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Long non-coding RNA NEAT1 promoted ovarian cancer cells' metastasis through regulation of miR-382-3p/ROCK1 axial

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Long non-coding RNA (lncRNA) are extensively involved in various malignant tumors, including ovarian cancer (OC). In the present study, we focused on the expression and function of nuclear enriched abundant transcript 1 (NEAT1) in OC cells' metastasis. We demonstrated that NEAT1 was upregulated in OC tissue specimens and cell lines. In addition, we revealed that depression of NEAT1 inhibited OC cells' metastasis and the expression of Rho associated coiled-coil containing protein kinase 1 (ROCK1), which is a metastasis-related gene. Using online predictive software and a series of luciferase assays, we demonstrated that both NEAT1 and ROCK1 were the targets of microRNA-382-3p (miR-382-3p) and share similar microRNA responding elements (MRE). Furthermore, we illustrated that NEAT1 and miR-382-3p inhibited each other in a reciprocal manner. Finally, through antisense experiments we demonstrated that NEAT1 promoted ROCK1-mediated metastasis by functioning as a ceRNA of miR-382-3p. In summary, the findings of this study revealed that NEAT1 promoted OC cells' metastasis through regulating the miR-382-3p/ROCK1 axial. The present study might provide a new target for treating OC.

KEYWORDS
lncRNA NEAT1, metastasis, miR-382-3p, ovarian cancer, ROCK1

1 | INTRODUCTION

Ovarian cancer (OC) is the fifth leading cause of cancer death among women worldwide.[1] Difficulty in early detection of OC and rapid dissemination to the peritoneum, the omentum and the organs located in the peritoneal cavity contribute to the poor survival rates for OC.[2] Importantly, distant metastasis in OC is consistent with stage IV disease and is a late complication that occurs in approximately one-third of OC patients; prognosis of OC after documentation of distant metastases is poor.[3] Therefore, seeking out an available metastasis-related molecule and identifying its working mechanism might provide a useful target in molecular treatment of OC.

Long non-coding RNA (lncRNA) are a group of transcribed RNA molecules with lengths of more than 200 nucleotides that are not protein-coding and are widely involved in various cancer-related biological progresses.[4] The functions of lncRNA include chromatin regulation, histone modification, chromatin remodeling, genomic imprinting and working as a ceRNA in posttranscriptional regulation.[5-9] Leonardo Salmena's (2011) competitive endogenous RNA (ceRNA) hypothesis proposes that all types of RNA transcripts communicate through a new "language" mediated by micro-RNA-binding sites ("microRNA response elements," or "MRE").[10] A member of the lncRNA, nuclear enriched abundant transcript 1 (NEAT1) is reported as an oncogene in various malignant tumors, including hepatocellular carcinoma, non-small cell lung cancer, nasopharyngeal carcinoma, breast cancer and OC.[11-16] An (2017) report that NEAT1 contributed

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to paclitaxel (PTX) resistance of OC cells through upregulating ZEB1 expression by sponging of microRNA-194 (miR-194).[11] Ding (2017) report that NEAT1 promoted OC cells' proliferation and apoptosis through regulation of microRNA-34a-5p (miR-34a-5p)/B-cell lymphoma-2 (BCL2).[17] Fu (2017) MC found that NEAT1 promoted metastasis and epithelial-mesenchymal transition (EMT) through inhibiting microRNA-34a-5p (miR-34a-5p) in hepatoblastoma cells.[18] To date, whether NEAT1 could affect OC metastasis and its specific working mechanism remains elusive.

In the present study, we focused on the expression level and the function of NEAT1 in OC cell migration and invasion. In addition, we demonstrated that NEAT1 promoted OC cells' migration/invasion by acting as a ceRNA of miR-382-3p.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue samples

A total of 67 OC specimens and paired para-tumor specimens (normal ovarian tissues in stages I and II and normal tissues adjacent to implant focus of omentum in stages III and IV) were collected with the permission of patients during tumorectomy in the First Affiliated Hospital of Dalian Medical University between September 2012 and September 2017. Written informed consent was provided by the patients whose tissues were used in the present study. The Institute Research Medical Ethics Committee of the First Affiliated Hospital of Dalian Medical University granted approval for this study. All 67 cases had definitive pathological diagnoses and the clinical stage of these patients was determined according to the International Federation of Obstetrics and Gynecology (FIGO) criteria.

2.2 | Cell culture

Human OC cell lines ES2 and SKOV3 and human normal ovarian surface epithelial cells, IOSE80, were purchased from the ATCC (Manassas, VA, USA) and cultured in McCoy's 5a Modified Medium (ATCC), McCoy's 5a Modified Medium and DMEM (Gibco, El Paso, TX, USA), respectively. Human OC cell line HO8910 was purchase from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and was cultured in DMEM (Gibco). All mediums were supplemented with 10% (v/v) FBS (Sigma, St. Louis, MO, USA), 100 IU/mL penicillin (Baomanbio, Shanghai, China) and 100 mg/mL streptomycin (Baomanbio). All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

2.3 | Reverse transcription and quantitative real-time PCR

The procedure was carried out as previously described.[19] Trizol Reagent (Invitrogen, Carlsbad, CA, USA) was applied to isolate total RNA from tissue specimens and cells, respectively. Synthesis of cDNA was performed using a Takara RNA PCR Kit (Takara, Dalian, China) according to the manufacturer's protocol. PCR reactions containing SYBR Premix Ex Taq II (Takara) were performed according to the manufacturer's manual. Primer sequences were synthesized by RiboBio (RiboBio, Guangzhou, China); see Table 1.

2.4 | Plasmids transfection

NEAT1 silencing plasmids, NEAT1 smart silencer (NEAT1 silencer) and negative control smart silencer (NC silencer), miR-382-3p mimics and mimic control, and miR-382-3p inhibitor and inhibitor control were chemically synthesized by RiboBio (RiboBio) for further upregulation and downregulation of miR-382-3p. Wild and mutant type NEAT1 overexpression plasmids wt-pcDNA-NEAT1 and mut-pcDNA-NEAT1 were synthesized by GenePharma (GenePharma, Shanghai, China). When OC cells SKOV3 and HO8910 reached 70% confluence, the aforementioned plasmids were transfected into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions to construct NEAT1 or miR-382-3p overexpression/knockdown OC cell models.

