COUP-TFII promotes epithelial-mesenchymal transition by inhibiting miR-34a expression in colorectal cancer

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Abstract. Chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) expression is upregulated in colorectal cancer and is associated with its progression and a poor prognosis. The aim of the present study was to determine whether COUP-TFII regulates colorectal cancer cell (CRC) invasion and migration by inhibiting microRNA (miR)-34a. Transwell system and wound healing assays were performed to examine cell invasiveness and migration, respectively. Reverse transcription polymerase chain reaction and western blotting were used to detect the RNA and protein levels of target molecules, respectively. The results revealed that COUP-TFII knockdown significantly inhibited CRC invasion and migration. In addition, the expression of miR-34a, a well-known tumor suppressor was revealed to be inversely correlated with COUP-TFII expression. The miR-34a mimic significantly reduced CRC invasion and migration abilities, while the miR-34a inhibitor enhanced CRC invasion and migration activity. There was no significant difference between the negative small interfering RNA and miR-34a inhibitor groups following knockdown of COUP-TFII. Furthermore, western blotting demonstrated that miR-34a mimics inhibited the epithelial-mesenchymal transition (EMT) process of CRCs, while the miR-34a inhibitor had the opposite effect. Taken together, the results demonstrate that miR-34a regulates CRC invasion and migration by examining the mechanism by which COUP-TFII regulates EMT.

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Introduction

Colorectal cancer (CRC) is a very common malignancy associated with a high mortality rate, and it has become a major health problem worldwide (1). Due to advances in screening techniques and surgical management, the mortality rate of CRC has reduced considerably in developed countries (2). However, as is the case in other organ malignancies, recurrence and metastasis in CRC counteracts these improvements in prognosis following resection. The 5-year overall survival rate of patients with CRC is nearly 90% when the CRC is localized, but it reduces to <70% once distant metastases occurs (3). It is well known that the development of CRC metastasis is an extremely complex process with multiple stages and various molecular mechanisms. Therefore, it is urgently necessary to obtain a greater understanding of the factors involved in invasion and migration, and to confirm novel prognostic biomarkers to improve the survival rate in CRC.

Chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII), also known as nuclear receptor subfamily 2 group F member 1, is a nuclear orphan receptor that possesses two highly conserved motifs (a DNA-binding domain and a putative ligand-binding domain), and it belongs to the steroid/thyroid hormone receptor super-family (4). Biochemical studies have confirmed that COUP-TFII commonly exists in its dimeric form, has a high affinity to down-regulator of transcription 1, and competes for other nuclear receptors activated by hormone responses, which results in the suppression of a large number of genes (5,6). In addition, COUP-TFII forms DNA-binding heterodimers with retinoid X receptor, competes for various nuclear receptors,

and consequently reduces hormone responsiveness (7). Apart from acting as a repressor, COUP-TFII also activates the promoters of a vast number of genes by interacting with known co-activators such as p300 or other transcription factors (8). However, no data published thus far has shown whether COUP-TFII regulates the expression of non-coding RNA, including long non-coding RNA and microRNA (miR/miRNA). COUP-TFII is mainly expressed in early embryonic tissues and serves an important role in regulating various developmental processes such as peripheral and central nervous system developments (9).

Epithelial-mesenchymal transition (EMT) is considered the main process by which various cancers progress, including CRC, which gains metastatic features as tumor cells transition from having an epithelial morphology to an elongated, fibroblast-like morphology with depolarization and cell-cell disconnection. In addition, EMT promotes the development of drug resistance and stemness, which are significant inhibitors of the successful treatment of cancer (10). COUP-TFII expression is often downregulated shortly after birth, but several recent reports have shown that COUP-TFII expression is significantly increased in various cancer tissues when compared with corresponding non-cancerous tissues and is correlated with cancer development (11,12). Qin *et al* (13,14) demonstrated that by regulating two major angiogenic signaling pathways, vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR)-2 and angiopoietin (Ang)-1/tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2 (Tie2), ectopic COUP-TFII expression serves a crucial role in promoting angiogenesis in xenograft mouse models. Furthermore, COUP-TFII was reported to suppress the expression of several tumor suppressors such as BRCA1 to promote tumor cell proliferation and inhibit apoptosis (15). Although COUP-TFII is detected in the mesenchyme and associated with mesenchymal differentiation to epithelium (16), previous results have suggested that the paradoxical effect of this receptor may regulate the EMT process in cancer. Bao *et al* (17) demonstrated that upregulation of COUP-TFII expression is associated with the overexpression of Snail family transcription repressor 1 (Snail1), an important enhancer of EMT. This finding was corroborated by Zhang *et al* (18) who demonstrated that miRNA-382 against COUP-TFII led to the inhibition of Snail1 expression. Conversely, a high nuclear receptor subfamily 2 group F member 2 transcript level was revealed to be negatively associated with the transforming growth factor (TGF)-β signaling pathway and EMT in breast cancer (19).

