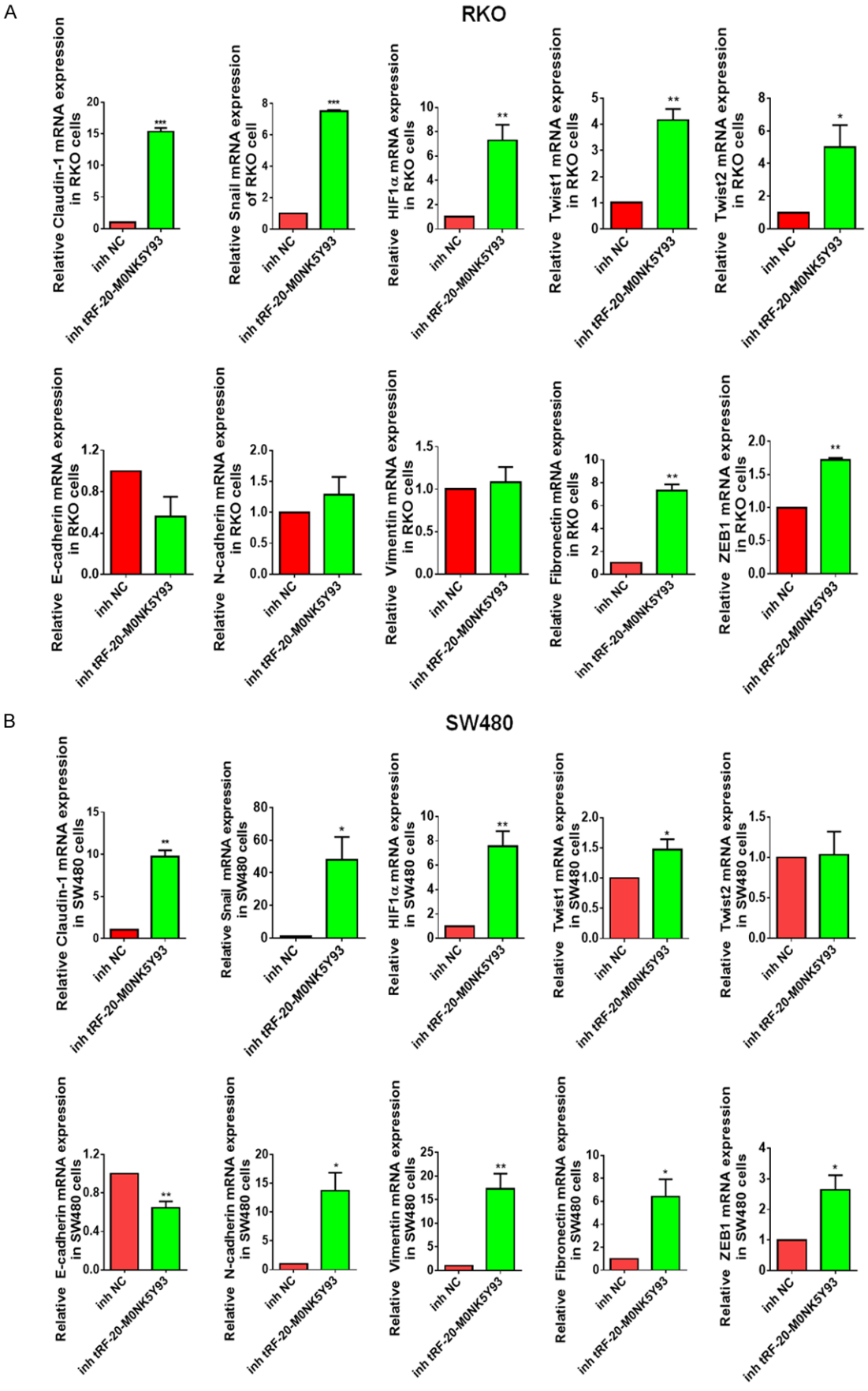


Figure 4. The effects of the tRF-20-M0NK5Y93 inhibitor on the protein expression levels of EMT-related molecules in RKO and SW480 cells. The protein levels of Claudin-1, Snail, HIF1α, Twist1, Twist2, E-cadherin, N-cadherin, Vimentin, Fibronectin and ZEB1 were detected by Western blot assays in RKO cells (A) and SW480 cells (B) after transfection with tRF-20-M0NK5Y93 inhibitor. All these assays were repeated more than three times. *P < 0.05, **P < 0.01, ***P < 0.001.