TABLE 1 Primer and oligonucleotides sequences used in the present research

Name	Sequence (5′->3′)
NEAT1 Forward primer	CAGGGTGTCCTCCACCTTTA
NEAT1 Reverse primer	AAACCAGCAGACCCCTTTTT
ROCK1 Forward primer	AAAGCCTTACTGTCGATTGCC
ROCK1 Reverse primer	AGGGTAATGCAACTTCCACTG
NR2F2 Forward primer	CTCCCACCCCTCACAACTAA
NR2F2 Reverse primer	GCGCCCAATACGACCAAATC
GAPDH Forward primer	CTCTGCTCCTCCTGTTCGAC
GAPDH Reverse primer	GCGCCCAATACGACCAAATC
miR-382-3p RT primer	CTCAACTGGTGTCGTGGAGTCGGCAA TTCAGTTGAGAAGTGTT
miR-382-3p Forward primer	ACACTCCAGCTGGGAATCATTCACGGACA
miR-340 RT primer	CTCAACTGGTGTCGTGGAGTCGGCAATT CAGTTGAGCTACCAT
miR-340 Forward primer	ACACTCCAGCTGGGCAGTGGTTTTACCCTA
miR-150 RT primer	CTCAACTGGTGTCGTGGAGTCGGCAATT CAGTTGAGCACTGGT
miR-150 Forward primer	ACACTCCAGCTGGG UCUCCCAACCCUU GUA
miRNAs Reverse primer	CCAGTGCAGGGTCCGAGGT
U6 Forward primer	CTCGCTTCGGCAGCACA
U6 Reverse primer	AACGCTTCACGAATTTGCGT
miR-382-3p mimic	AATCATTCACGGACAACACTT
Mimic control	UUCUCCG AACGUGUCACGUTT
miR-382-3p inhibitor	AAGTGTTGTCCGTGAATGATT
Inhibitor control	CAGUACUUUUGUGUAGUACAA

2.5 | Transwell assay

The procedure was carried out as previously described.[20] In short, SKOV3 and HO8910 cells were seeded on uncoated (for migration assays) and Matrigel-coated (for invasion assays) upper chambers (BD Bioscience, New Jersey, USA), respectively. Culture medium without and with 10% FBS were supplemented into the upper and lower wells, respectively, and incubated for a further 24 hours, followed by wiping of the non-migrated or non-invaded cells. Then the filters were fixed in 90% alcohol, followed by crystal violet staining. Five random fields were counted per chamber using an inverted microscope (Olympus, Tokyo, Japan).

2.6 | Scratch assay

After transfection for 48 hours, the sub-confluent cell monolayers were formed into 3 parallel lines with a P-200 pipette tip. The detached cells were gently washed off twice, and the medium was then replaced with 1% FBS complete medium. To visualize wound healing, images were taken at 0 and 24 hours. The percentage of wound closure (Original width − Width after cell migration/Original width) was calculated.

2.7 | Western blot analysis

Total proteins were isolated using radio immunoprecipitation assay (RIPA) lysis buffer (Sigma, St. Louis, MO, USA) and qualified by a BCA detecting kit (Keygen, Nanjing, China) according to the manufacturer's protocol. Proteins samples were subjected to 10% SDS-PAGE, transferred onto a PVDF membrane, then incubated with ROCK1 (Abcam, Cambridge, MA, UK; dilution rates of 1:2000), nuclear receptor subfamily 2, group F, member 2 (NR2F2, Abcam, concentration of 2 μg/mL) and GAPDH antibodies (Abcam, dilution rates of 1:500) at 4°C overnight, respectively. The following day, the membranes were incubated with secondary antibodies (Abcam, dilution rates of 1:2000) at room temperature for 1 hours. Protein bands were detected on X-ray film using an enhanced chemiluminescence detection system.

2.8 | RNA fish

The procedure was performed as previously described by using a Ribo FISH Hybridization Kit (RiboBio). Briefly, SKOV3 and HO8910 cells seeded on the glass coverslips (0.8 × 0.8 cm) were cultured to 60%-70% confluence. The coverslips were washed with a solution of .5% Triton X-100 in 1× PBS. The coverslips were incubated with anti-NEAT1 or anti-miR-382-3p oligodeoxy-nucleotide probes (RiboBio) with a hybridization solution containing 1% blocking solution in a humid chamber at 37°C overnight. The following day, the coverslips were rinsed 3 times for 15 minutes (5 minutes each time) at 42°C with a solution of .1% Tween-20 in 4× sodium citrate buffer (SSC), once for 5 minutes in 2× SSC and once for 5 minutes in 1× SSC in dark conditions. Finally, after rinsing with 1× PBS for 5 minutes 3 times at room temperature and re-staining by DAPI (Cell Signaling Technologies, Danvers, USA), the coverslips were observed and photographed using a fluorescent microscope (Leica, Wetzlar, Germany). Images were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, USA).

2.9 | Dual luciferase reporter assay

The procedure was carried out as previously described. Wild and mutant reporter plasmids of NEAT1 (wt-NEAT1 and mut-NEAT1) and ROCK1 (wt-ROCK1 and mut-ROCK1), which contained wild or mutant miR-382-3p binding sites, were synthesized by GenePharma (GenePharma, Shanghai, China). When OC cell lines SKOV3 and HO8910 achieved 70% confluence, the synthesized reporter plasmids were co-transfected with miR-382-3p mimics or mimic control by using Lipofectamine 2000 (Invitrogen), respectively. After 48 hours, the changes in the fluorescence in each group were detected using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol.

2.10 | Statistical analysis

All experiments were repeated in triplicate and all data from 3 independent experiments were expressed as mean ± SD. GraphPad Prism V5.0 (GraphPad Software, USA) software and SPSS 19.0 statistical software were used for statistical analysis. Correlation between NEAT1 and clinicopathological features of OC patients was analyzed using Pearson's χ^2-test or the Mann–Whitney *U*-test. Survival analysis was performed using the log-rank test in GraphPad Prism 5.0. Differences in the 2 groups were analyzed using Student's *t* test or one-way ANOVA. Differences were considered significant or very significant if *P*-value <.05 or .01, respectively.

3 | RESULTS

3.1 | NEAT1 was overexpressed and correlated with poor prognosis in ovarian patients

The expression of NEAT1 in ovarian tissue specimens was first determined. Upregulated NEAT1 were found in most ovarian tissues (53/67, 79.10%) when compared with para-tumor tissue specimens, as detected by quantitative RT-PCR (qRT-PCR) (see Figure 1A and B). In addition, a representative elevation of NEAT1 was positively correlated with pathological grades of human ovarian tissue specimens as presented by in situ hybridization (ISH) analysis (Figure 1D). Furthermore, we found that elevated NEAT1 was more commonly presented in ovarian tissue specimens with distant metastasis when compared with that without distant metastasis (Figure 1C). Furthermore, the expression of NEAT1 at cellular level was determined by qRT-PCR in a human normal ovarian surface epithelial cell line, IOSE80, and 3 OC cell lines, ES2, HO8910 and SKOV-3. As the findings show in Figure 1E, increased NEAT1 was presented in the 3 OC cell lines ES2, HO8910 and SKOV-3 as comparing with IOSE80.