miRNAs are non-coding RNAs of 18-22 nucleotides in length that negatively regulate gene expression at the post-transcriptional level by directly binding with the 3'-untranslated regions of target mRNAs to induce mRNA degradation or suppress mRNA translation (20). miRNAs serve a key role in cell growth and metastasis in colorectal cancer (21). miR-34a is a known tumor suppressor that takes part in the proliferation, migration and metastasis of tumor cells. It has also been reported that miR-34a could inhibit cell migration and invasion in various cancer cell types, such as breast cancer, laryngeal carcinoma and human glioma (22-24).

Therefore, the aim of the present study was to further understand the role of COUP-TFII in CRC migration and the mechanism underlying the EMT process to prevent the invasion and migration of CRC by inhibiting miR-34a expression.

Materials and methods

Cell lines and antibodies. Three human CRC cell lines (HCT116, HT29 and LOVO) were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in 1640 complete medium containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% streptomycin and penicillin in a humidified incubator with 5% CO_2 at 37˚C. Primary antibodies against COUP-TFII (cat. no. ab50487, Abcam, Cambridge, MA, USA; dilution 1:1,000), GAPDH (cat. no. 5174), E-cadherin (cat. no. 14472; dilution 1:1,000) and Vimentin (cat. no. 5741; dilution 1:1,000), and goat anti-rabbit horseradish peroxidase (HRP)-conjugated (cat. no. 7074; dilution 1:2,000) and goat anti-Mouse HRP-conjugated secondary antibodies (cat. no. 7076; dilution 1:2,000) (Cell Signaling Technology, Inc., Danvers, MA, USA) were utilized in the present study.

Small interfering (si)-RNA transfection. LOVO, HCT116, and HT29 cells ($1x10^5$) in the logarithmic phase of growth were suspended in 2 ml of 1640 complete medium and subsequently plated in a 6-well plate for 24 h at 37˚C prior to transfection. Then, 50 nM siRNAs for target genes or a scramble control (Shanghai GenePharma Co., Ltd., Shanghai, China) were transfected into the cell monolayers with 20-30% confluence using the Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. The sequences of siRNAs were as follows: COUP-TFII-homo-2445 sense, 5'-GGCCGUAUAUGG CAAUUCATT-3' and antisense, 5'-UGAAUUGCCAUAUAC GGCCTT-3'; COUP-TFII-homo-1971 sense, 5'-GCGAGC UGUUUGUGUUGAATT-3' and antisense, 5'-UUCAACACA AACAGCUCGCTT-3'; COUP-TFII-homo-2100 sense, 5'-GG AUCUUCCAAGAGCAAGUTT-3' and antisense, 5'-ACU UGCUCUUGGAAGAUCCTT-3'; scramble control sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-AC GUGACACGUUCGGAGAATT-3'. Subsequent experiments were performed 6 h post-transfection.

RNA oligoribonucleotides and transfection. The miR-34a mimic, inhibitor (5 nM) and negative control siRNA (as aforementioned) were synthesized by Shanghai GenePharma Co., Ltd. Transfection was conducted using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's instructions. Subsequent experiments were performed following 6 h. The sequences were as follows: miR-34a mimic sense, 5'-UGGCAGUGUCUUAGCUGGUUGU-3' and antisense, 5'-AACCAGCUAAGACACUGCCAUU-3'; miR-34a inhibitor, 5'-ACAACCAGCUAAGACACUGCCA-3'.

Cell migration and invasion assays. Migration and invasion assays were performed using the Transwell system (24-well insert; 8.0-μm pores). For this, $5x10^4$ CRC cells transfected with siRNAs were suspended in 200 μl 1640 complete medium without FBS and plated in the upper chamber. For