A
RKO
Relative Claudin-1 mRNA expression in RKO cells
Relative Snail mRNA expression of RKO cell
Relative HIF1α mRNA expression in RKO cells
Relative Twist1 mRNA expression in RKO cells
Relative Twist2 mRNA expression in RKO cells
Relative E-cadherin mRNA expression in RKO cells
Relative N-cadherin mRNA expression in RKO cells
Relative Vimentin mRNA expression in RKO cells
Relative Fibronectin mRNA expression in RKO cells
Relative ZEB1 mRNA expression in RKO cells
B
SW480
Relative Claudin-1 mRNA expression in SW480 cells
Relative Snail mRNA expression in SW480 cells
Relative HIF1α mRNA expression in SW480 cells
Relative Twist1 mRNA expression in SW480 cells
Relative Twist2 mRNA expression in SW480 cells
Relative E-cadherin mRNA expression in SW480 cells
Relative N-cadherin mRNA expression in SW480 cells
Relative Vimentin mRNA expression in SW480 cells
Relative Fibronectin mRNA expression in SW480 cells
Relative ZEB1 mRNA expression in SW480 cells
inh NC
inh tRF-20-M0NK5Y93

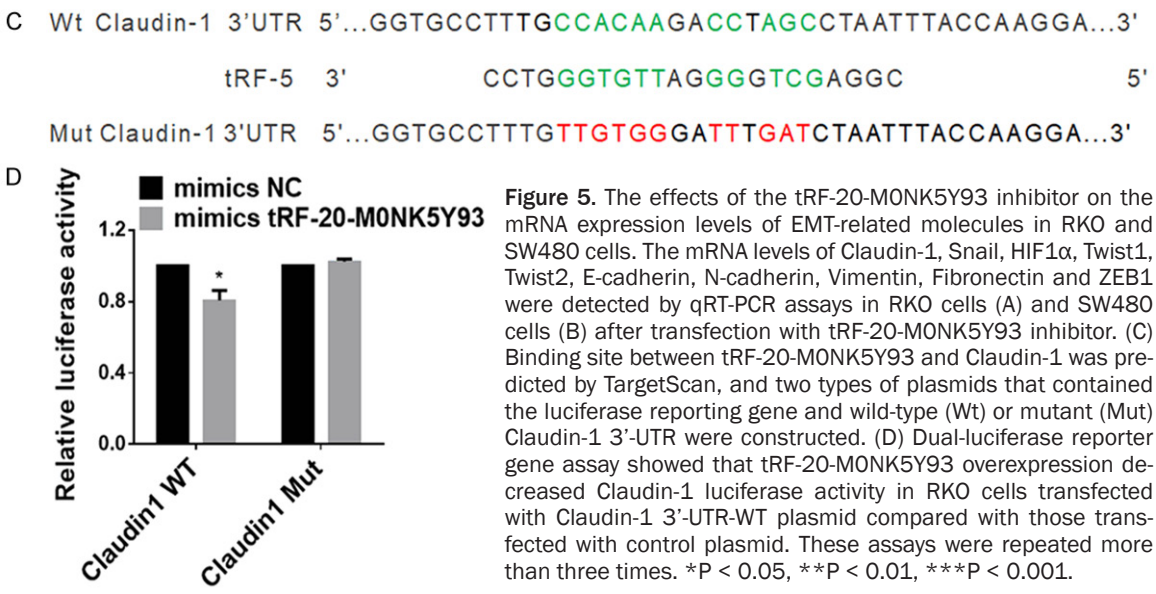


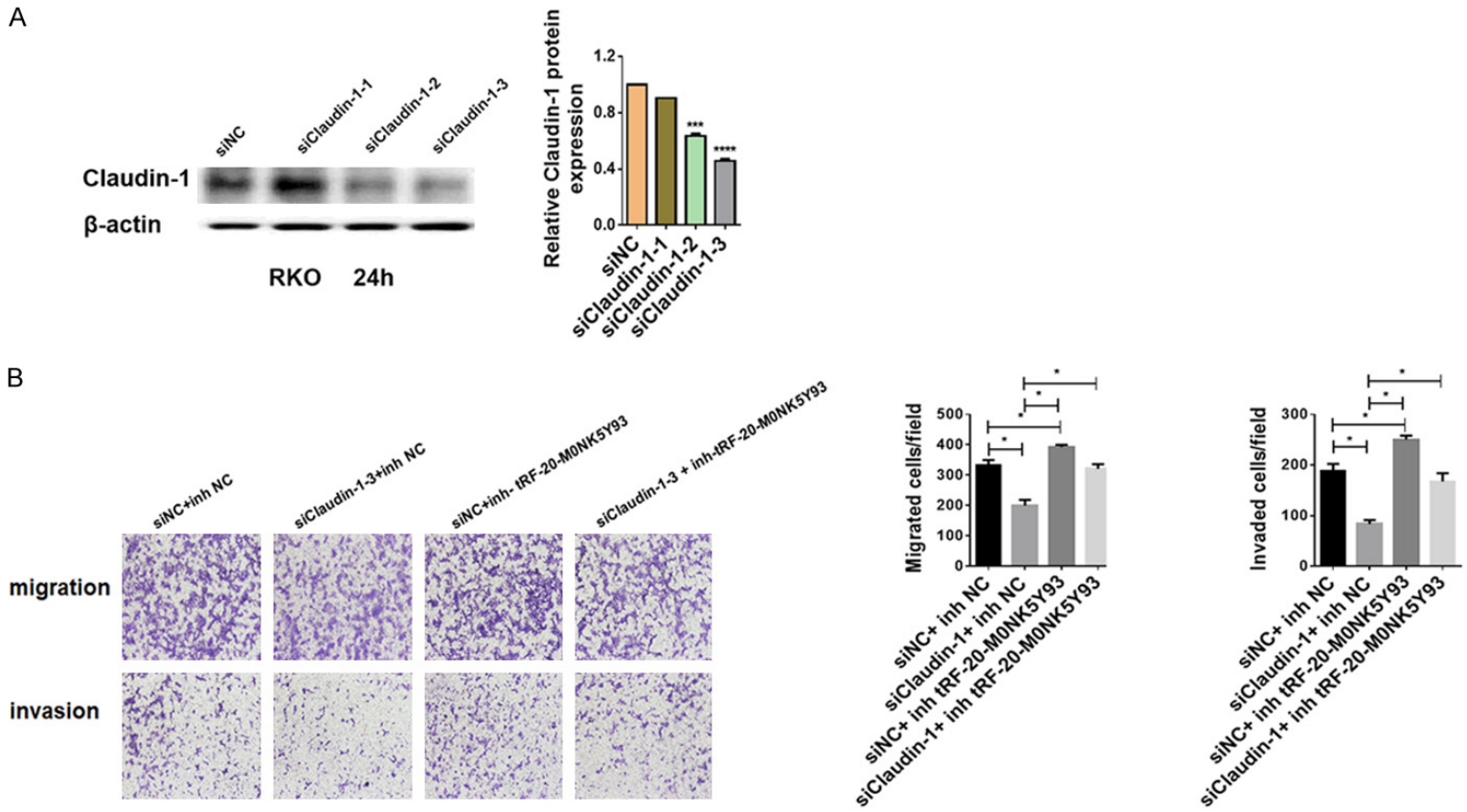