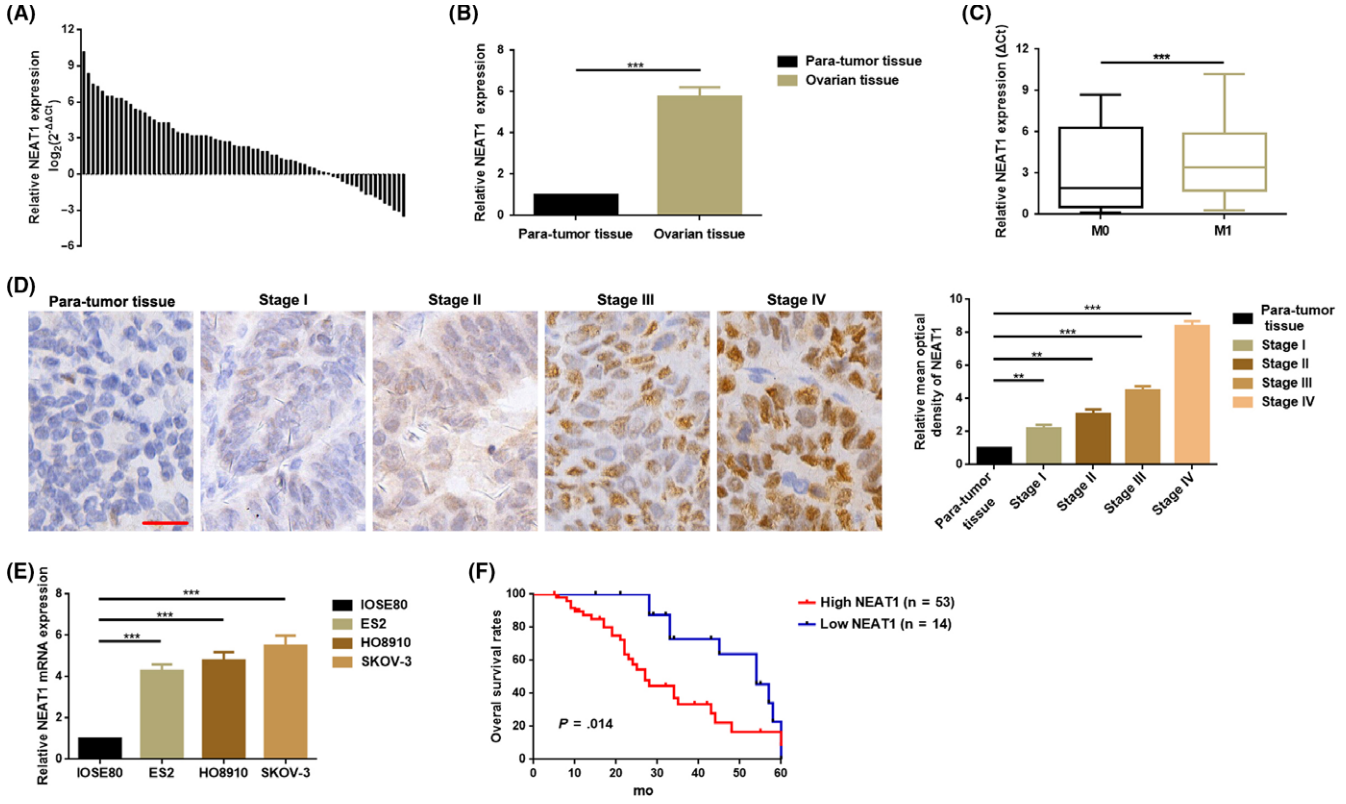

FIGURE 1 NEAT1 was overexpressed and correlated with poor prognosis in ovarian cancer. A,B, Expression of NEAT1 in ovarian cancer (OC) tissue specimens was determined by quantitative RT-PCR (qRT-PCR) assay; data are shown as $\log_2 (2^{-\triangle\triangle Ct})$ method (A) and $\triangle$Ct method (B), respectively. ***$P < .001$ as normalized and compared with para-tumor tissue group. C, NEAT1 was significantly upregulated in patients with distant metastasis, as confirmed by qRT-PCR assay. **$P < .01$ as compared to M_0 group. D, Optical density of NEAT1 was gradually elevated with advanced staging as measuring by an in situ hybridization assay. **$P < .01$ and ***$P < .001$ as normalized and compared with para-tumor tissue group, respectively. E, NEAT1 expression was elevated in OC cell lines ES2, HO8910 and SKOV3. ***$P < .001$ as normalized and compared with IOSE80 group. F, The overall survival (OS) in the patients with high NEAT1 (n = 53) was significantly shorter than that in the patients with low NEAT1 (n = 14); $P = .014$ as determined by Kaplan–Meier analyses. Data are shown as mean ± SD based on 3 independent experiments

Finally, we analyzed the correlation between the elevated NEAT1 and the clinicopathological features in the ovarian patients. As the results show in Figure 1E and Table 2, highly-expressed NEAT1 was closely correlated with a shorter survival rate (determined by Kaplan–Meier analysis), a poor differentiated degree ($P = .002$), a bigger tumor size ($P = .025$), an advanced FIGO stage ($P = .010$) and significant peritoneal metastasis ($P = .018$).

3.2 | Depression of NEAT1 suppressed migration/invasion and ROCK1 expression in SKOV3 and HO8910 cells

In this subsection, we describe a loss of function experiment to examine the role NEAT1 plays in metastasis in SKOV3 and HO8910 cells. We first verified that a transfection of NEAT1 silencer inhibited NEAT1 expression remarkably in SKOV3 and HO8910 cells using a qRT-PCR assay (Figure 2A). We then performed a transwell assay and a scratch assay to evaluate the metastatic ability changes in SKOV3 and HO8910 cells. As demonstrated in Figure 2B and C, depression of NEAT1 (transfection of NEAT1 silencer) resulted in a remarkable suppression of migration and invasion ability in SKOV3 and HO8910 cells as compared with NC silencer ($P < .01$). A further scratch assay verified the same tendency (Figure 2D,E) ($P < .01$).

ROCK1 was reported as a metastasis-related gene in various malignant tumors, including OC. Here, we also evaluated the effect NEAT1 working on ROCK1 expression. As demonstrated in Figure 2F, depression of NEAT1 significantly inhibited ROCK1 expression at protein level ($P < .01$).