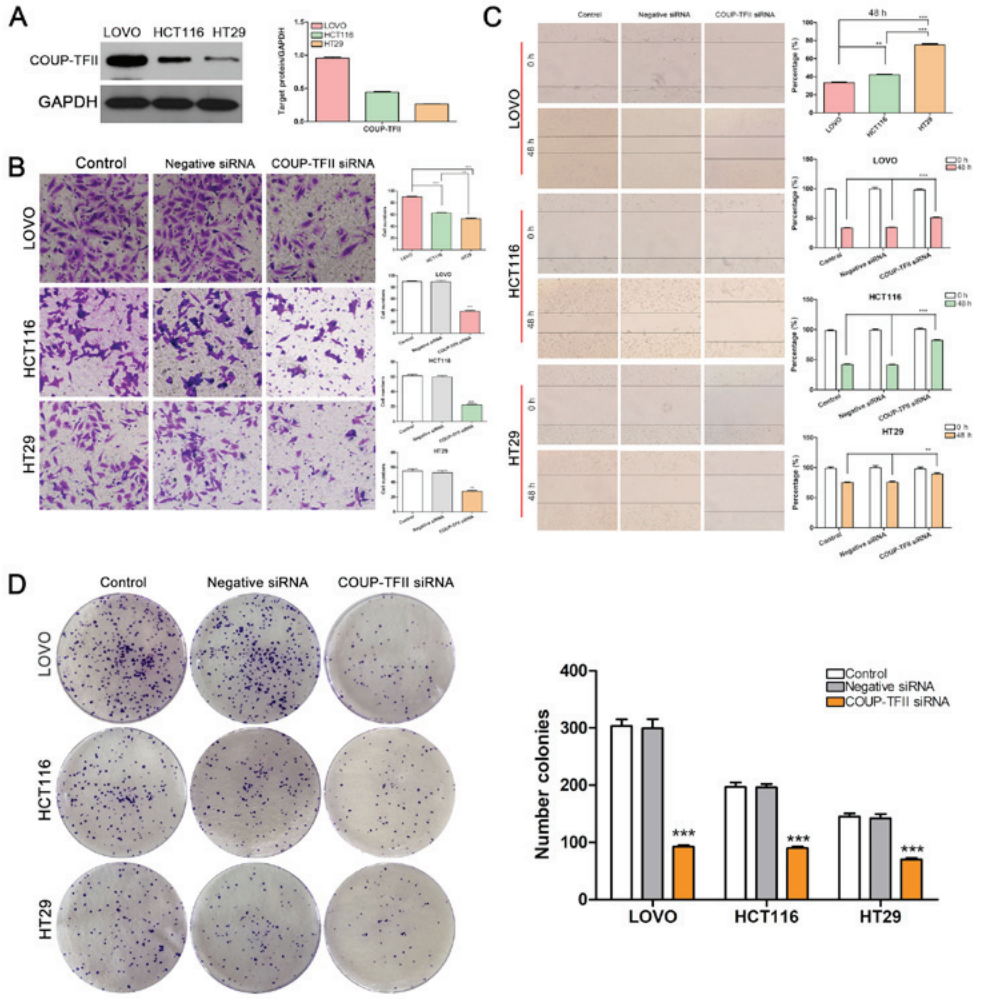

Figure 1. COUP-TFII knockdown significantly reduces the migration and invasion of CRC cell lines. (A) Western blotting was performed to detect COUP-TFII expression in CRC cells. COUP-TFII expression was high in LOVO cells, moderate in HCT116 cells, and low in HT29 cells. (B) COUP-TFII knockdown enhanced the number of cells that passed through the Matrigel membrane (magnification, x100). (C) The percentage of wound healing in CRC cells was significantly reduced in CRC cells compared with the control group (magnification, x100). (D) The colony-formation ability of HCT116, HT29, and LOVO cells was inhibited with COUP-TFII knockdown. **P<0.01 and ***P<0.001 vs. Control (as indicated). COUP-TFII, chicken ovalbumin upstream promoter-transcription factor II; CRC, colorectal cancer cell; siRNA, small interfering RNA.

invasion assays, this chamber was coated with Matrigel. The bottom chamber was filled with 500 μl 1640 complete medium supplemented with 10% FBS, in order to drive cell translocation. Following incubation at 37˚C for 24, 48 and 72 h, the cells on the upper surface of the chamber were scraped off using cotton swabs, while cells in the lower chamber were fixed with 95% methanol for 20 min at room temperature then stained with 0.4% crystal violet at room temperature for 5 min. The number of invaded and migrated cells were then counted under a light microscope (magnification, x100; Olympus Corporation, Tokyo, Japan).

Wound scratch assay. CRC cells (1x10^5) transfected with siRNAs in the logarithmic phase of growth were suspended in 2 ml 1640 complete medium without FBS and added to 6-well plates. The CRC monolayers with 80-90% confluence were scratched with 100 μl pipettes. The distance to which the cells migrated to was recorded at 0 and 48 h following scratching.

Colony forming assay. A total of 600 cells in the log phase were suspended in 2 ml 1640 complete medium supplemented with 10% FBS and added to 6-well plates in a humidified incubator with 5% CO_2 at 37˚C. Following 1 week, the colonies on the plates were fixed with 95% methanol for 10 min at room temperature and then stained with 0.4% crystal violet at room temperature for 5 min. The number of colonies was then counted under a light microscope (Olympus Corporation).