Figure 5. The effects of the tRF-20-M0NK5Y93 inhibitor on the mRNA expression levels of EMT-related molecules in RKO and SW480 cells. The mRNA levels of Claudin-1, Snail, HIF1α, Twist1, Twist2, E-cadherin, N-cadherin, Vimentin, Fibronectin and ZEB1 were detected by qRT-PCR assays in RKO cells (A) and SW480 cells (B) after transfection with tRF-20-M0NK5Y93 inhibitor. (C) Binding site between tRF-20-M0NK5Y93 and Claudin-1 was predicted by TargetScan, and two types of plasmids that contained the luciferase reporting gene and wild-type (Wt) or mutant (Mut) Claudin-1 3'-UTR were constructed. (D) Dual-luciferase reporter gene assay showed that tRF-20-M0NK5Y93 overexpression decreased Claudin-1 luciferase activity in RKO cells transfected with Claudin-1 3'-UTR-WT plasmid compared with those transfected with control plasmid. These assays were repeated more than three times. *P < 0.05, **P < 0.01, ***P < 0.001.

that in the control group (**Figure 6B**). The data from the Transwell assay indicated that the downregulation of Claudin-1 could reverse the promotive effect of tRF-20-M0NK5Y93 inhibition on the migration and invasion of RKO cells (**Figure 6B**). And downregulation of Claudin-1 reversed tRF-20-M0NK5Y93 inhibitor-induced EMT in RKO cells and indeed regulated the key EMT-involved molecules, whether in mRNA expression levels (**Figure 6C**) or in protein levels (**Figure 7**).