3.3 | NEAT1 was directly targeting to miR-382-3p

Recently, lncRNA were reported as working as a ceRNA of certain miRNA to exert their function.[21] Using the online predictive software DIANA-LncBase (www.microrna.gr/LncBase) and TargetScan (http://www.targetscan.org/), we found that NEAT1 and ROCK1 shared the same binding sites for microRNA-382-3p (miR-382-3p) in their 3′ untranslated regions (3′-UTR), respectively (Figure 3A). We revealed that upregulation and downregulation of NEAT1 could

TABLE 2 Association of NEAT1 expression with clinicopathological features of ovarian cancer

Features	Number of cases	NEAT1 High	NEAT1 Low	*P*-value[a]
Age at diagnosis (y)				
<50	34	28	6	.358
≥50	33	25	8	
Histological subtype				
Serous	34	26	8	.914
Mucinous	5	4	1	
Endometroid	12	9	3	
Clear cell	9	8	1	
Other	7	6	1	
FIGO stage				
Stage I	14	7	7	.010
Stage II	15	11	4	
Stage III	15	14	1	
Stage IV	23	21	2	
Differentiated degree				
Well differentiated (G1)	15	7	8	.002
Moderately differentiated (G2)	16	13	3	
Poorly differentiated (G3)	36	33	3	
Residual tumor diameter (cm)				
<1	30	20	10	.025
≥1	37	33	4	
Peritoneal metastasis				
Absent	29	19	10	.018
Present	38	34	4	

[a]*P*-value was obtained from Pearson χ^2-test or Fisher's exact test.

negatively affect miR-382-3p expression. Meanwhile, an elevation or a depression of miR-382-3p also negatively affected NEAT1 expression. These phenomenon revealed a reciprocal repression effect between NEAT1 and miR-382-3p (Figure 3B,C). Furthermore, using an RNA FISH assay, we confirmed that NEAT1 and miR-382-3p were both co-localized in the nucleus in SKOV3 and HO8910 cells (Figure 3E,F), which indicated the interacting foundation between NEAT1 and miR-382-3p. Finally, a dual luciferase assay was constructed to verify the potential targeting effect between NEAT1 and miR-382-3p. Compared with mimic control, co-transfection of wt-NEAT1 and miR-382-3p mimics resulted in a notable weakening of fluorescence (see Figure 3D,G,H). When we mutated the theoretical binding sites NEAT1 might provide for miR-382-3p, the weakened fluorescence re-strengthened ($P < .01$). These outcomes indicated that NEAT1 could directly target miR-382-3p.

3.4 | MiR-382-3p suppressed migration/invasion by targeting ROCK1 in SKOV3 and HO8910 cells

In the above sections, we verified that NEAT1 could regulate migration/invasion and ROCK1 expression and that NEAT1 was a target of miR-382-3p. In addition, according to previous research, miR-382 and ROCK1 were both involving in OC malignancies.[22-24] Furthermore, as demonstrated in Figure 3A, NEAT1 and ROCK1 shared the same binding sites for miR-382-3p; therefore, we wondered whether there was any potential relationship between miR-382-3p and ROCK1 and OC cells' migration/invasion. We first confirmed that an increase and a decrease of miR-382-3p negatively regulated ROCK1 expression at mRNA and protein levels (Figure 4A,B). Second, we established the inhibitive effect of miR-382-3p working on migration/invasion in SKOV3 and HO8910 cells. Meanwhile, we found that the inhibitive effect of miR-382-3p did could be reversed by a wild ROCK1 overexpression plasmid wt-pcDNA-ROCK1 but not by a mutant type (mut-pcDNA-ROCK1) (Figure 4C,D). Finally, using a constructed dual luciferase assay, we confirmed that miR-382-3p could target ROCK1 3′-UTR with the predicted binding sites (Figure 4E,F). In brief, in this subsection, we established that miR-382-3p could target ROCK1 and suppress its mediated migration/invasion in SKOV3 and HO8910 cells.

3.5 | NEAT1 promoted ROCK1-mediated migration/invasion by acting as a ceRNA of miR-382-3p

As demonstrated above, NEAT1, miR-382-3p and ROCK1 were all involved in ovarian cells' migration/invasion, so we wondered whether the potential ceRNA mechanism exists among NEAT1, miR-382-3p and ROCK1. Wild and mutant NEAT1 overexpression plasmids (wt-pcDNA-NEAT1 and mut-pcDNA-NEAT1), which contained the wild and the mutant miR-382-3p binding sites, were applied to examine the regulatory network among NEAT1, miR-382-3p and ROCK1. As demonstrated in Figure 5A, it was wt-pcDNA-NEAT1 and not wt-pcDNA-NEAT1 that could suppress miR-382-3p expression, and the suppressive effect which wt-pcDNA-NEAT1 had demonstrated could be reversed by an elevation of miR-382-3p (co-transfection of wt-pcDNA-NEAT1 and miR-382-3p mimics). Meanwhile, we found that only wt-pcDNA-NEAT1 could promote ROCK1 expression, and when the theoretical binding sites for miR-382-3p were mutated, the facilitative effect of NEAT1 on ROCK1 disappeared. More convincingly, the facilitative effect of NEAT1 on ROCK1 could be attenuated by an elevation of miR-382-3p (co-transfection of wt-pcDNA-NEAT1 and miR-382-3p mimics) (Figure 5B). As reported previously, ROCK1 was also targeted by other miRNA like miR-340, miR-150.[25,26] Here, we measured the expression of miR-340 and miR-150 as well by qRT-PCR. As shown in Figure 5C and D, the expression of miR-340 and miR-150 were not affected by the abovementioned interventions. Furthermore, we determined the expression level of another previously reported downstream target of miR-382-3p: nuclear receptor subfamily 2, group F, member 2 (NR2F2).[27,28] As shown in Figure 5E, transfection of wt-pcDNA-NEAT1 also increased the NR2F2 expression in mRNA level and the promoting effect could be reversed by themiR-382-3p mimics. All the outcomes above strongly indicated that the function of NEAT1 working on ROCK1 was achieved through acting