Western blotting. Protein was extracted from CRC cells using Radioimmunoprecipitation Assay buffer (Beyotime Institute of Biotechnology, Jiangsu, China) supplemented with protease/phosphatase inhibitors (Cell Signaling Technology, Inc.). Total protein concentration was determined using the bicinchoninic acid assay, and the protein sample was then denatured by boiling at 95˚C for 10 min. Equal amounts (40 μg) of protein were subjected to 10% SDS-PAGE and then transferred to 0.45 μm polyvinylidene fluoride membranes (EMD Millipore, Bedford, MA, USA) by electroblotting at 350 mA for 90 min. Following membrane blocking with 5% nonfat milk at 37˚C for 2 h, they were immersed in 10 ml of TBS/0.1% Tween-20 containing 0.1% of the aforementioned primary antibodies and 5% FBS (Gibco; Thermo Fisher

Scientific, Inc.) overnight at 4˚C. The membranes were washed three times with TBS/0.1% Tween-20 and then incubated with the aforementioned secondary antibodies for 1 h at room temperature. Protein expression was analyzed using SuperSignal West Pico Chemiluminescent Substrate (Pierce; Thermo Fisher Scientific, Inc.). The bands were quantified by densitometry using ImageLab 5.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). GADPH was used as the internal control.

Statistical analysis. Data were presented as the mean ± standard deviation of triplicate experiments and analyzed using one-way analysis of variance followed by Tukey's post hoc test. Data were analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). TargetScan 7.2 (www.targetscan.org) software was used to predict miRNAs associated with COUP-TFII. P<0.05 was considered to indicate a statistically significant difference.

Results

COUP-TFII knockdown suppresses the migration and invasion of CRC cell lines. The present study performed western blotting to determine the level of COUP-TFII expression in the three CRC cell lines and revealed that this receptor was strongly expressed in LOVO cells, moderately expressed in HCT116 cells, and weakly expressed in HT29 cells (Fig. 1A). Notably, the cells' ability to migrate and invade exhibited a similar trend as COUP-TFII expression (P<0.01 and P<0.001 vs. Control; Fig. 1B and C). Once COUP-TFII expression was knocked down using siRNA, the number of cells that passed through the Matrigel membrane and the percentage of wound healing were significantly reduced in CRC cells when compared with control cells (Fig. 1B and C). These results demonstrated that COUP-TFII expression was associated with CRC cell invasion and migration. Furthermore, COUP-TFII knockdown impaired the colony-forming ability of HCT116, HT29 and LOVO cells (P<0.001 vs. Control; Fig. 1D).

miR-34a suppresses CRC invasion and migration via a process mediated by COUP-TFII. TargetScan (www.targetscan.org) software was used to predict miRNAs associated with COUP-TFII (Fig. 2A). RT-qPCR revealed that miR-34a expression in CRC cells was inversely associated with COUP-TFII expression; miR-34a was highly expressed in HT29 cells, moderately expressed in HCT116 cells, and the lowest expression was observed in LOVO cells (Fig. 2B). In addition, a decrease in COUP-TFII expression led to a significant increase in miR-34a expression (P<0.001 vs. Control; Fig. 2C). Furthermore, miR-34a mimics could decrease the expression of COUP-TFII and the miR-34a inhibitor could increase COUP-TFII expression (Fig. 2D). These results indicated that COUP-TFII and miR-34a may regulate each other (Fig. 2C and D). Since miR-34a is an important suppressor in CRC (25), and its role in CRC invasion and migration has also been explored, miR-34a mimics and inhibitors were used to up- or downregulate its expression (Fig. 3A). As expected, transfection with the miR-34a inhibitor increased the invasion and migration abilities of CRC cells, while miR-34a mimics had the opposite effect (P<0.05, P<0.01 and P<0.001 vs. Control; Fig. 3B and C).