Discussion

Due to trends in multidisciplinary therapies, the mortality of CRC has steadily declined over the past 20 years [23, 24]. However, approximately 25% of CRC patients have tumour metastasis at the time of initial diagnosis [25, 26]. Thus, new tumour markers and molecular targets for diagnosis and therapies are needed. The metastasis of CRC cells is a complex process involving multiple factors and the regulation of numerous genes [4]. tRFs, tRNA-derived fragments, have been demonstrated to be associated with many types of cancers, including lung cancer, prostate cancer, breast cancer, ovarian cancer, B-cell lymphoma and chronic lymphocytic leukaemia [10, 12, 27-30]. Therefore, studies on tRFs in CRC may improve our understanding of the mechanisms related to CRC occurrence and progression, which may help design promising therapeutic approaches for CRC. In the present study, we discovered that tRF-20-M0NK5Y93 inhibited CRC progression, cell invasion and metastasis by targeting Claudin-1. In contrast, inhibiting the expression of Claudin-1 by siRNA reversed those phenotypes.

Most studies have focused on the relationship between tRFs and tumour proliferation, while our findings verified that there was no correlation between tRFs and CRC proliferation. However, an increasing number of studies have shown that ncRNAs are key regulators of cancer-related processes. For example, a previous study noted that miRNAs can play a critical role in EMT [31]. CircRNAs can act as miRNA sponges to regulate miRNA activity. Dou et al. [32] studied the protein level of specific RNA-binding protein (RBP) and the level of circRNA and found the potential role of circRNA in EMT regulation. Notably, likely miRNAs and tRFs play different roles in the processes of cancers, either cancer-promoting or cancer-suppressing effects, which might depend on the differential biological functions of corresponding genes. For instance, in high-grade serous ovarian cancer (HGSOC), Zhang et al. [33] reported that tRF-03357 supported SK-OV-3 cell migration and invasion, possibly partly inhibiting the expression of HMBOX1. In CRC, Huang et al. [34] found that a fragment named tRF/miR-1280, which is derived from tRNA Leu and a pre-miRNA, suppressed CRC progression by restraining the function of cancer stem cell-like cells (CSCs) by affecting the


A
siNC
siClaudin-1-1
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Claudin-1
β-actin
RKO 24h
Relative Claudin-1 protein expression
1.2
0.8
0.4
0.0
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siNC
siClaudin-1-1
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siClaudin-1-3
B
siNC+inh NC
siClaudin-1-3+inh NC
siNC+inh- tRF-20-M0NK5Y93
siClaudin-1-3 + inh-tRF-20-M0NK5Y93
migration
invasion
Migrated cells/field
500
400
300
200
100
0
siNC+ inh NC
siClaudin-1+ inh NC
siNC+ inh tRF-20-M0NK5Y93
siClaudin-1+ inh tRF-20-M0NK5Y93
Invaded cells/field
300
200
100
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C