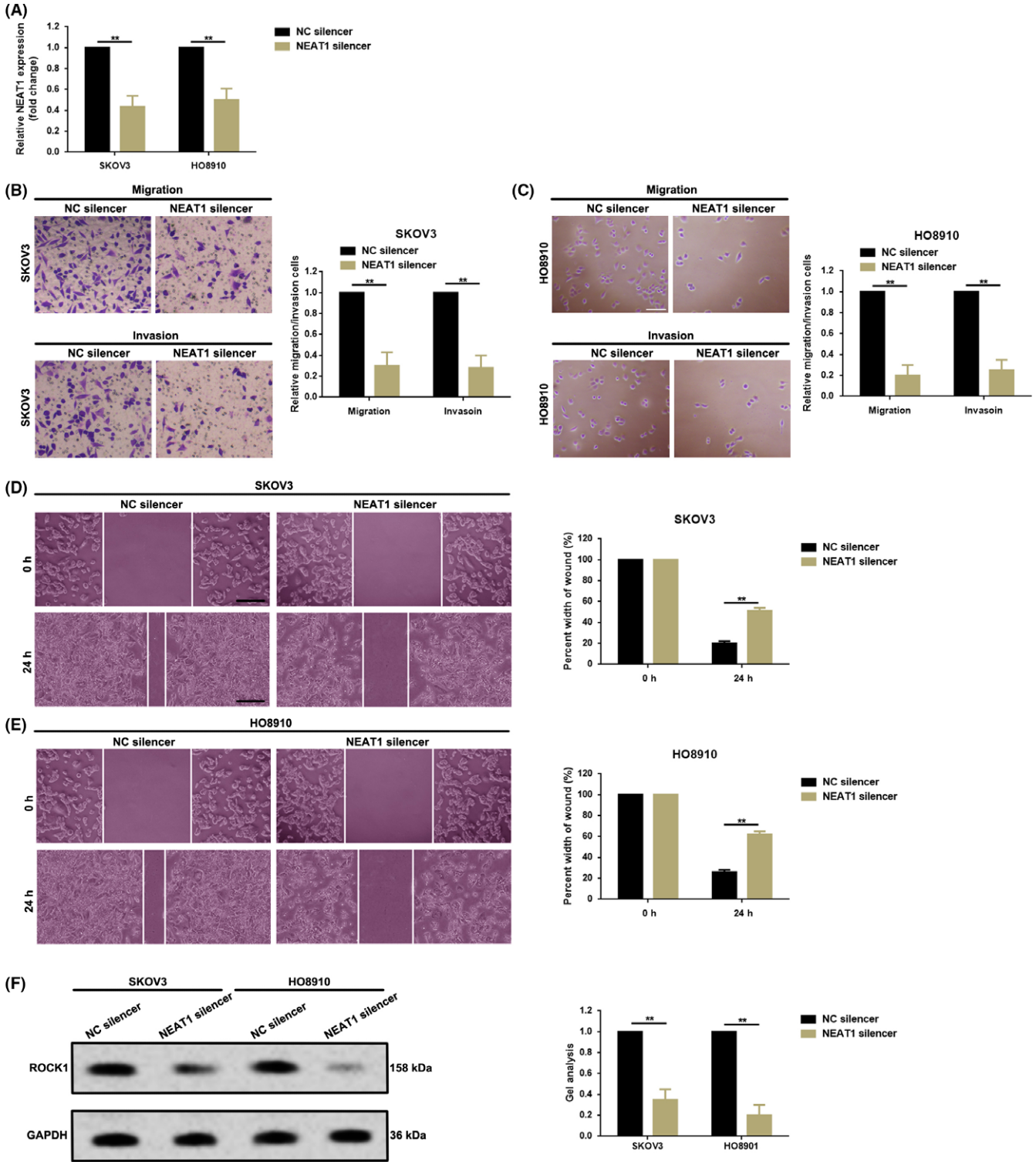

FIGURE 2 Depression of NEAT1 suppressed metastasis and ROCK1 expression in SKOV3 and HO8910 cells. A, NEAT1 was depressed by transfection of NEAT1 silencer as qualified by a quantitative RT-PCR assay. B,C, Depression of NEAT1 by transfection of NEAT1 silencer significantly suppressed SKOV3 (B) and HO8910 (C) cells' migration and invasion abilities as determined by a transwell assay. D,E, A scratch assay was applied to determine the inhibitive effect due to depression of NEAT1 on SKOV3 (D) and HO8910 (e) cells' migration ability. F, Depression of NEAT1 suppressed ROCK1 expression as measured by a western blot assay. $^{**}P < .01$ as normalized and compared with NC silencer group. Data were shown as mean ± SD based on 3 independent experiments

as a ceRNA of miR-382-3p. Finally, we re-executed the transwell assay to demonstrate the ceRNA mechanism of the NEAT1/miR-382-3p/ROCK1 axial working on migration/invasion in SKOV3 and HO8910 cells. As shown in Figure 5F and G, it was wt-pcDNA-NEAT1 and not wt-pcDNA-NEAT1 that promoted SKOV3 and HO8910 cells' migration/invasion, and the promoting effect could be