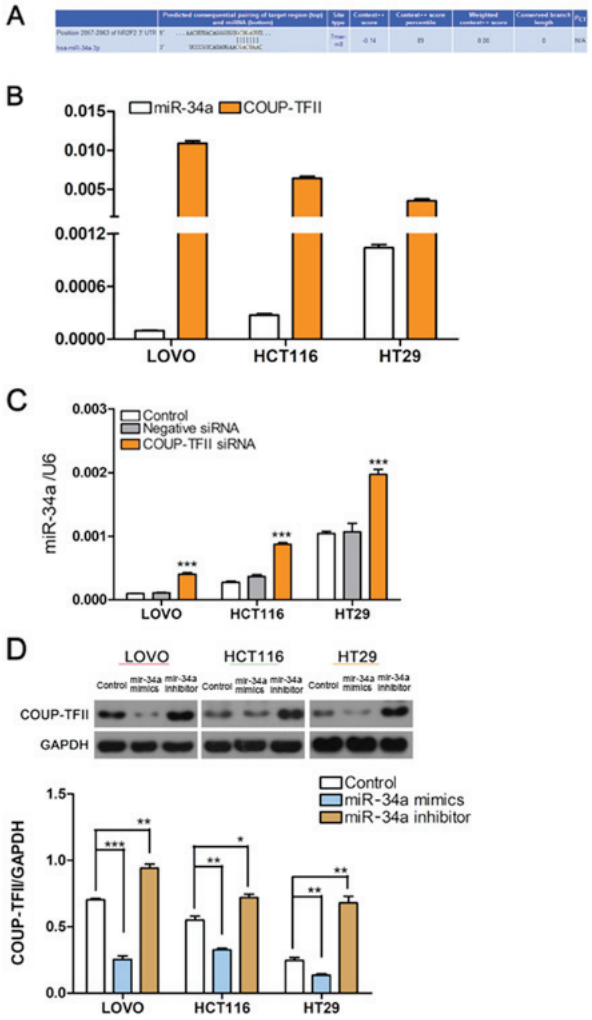

Figure 2. miR-34a expression is inversely associated with COUP-TFII expression. (A) Predicted matches for miR-34a to the COUP-TFII 3'-untranslated region by TargetScan. (B) miR-34a was highly expressed in HT29 cells, moderately expressed in HCT116 cells, and had low expression in LOVO cells. (C) COUP-TFII knockdown significantly increased miR-34a expression. ***P<0.001 vs. Control. (D) Western blot analysis determined the expression of COUP-TFII following transfection with miR-34a mimics or inhibitor. *P<0.05, **P<0.01 and ***P<0.001, as indicated. COUP-TFII, chicken ovalbumin upstream promoter-transcription factor II; miR, microRNA; siRNA, small interfering RNA.

miR-34a reverses the effect of COUP-TFII on CRC cells. To determine whether miR-34a is essential in maintaining the effects of COUP-TFII on the promotion of CRC invasion and migration, an miR-34a inhibitor and siRNA targeting COUP-TFII were transfected into CRC cells, respectively. The results revealed that the number of cells that passed through the Matrigel membrane and the percentage of wound healing did not differ between the miR-34a inhibitor group and the COUP-TFII-miR-34a joint knockdown group (Fig. 4A and B). Thus, miR-34a reversed the impairment of COUP-TFII knockdown on CRC cell invasion and migration, and could be regulated by COUP-TFII.

COUP-TFII knockdown reduces EMT in CRC cell lines. Furthermore, Vimentin expression was high in LOVO cells, moderate in HCT116 cells and low in HT29 cells; however, E-cadherin expression exhibited the opposite trend and