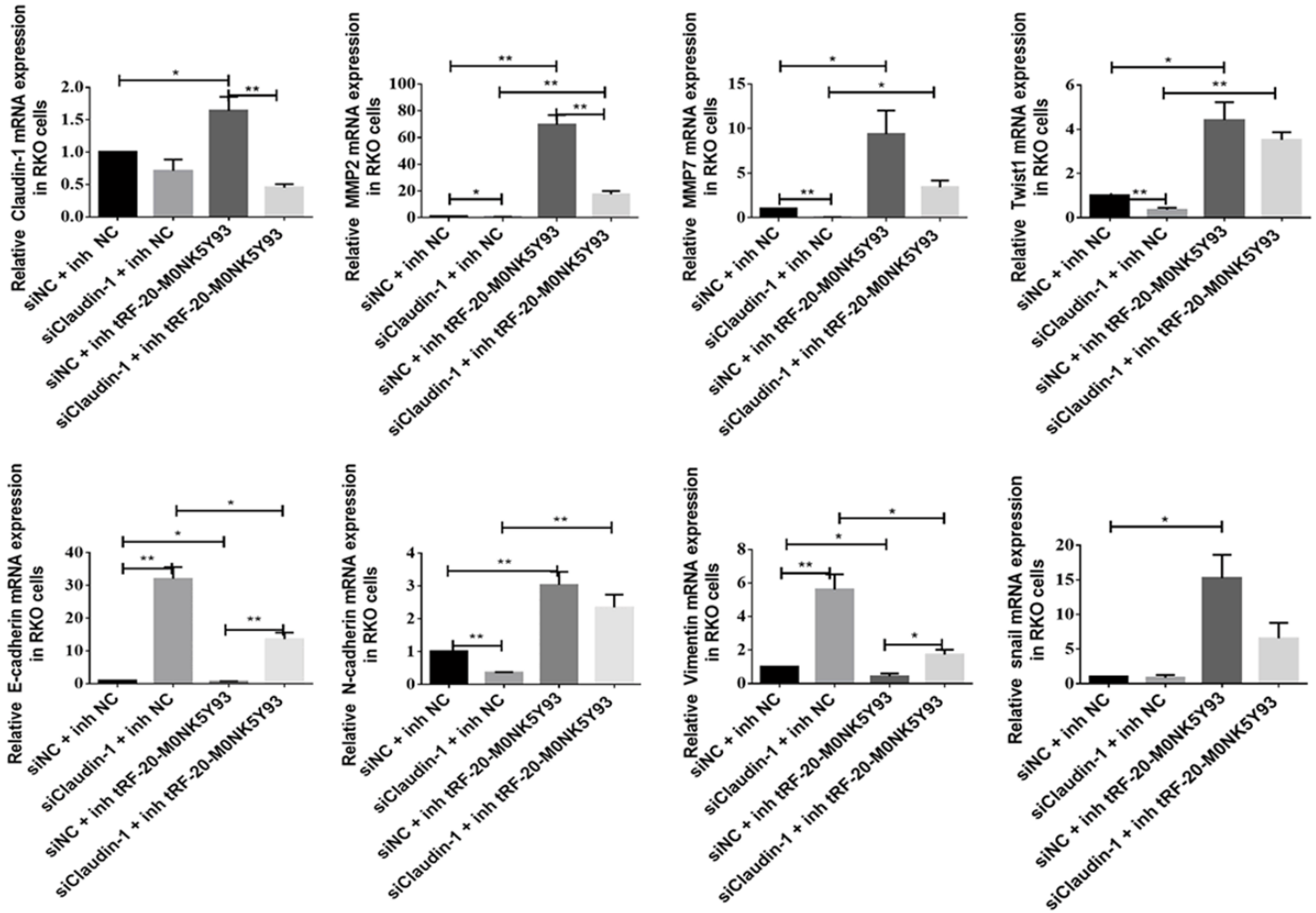

Figure 6. Downregulation of Claudin-1 is a key process tRF-20-M0NK5Y93 acts as a tumor suppressor in CRC. A. RKO cells were transfected with NC or three siRNA against Claudin-1. The protein levels of Claudin-1 in RKO cells were determined by Western blotting. B. The migration and invasion of RKO cells with tRF-20-M0NK5Y93 inhibitor or Claudin-1 silence alone or in combined were assessed by Transwell assays. C. qPCR assay was used to detect the mRNA expression of EMT-related molecules in RKO cells with tRF-20-M0NK5Y93 inhibitor or Claudin-1 silence alone or in combined. All assays were repeated more than three times. *P < 0.05, **P < 0.01.