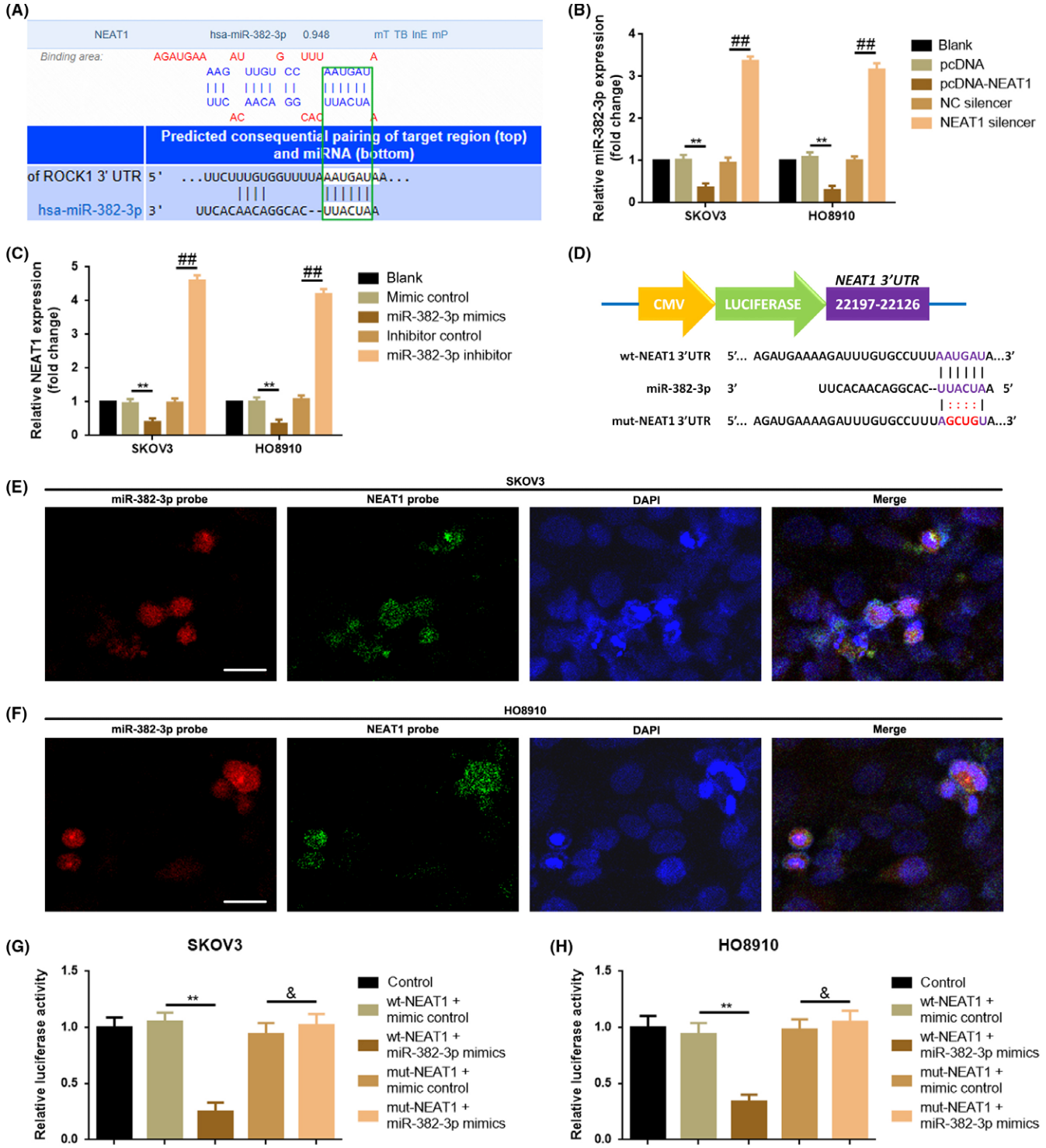

FIGURE 3 NEAT1 was directly targeting miR-382-3p. A, NEAT1 and ROCK1 shared similar microRNA responding elements (MRE) for miR-382-3p predicted by DIANA-LncBase and Targetscan. B,C, A reciprocal suppressive effect between NEAT1 and miR-382-3p was verified by a quantitative RT-PCR assay. $^{**}P < .01$ as normalized and compared with pcDNA group or mimic control group, and $\#\#P < .01$ as normalized and compared with NC silencer group or inhibitor control group, respectively. D, Diagram of the constructed NEAT1 reporter plasmid containing a wild or a mutant MRE for miR-382-3p. E,F, NEAT1 and miR-382-3p were localized to a similar position in nucleus of SKOV3 (E) and HO8910 (F) cells as presented by an RNA FISH assay. G,H, A luciferase assay demonstrated that miR-382-3p could directly target NEAT1 3′-UTR. Data were normalized to the control group, and $^{**}P < .01$ as compared with wt-NEAT1+ miR-382-3p mimics group while $\&P > .05$ as compared with mut-NEAT1+ miR-382-3p mimics group. Data are shown as mean ± SD from 3 independent experiments