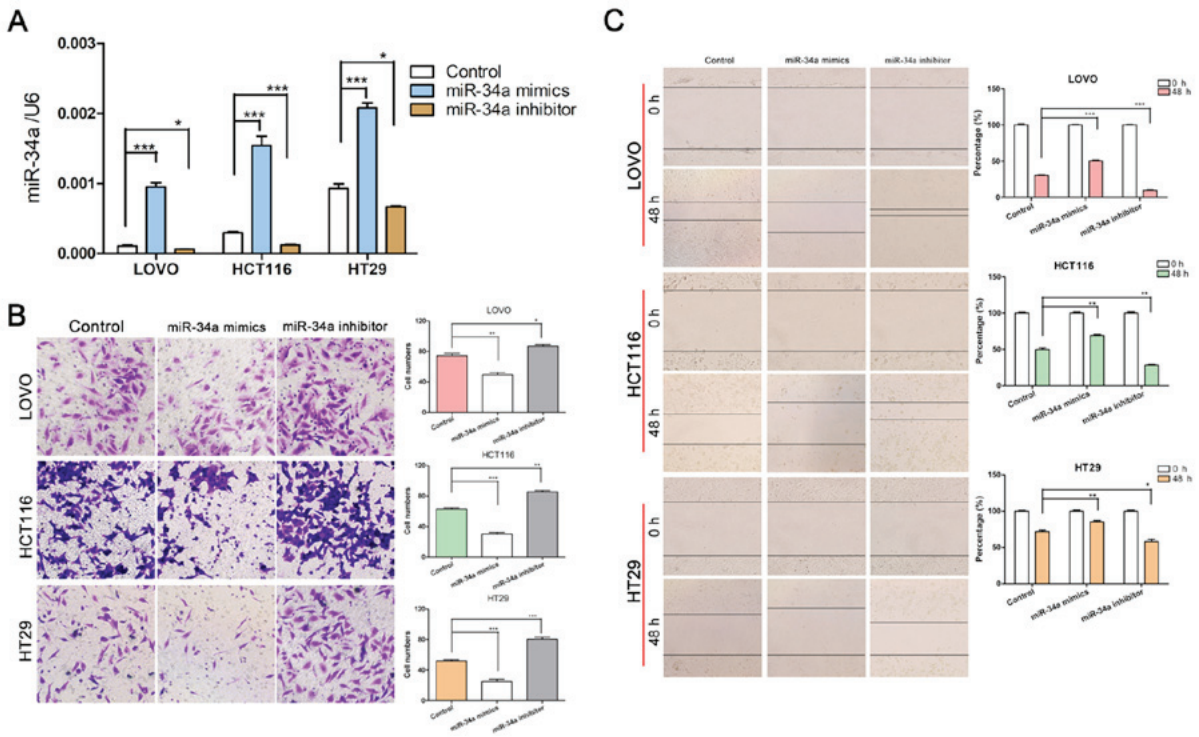

Figure 3. Effect of miR-34a inhibitor and mimics on CRC cells. (A) miR-34a mimics and inhibitors were used to alter miR-34a expression. *P<0.05 and ***P<0.001, as indicated. (B) Images and quantification of CRC cell invasion following treatment with miR-34a mimics, miR-34a inhibitor or control. The miR-34a inhibitor promoted CRC cell invasion, but miR-34a mimics produced the opposite effect (magnification, x100). (C) Wound healing assay revealed that the miR-34a mimics reduced cell migration capabilities and the miR-34a inhibitor increased it when compared with the control (magnification, x100). *P<0.05, **P<0.01 and ***P<0.001, as indicated. miR, microRNA; CRC, colorectal cancer cell.

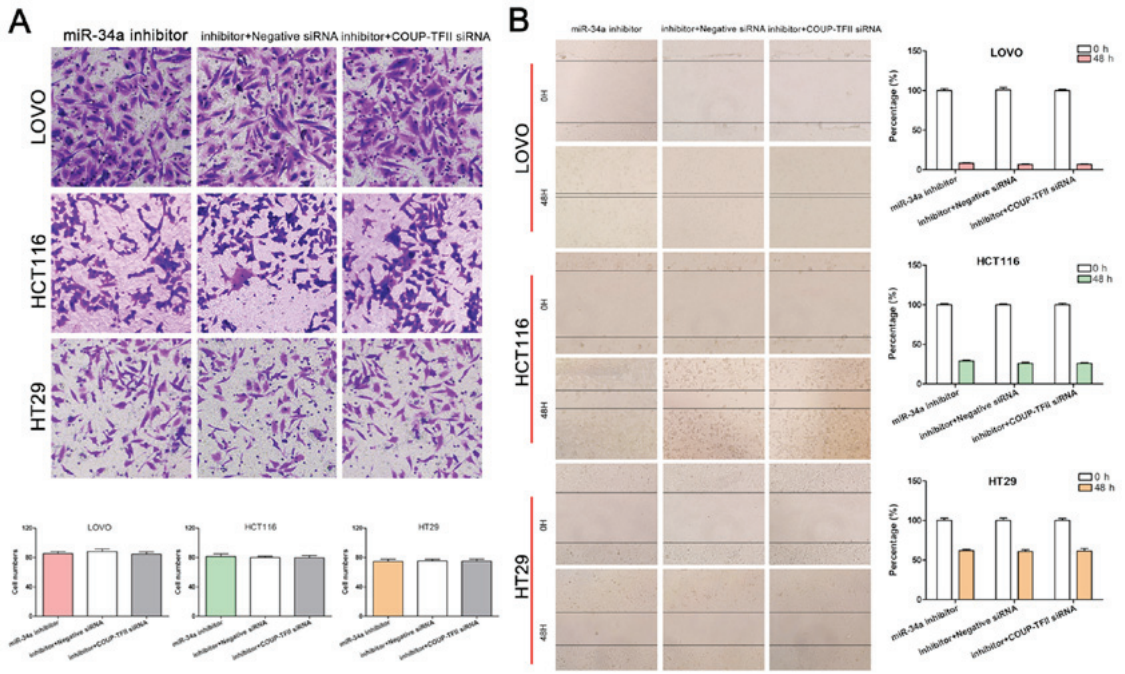

Figure 4. Effect of the miR-34a inhibitor on the invasiveness and migration of CRC cells transfected with COUP-TFII-miR-34a. (A and B) miR-34a inhibitor transfection did not significantly affect the number of cells passing through the (A) Matrigel membrane nor (B) the percentage of wound healing in CRC cells transfected with COUP-TFII siRNA+miR-34a inhibitor when compared with negative siRNA+miR-34a inhibitor at 0 h (control) cells (magnification, x100). miR, microRNA; CRC, colorectal cancer cell; COUP-TFII, chicken ovalbumin upstream promoter-transcription factor II; siRNA, small interfering RNA.