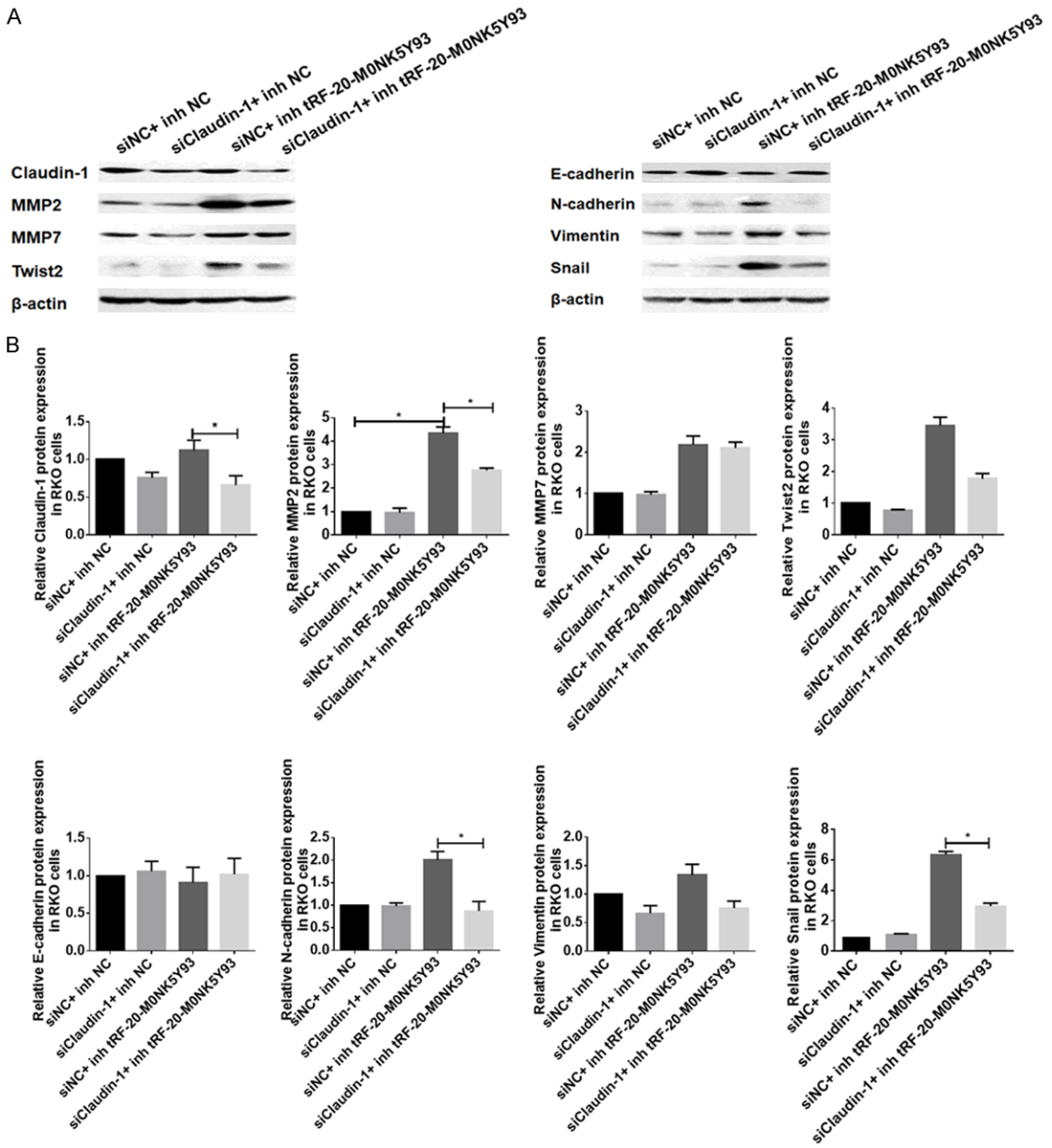

Figure 7. Western blotting assay was used to detect the mRNA expression of EMT-related molecules in RKO cells with tRF-20-M0NK5Y93 or Claudin-1 silence alone or in combined. All assays were repeated more than three times. All assays were repeated more than three times. *P < 0.05.

Notch signalling pathway. Some tRFs may act as tumour suppressors by combining with particular RBPs. One recent study identified a novel class of tRFs that decrease the stability of multiple carcinogenic transcripts to inhibit cancer progression by interacting with the RBP YBX1 in breast cancer [10]. In our study, we identified a human-specific tRNA fragment called tRF-20-M0NK5Y93 and explored the specific role and regulatory mechanism of tRF-20-M0NK5Y93 in the EMT of CRC. Functional assays verified that upregulation of tRF-20-M0NK5Y93 restrained CRC cell migration and invasion and downregulation of tRF-20-M0-NK5Y93 showed the opposite results, which supported the hypothesis that tRF-20-M0NK5Y93 functions as a tumour-suppressive molecule in vitro. Furthermore, we evaluated

the changes in representative molecules of EMT in RKO and SW480 cells transfected with the tRF-20-M0NK5Y93 inhibitor. The data showed that the inhibition of tRF-20-M0NK-5Y93 could lead to the dramatic upregulation of the expression of MMP-2, MMP-7, Claudin-1, Snail and Fibronectin in RKO cells and MMP2, MMP7, Claudin-1 and Fibronectin in SW480 cells, which suggested that the downregulation of tRF-20-M0NK5Y93 in colorectal tumours facilitates the acquisition of a variety of oncogenic characteristics, including cell invasion and migration.