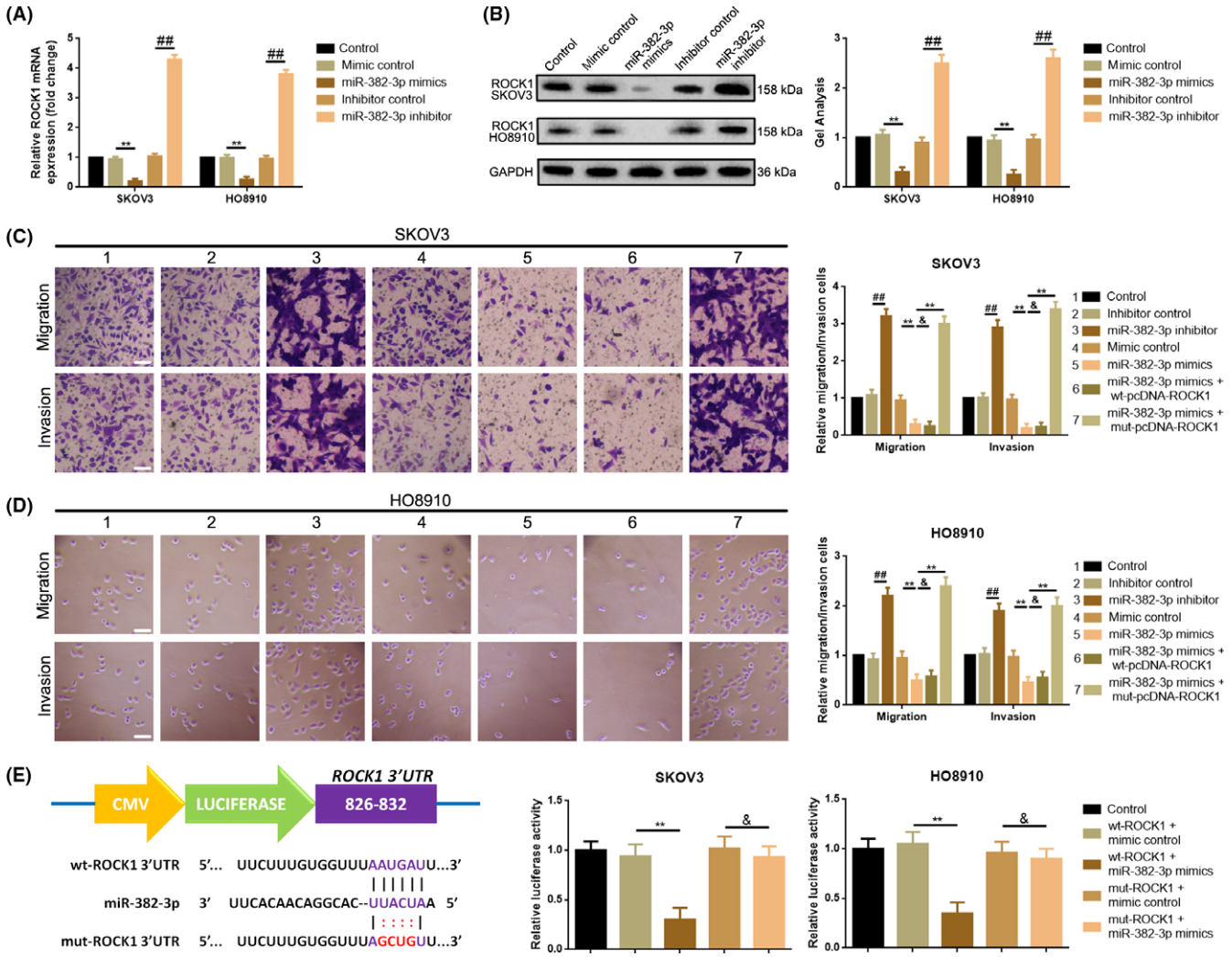

FIGURE 4 MiR-382-3p suppressed migration/invasion by targeting ROCK1 in SKOV3 and HO8910 cells. A,B, ROCK1 expression was inversely regulated by miR-382-3p as determined separately by a quantitative RT-PCR assay (A) and a western blot assay (B). Data were normalized to a control group, and $^{**}P < .01$ as compared with the mimics control group and $^{\#\#}P < .01$ as compared with the inhibitor control group. C,D, MiR-382-3p negatively affected migration and invasion and the suppressive effect miR-382-3p mimics presented was attenuated by a wt-pcDNA-ROCK1 but not by a mut-pcDNA-ROCK1 in SKOV3 and HO8910 cells, individually. Data were normalized to the control group, and $^{\#\#}P < .01$ compared with the inhibitor control group, while $^{**}P < .01$ and $\&P > .05$ compared with the miR-382-3p mimics group. E, Diagram (left) of the constructed ROCK1 reporter plasmid containing a wild or a mutant MRE for miR-382-3p and ROCK1 was a target of miR-382-3p as qualified by a luciferase assay (middle and right). Data were normalized to the control group, and $^{**}P < .01$ compared with the wt-ROCK1+ miR-382-3p mimics group, while $\&P > .05$ compared with the mut-ROCK1+ miR-382-3p mimics group. Data are shown as mean ± SD from 3 independent experiments

attenuated by an elevation of miR-382-3p. We drew the conclusion that NEAT1 promoted OC metastasis by regulating ROCK1 by acting as a ceRNA of miR-382-3p.

4 | DISCUSSION

Growing evidence has shown that lncRNA are not junk transcripts and play regulatory roles in multiple aspects of biological processes, including gene expression regulation, initiation and maintenance of protein complexes, subcellular architecture and cancer metastasis.[29-32] LncRNA NEAT1, which is transcribed by RNA polymerase II, is located at chromosome 11q13.1 and is extensively involved in various intracellular events like to build nuclear bodies, epigenetic event regulation and gene expression.[33] Zheng (2017) found that NEAT1 promoted hepatocellular carcinoma cells' EMT, migration and invasion capacities by stimulating the activation of HIF-2α.[16] Sun (2016) found that NEAT1 modulated the expression of the E2F transcription factor 3 (E2F3) gene by acting as a ceRNA and promoted non-small cell lung cancer (NSCLC) cells' proliferation and metastasis.[34] To date, related research on NEAT1 and OC is rare.[35,36] In the present study, we found that NEAT1 was upregulated in OC tissue specimens and in OC cell lines. In addition, an elevation of NEAT1 was closely related to OC patients' clinicopathological features,