had low expression in LOVO cells, moderate expression in HCT116 cells, and high expression in HT29 cells (Fig. 5A). The results also demonstrated that miR-34a knockdown reduced E-cadherin expression and increased Vimentin expression in CRC cells (P<0.05, P<0.01 and P<0.001 vs. Control; Fig. 5B). Conversely, COUP-TFII inhibition significantly increased

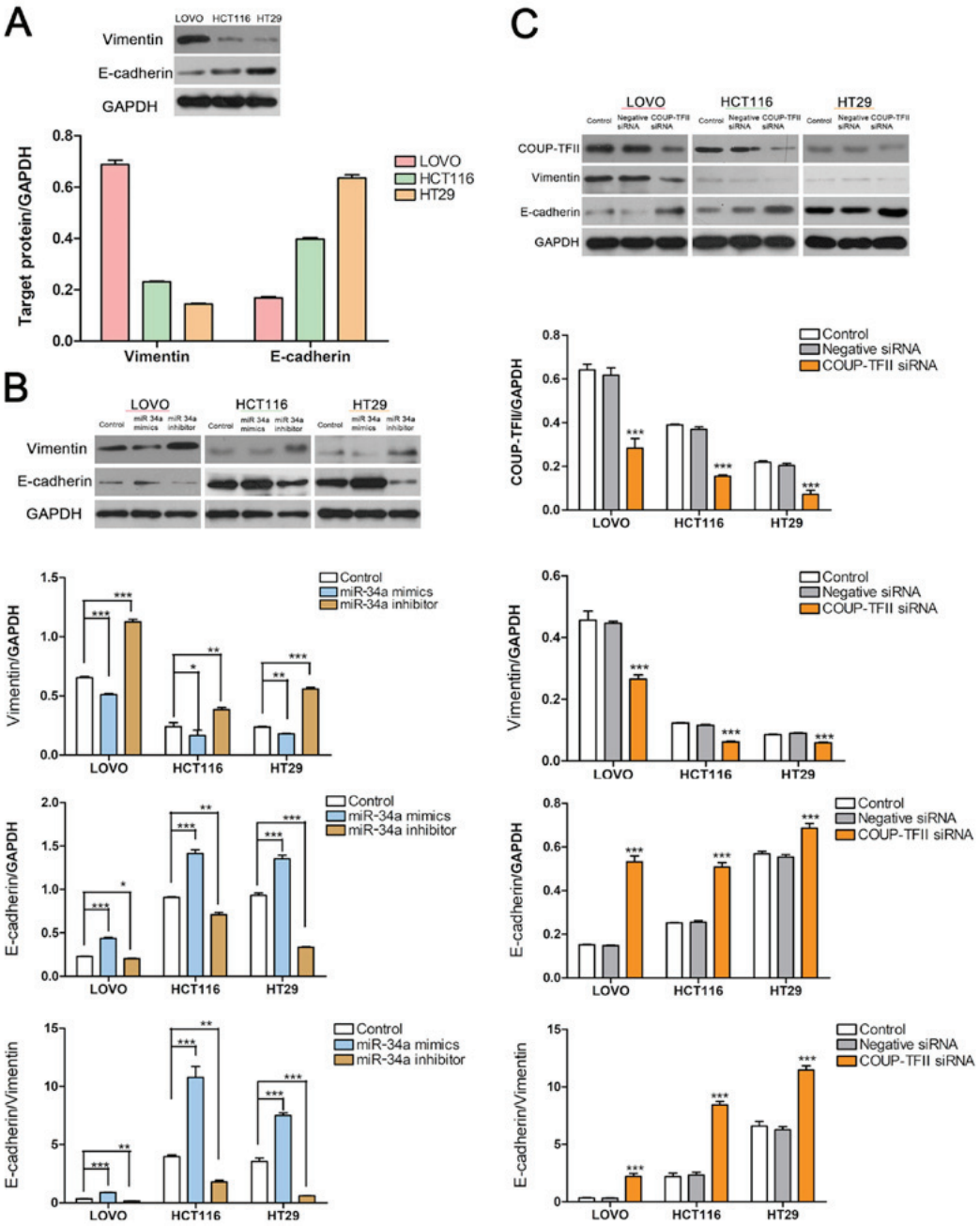

Figure 5. COUP-TFII knockdown reduces EMT in CRC cell lines. (A) The level of EMT was high in LOVO cells, moderate in HCT116 cells, and low in HT29 cells, as indicated by the protein levels of Vimentin and E-cadherin. (B) miR-34a knockdown decreased the expression of E-cadherin and increased the expression of Vimentin, but miR-34a overexpression induced the opposite effect. *P<0.05, **P<0.01 and ***P<0.001, as indicated. (C) Inhibited COUP-TFII expression significantly increased E-cadherin expression but decreased the expression of Vimentin. ***P<0.001 vs. Control. COUP-TFII, chicken ovalbumin upstream promoter-transcription factor II; siRNA, small interfering RNA; EMT, epithelial-mesenchymal transition; CRC, colorectalcancer cell; miR, microRNA.