Claudin-1 was first cloned from chicken liver, and its key role in tight junctions (TJs) was characterized [35]. Claudin-1, as one of the distinguished members of the Claudin family proteins, plays an essential role in the composition of TJs. In this study, we identified and verified that downregulation of Claudin-1 suppressed the migration and invasion of RKO cells and partially decreased the expression of EMT-related molecules, indicating that Claudin-1 promote the migration and invasion of CRC cells. And our study showed that Claudin-1 was a downstream functional target of tRF-20-M0NK5Y93 using a dual-luciferase assay, which is particularly vital for further exploration of the antitumour mechanism of tRF-20-M0NK5Y93 in CRC progression. Therefore, the results indicate that Claudin-1 is a target gene of tRF-20-M0NK5Y93 in vitro. In addition, results showed that Claudin-1 expression was increased at both the protein and mRNA levels, respectively, in CRC cells after the downregulation of tRF-20-M0NK5Y93.

In CRC, Claudin-1 reduces the expression of E-cadherin by upregulating the repressor molecular ZEB-1 in colon cancer cells, thereby increasing cell invasion [21, 36]. A previous study also suggested that after inhibition of Claudin-1 expression, transcription of E-cadherin increased, while the expression of mesenchymal markers decreased [21, 36]. Although Claudin-1 is a tumour suppressor in some cancers [37, 38], a large number of studies, as indicated above, show that Claudin-1 is overexpressed in CRC cells compared with normal controls and promotes cell invasion and metastasis [20, 21, 39-42]. We confirmed the biological role of Claudin-1 in CRC by transfecting cells with siRNA targeting Claudin-1. Transwell assay results indicated that the knockdown of Claudin-1 led to the inhibition of tumour invasion and migration.

Many studies found that ncRNA modulates cancer cell metastasis by interacting with Claudin-1. Mahati et al. [43] found that miR-29a inhibited liver cancer cell proliferation and migration partly by downregulating Claudin-1 by binding to its 3'-UTR. A previous study indicated that miR-155 overexpression increased the expression of Claudin-1 compared with that in the control group, thereby increasing the expression level of ZEB-1 and inhibiting E-cadherin, promoting colorectal cell invasion and migration [44]. In the present study, functional analysis also showed that tRF-20-M0NK5Y93 could inhibit tumour metastasis in vitro. Transfection with tRF-20-M0NK5Y93 inhibitor significantly upregulated Claudin-1 expression at the mRNA and protein levels in CRC cells. Bioinformatics analysis and dual-luciferase reporter assays showed that tRF-20-M0NK5Y93 could target the Claudin-1 gene directly. Thus, these above findings indicate that the downregulation of tRF-20-M0NK5Y93 expression promotes the invasion and migration of CRC cells partly through the regulation of Claudin-1 expression. Notably, the downregulation of Claudin-1 could reverse tRF-20-M0NK5Y93 inhibitor-mediated promotion and partially reversed tRF-20-M0NK5Y93 inhibitor-induced EMT-involved molecules, further demonstrating that tRF-20-M0NK5Y93 regulates tumour invasion and migration by targeting Claudin-1. Furthermore, based on previous data [36], we suggest that decreased expression of tRF-20-M0NK5Y93 upregulates Claudin-1 and then correspondingly regulates the expression of ZEB-1 and E-cadherin, ultimately promoting CRC cell invasion and migration.

Conclusion

Our study aimed to determine the relationship between tRFs and the malignant biological behaviour of CRC cells in the tumour microenvironment. In many tissues, tRFs account for a large proportion of the small RNAs, and their quantity is substantially higher than that of microRNAs, which strongly predicts their critical physiological functions and biomarker application potential. If tRFs could be systematically

researched and transformed as potential tumour suppressor molecules, then they may become novel biological markers for tumour diagnosis or provide a new avenue for the development of new drugs, offering an innovative strategy for inhibiting tumour metastasis that has broad application prospects in oncology.

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Disclosure of conflict of interest

None.

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