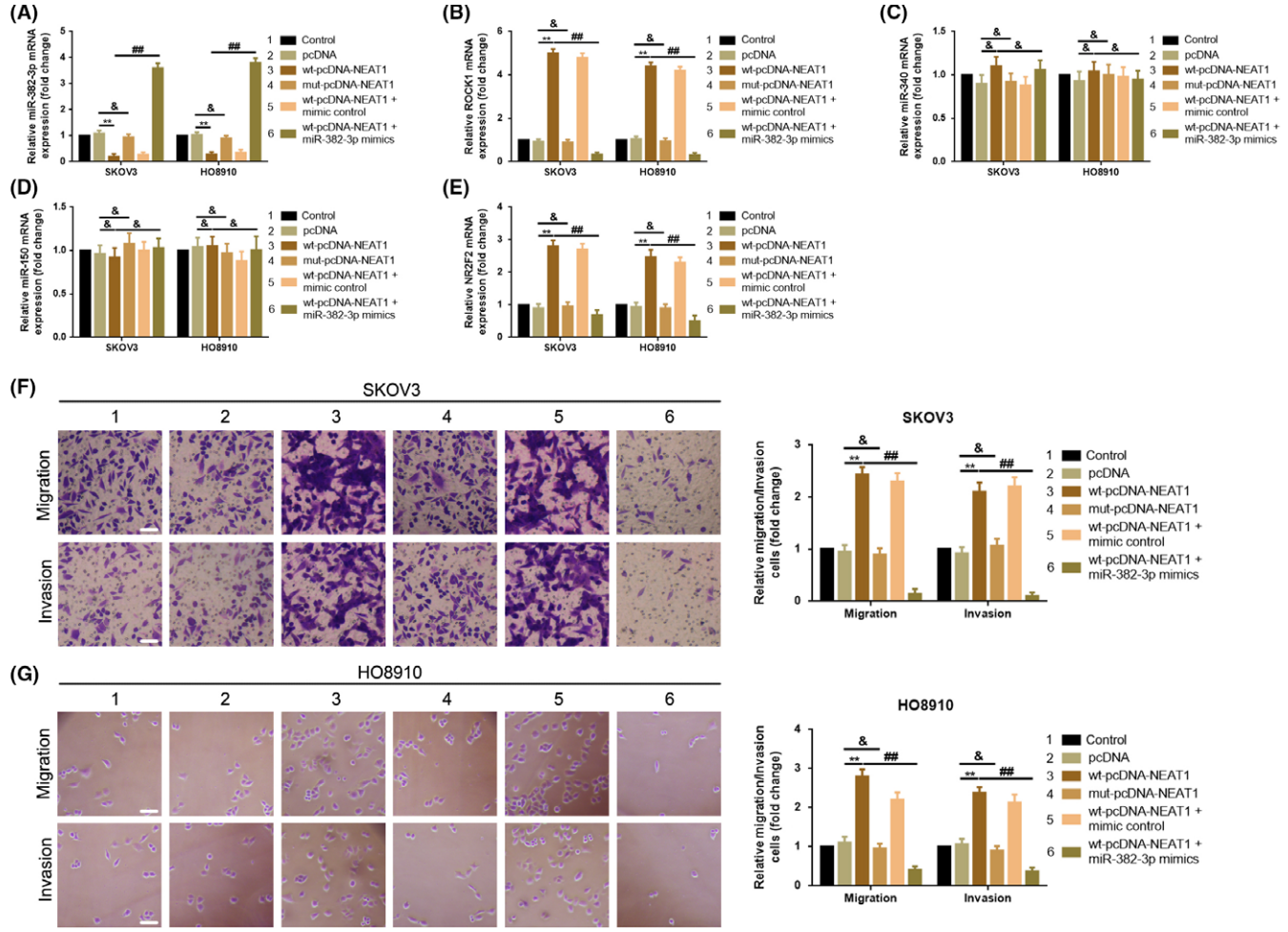

FIGURE 5 NEAT1 promoted ROCK1-mediated migration/invasion by acting as a ceRNA of miR-382-3p. A, A wild type but not a mutant type of NEAT1 overexpression plasmid suppressed miR-382-3p expression, and the suppressive effect was reversed by miR-382-3p mimics. B, ROCK1 expression was promoted by a wild type but not by a mutant type of NEAT1 overexpression plasmid, and the facilitative effect was attenuated by miR-382-3p mimics. &$P > .05$, **$P < .01$ comparing with the pcDNA group; ##$P < .01$ comparing with the wt-pcDNA-NEAT1 group. C,D, Neither the wild type or mutant type of NEAT1 overexpression plasmid affected the expression of miR-340 (C) and miR-150 (D), which were qualified by a quantitative RT-PCR assay. &$P > .05$ comparing with pcDNA group. E, The expression of NR2F2 presented the same trend as ROCK1 under different NEAT1 and miR-382-3p interventions. &$P > .05$, **$P < .01$ comparing with pcDNA group, ##$P < .01$ comparing with wt-pcDNA-NEAT1 group. F,G, Migration and invasion ability of SKOV3 (F) and HO8910 (G) were promoted by a wild type but not by a mutant type of NEAT1 overexpression plasmid, and the facilitative effect was attenuated by miR-382-3p mimics. &$P > .05$, **$P < .01$ comparing with pcDNA group; ##$P < .01$ comparing with wt-pcDNA-NEAT1 group. All data are shown as mean ± SD from 3 independent experiments and were normalized to the control group, individually

especially to a shorter survival rate, a poor differentiated degree, a bigger tumor size, an advanced FIGO stage and significant peritoneal metastasis. Through a loss of function test, we verified that depression of NEAT1 inhibited OC cells' metastatic ability, which indicating that NEAT1 functioned as an oncogene in OC. LncRNA are widely reported as working as a "sponge" or a "ceRNA" in the regulatory network which is comprised of lncRNA, miRNA and target genes. In the present study, we found a metastasis-related regulating network which is comprised of NEAT1, miR-38 OC 2-3p and ROCK1. In addition, we confirmed that both NEAT1 and ROCK1 were the targets of miR-382-3p. We revealed that NEAT1 could regulate ROCK1 and its mediated metastasis by working as a ceRNA of miR-382-3p.

As a member of microRNA (miRNA), miR-382 works as a tumor suppressor in various malignant tumors.[37-40] Zhang (2016) reports that miR-382 inhibited proliferation and metastasis through targeting chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) in prostate cancer cells.[27] Xu (2015) reports that miR-382 suppressed osteosarcoma metastasis and relapse by targeting Y box-binding protein 1(YB-1).[41] Here, through the transwell assay, we found that miR-382-3p could negatively affect SKOV3 and HO8910 cells' migration and invasion. The negative regulation effect could be reversed by a mutant ROCK1 cDNA, strongly indicating that miR-382-3p was involved in ROCK1-mediated migration/invasion. Furthermore, the constructed luciferase assay strongly verified that ROCK1 was a direct target of miR-382-3p. ROCK1, a direct target of miR-382-3p, which is verified in the present study, is widely reported as an oncogene in multiple cancers, including osteosarcoma, pancreatic cancer, papillary thyroid

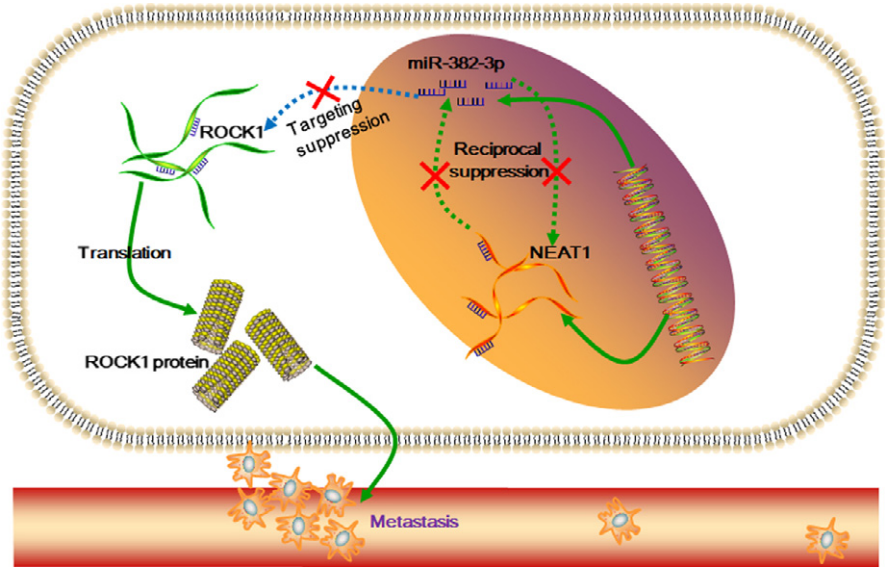

FIGURE 6 Schematic diagram of mechanism of this research. NEAT1 suppressed miR-382-3p expression by serving as a ceRNA of ROCK1 to promote ROCK1 expression and to facilitate its mediated metastasis in ovarian cancer

carcinoma, breast cancer and OC.[19,22,42-46] In the present study, we evaluated the effect that different expression levels of NEAT1 and miR-382-3p have on ROCK1 and its mediated migration/invasion. We found that elevation of NEAT1 and repression of miR-382-3p both promoted ROCK1 expression and its mediated migration/invasion. Through the online bioinformatic prediction, we revealed that NEAT1 and ROCK1 shared the same miRNA response elements (MRE) for miR-382-3p. In addition, we illustrated the reciprocal effect between NEAT1 and miR-382-3p. Furthermore, using a luciferase assay, we demonstrated that NEAT1 was a target of miR-382-3p. We revealed that it was wt-pcDNA-NEAT1 and not mut-pcDNA-NEAT1 that could directly affect ROCK1 and NR2F2: another reported downstream target of miR-382-3p. In addition, the facilitative effect of wt-pcDNA-NEAT1 on ROCK1 and NR2F2 could be reversed by miR-382-3p mimics. In addition, the unchanged expression level of miR-340 and miR-150 laterally approved that the regulatory effect of NEAT1 on ROCK1 was achieved through miR-382-3p pathway. As the diagram reveals in Figure 6, these outcomes strongly illustrated that NEAT1 regulated ROCK1 and its mediated migration/invasion by working as ceRNA of miR-382-3p.

The metastasis of OC is intricate. Multiple molecules and signal pathways may contribute to this complicated biological process. The present study illustrates that the NEAT1/miR-382-3p/ROCK1 axial might be a potential target in treating OC.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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