E-cadherin expression but decreased Vimentin expression (P<0.05, P<0.01 and P<0.001 vs. Control; Fig. 5C). Successful transfection was verified for miR-34a (Fig. 3A) and COUP-TFII (Fig. 5C) via RT-qPCR and western blotting.

Discussion

Among factors such as age, race and metastasis, which all negatively affect the prognosis of CRC patients, distant metastasis causes the maximum reduction in survival rate (1). Aberrant activation of the EMT process enables adherent epithelial carcinoma cells to become migratory, contributing to the early-stage dissemination of tumor cells from primary tumor tissues to novel organ sites through the blood (26). It has been reported that mesenchymal circulating tumor cells were significantly associated with disease progression in the patients with breast cancer (27). Furthermore, circulating tumor cells have frequently exhibited a reversible shift between the epithelial and mesenchymal phenotypes with therapy and disease progression (28).

A previous study revealed that synthetic steroid hormones could mediate miR-34a expression (29), and that steroid hormones and COUP-TFII competitively regulate transcription factor function. Therefore, the present

study investigated the association between miR-34a and COUP-TFII. The results revealed that miR-34a expression exhibited a reverse trend to COUP-TFII expression in CRC cell lines, and that COUP-TFII knockdown was associated with increased miR-34a expression. miR-34a is a well-known suppressor of multiple types of cancers and is considered a novel biomarker for diagnosis and prognosis prediction as well as being a therapeutic target (30,31). Transcription of miR-34a and COUP-TFII was reported to be competitively regulated by some common mechanisms. COUP-TFII was previously verified to be associated with the invasion and migration of many types of tumors including CRC (32), but its mechanism of action was largely unclear. Several genes associated with cancer development were also confirmed to be regulated by COUP-TFII (9). For example, ectopic COUP-TFII expression was revealed to promote angiogenesis in a tumor model by enhancing angiopoietin-1 expression and repressing VEGFR-1 expression (13,14). In the present study, miR-34a mimics could inhibit the CRC cell migration and invasion abilities. In addition, miR-34a siRNA transfection reversed the effect of COUP-TFII knockdown on CRC cell migration and invasion.

An accumulating body of clinical evidence has demonstrated that activation of EMT and overexpression of EMT-associated transcription factors in CRC promotes the metastasis of this type of cancer and limits long-term survival following resection. Slug and Vimentin expression were also revealed to be increased in CRC and these proteins were considered to be novel predictive biomarkers for lymph node metastasis and poor prognosis in CRC (33). Several previous studies have reported that various molecular mechanisms are involved in the EMT process. Wang *et al* (34) reported that in the TGF-β signaling pathway, high COUP-TFII expression was associated with negative mothers against decapentaplegic homolog 4 expression, while Zhang *et al* (19) showed that high COUP-TFII transcript levels inhibited TGF-β-dependent EMT. In addition, COUP-TFII suppressed the cadherin-11 to cadherin-6 switch, leading to the inactivation of EMT during the development of kidney cancer (35). To better determine the role of COUP-TFII in the EMT process of CRC and the associated mechanisms, the present study knocked down COUP-TFII expression using siRNA and confirmed that COUP-TFII significantly enhanced the migration and invasion abilities of CRC cells by promoting EMT. Furthermore, the expression of E-cadherin was increased and Vimentin expression was decreased following transfection with miR-34a mimics compared with negative siRNA. By contrast, inhibition of miR-34a decreased the expression of E-cadherin and promoted the expression of Vimentin, thereby increasing EMT in CRC cells. These results suggest that inhibition of miR-34a expression may have been the main mechanism underlying how COUP-TFII regulates EMT. In addition, miR-34a suppression could be essential to COUP-TFII in regulating other malignant behaviors as well.

In conclusion, the present study confirmed that COUP-TFII knockdown was negatively associated with the migration and invasiveness of CRC cells. High COUP-TFII expression competitively inhibited miR-34a transcription, thereby promoting the EMT process. The results suggest that control of EMT through the inhibition of COUP-TFII or restoration of miR-34a may be a novel therapeutic approach for CRC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XW and WC conceived the study. YB, YL and WF performed the experiments. HY, HG, YT and QS analyzed the data. WC wrote the manuscript. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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