



# Extracellular vesicles isolated from curcumin-medium weakened RKO cell proliferation and migration

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**Background:** Curcumin (Cur) is a natural phytochemical that is expected to become an indispensable drug for the treatment of colorectal cancer. A comprehensive understanding of the anti-tumor mechanism of Cur will provide a better reference for its clinical application. This study aimed to examine the effects of extracellular vesicles (EVs) isolated from Cur-medium on RKO colorectal cancer cell proliferation, apoptosis, and migration.

**Methods:** RKO cells were cultured in various concentrations of Cur-medium, and the EVs were isolated from the Cur-medium. The EVs were identified by transmission electron microscopy and western blotting. The effects of the EVs on RKO cell proliferation, apoptosis, and migration were analyzed, as was the expression of proliferating cell nuclear antigen (PCNA), Bax, vimentin, and E-cadherin. The expression of nuclear factor  $\kappa$ B (NF- $\kappa$ B) p65 in the EVs was also detected.

**Results:** Our results showed that the EVs isolated from the Cur-medium weakened RKO cell proliferation and migration but had no effect on cell apoptosis. Cur suppressed the expression of NF- $\kappa$ B p65 in the EVs. Overall, this study revealed that Cur exerts anti-tumor effects by suppressing NF- $\kappa$ B p65 in EVs to weaken RKO cell proliferation and migration.

**Conclusions:** In conclusion, the packaging of Cur into EVs is expected to become an indispensable treatment of colorectal cancer in the future.

**Keywords:** Curcumin (Cur); extracellular vesicles (EVs); colorectal cancer; proliferation; migration

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## Introduction

Cancer is the main cause of death in China. Data from the National Cancer Center of China in 2022 showed that rates of colorectal cancer had increased in the whole population in China, and colorectal cancer was the fifth leading cause of cancer-related death (1). Traditional Chinese medicine

has been shown to affect colorectal cancer cell proliferation, apoptosis, cell cycle, migration, invasion, autophagy, epithelial-mesenchymal transition (EMT), angiogenesis, and chemo-resistance by regulating multiple signaling pathways (2-4). Due to the complexity of colorectal cancer pathogenesis, with its multiple components, targets, and effects, traditional Chinese medicine is expected to lead to a

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breakthrough in the development of therapeutic colorectal cancer drugs.

Curcumin (Cur) is one of the most popular agents in traditional Chinese medicine. Cur is isolated from turmeric roots, and is a natural phytochemical with wide pharmacological activities (5), including anti-inflammatory, anti-bacterial, anti-viral, and anti-tumor activities (6,7). Studies have shown that Cur can reprogram the pro-tumor phenotype of cancer-associated fibroblasts (8) and inhibit the invasion and migration of carcinoma cells (9), effectively controlling carcinoma progression. Cur is thought to be a potent anti-tumor agent in colorectal cancer (10). A comprehensive understanding of the anti-tumor mechanism of Cur will provide a better reference for the clinical application of Cur.

In recent years, researchers have focused on extracellular vesicles (EVs), which function as messengers that exchange cargo between cells, enabling the transport of various signaling chemicals (11). The three primary types of EVs are exosomes, ectosomes, and apoptotic bodies. Ectosomes are mainly divided into microvesicles (0.2–1  $\mu\text{m}$ ) and large oncosomes (>1  $\mu\text{m}$ ) (12). The EVs used in this study represent exosomes and microvesicles. Tumor-derived EVs are powerful drivers of tumor progression (13,14). Remarkably, a study found that Cur was packaged into exosomes derived from pancreatic cancer cells treated with Cur (15); that is, Cur was packaged into EVs isolated from a Cur-medium. However, the effects of the EVs derived from the colorectal cancer cells treated with Cur on colorectal cancer cell proliferation, apoptosis, and migration have not been examined.

This study aimed to examine the effects of EVs

isolated from Cur-medium on colorectal cancer RKO cell proliferation, apoptosis, and migration. Our results demonstrated that EVs isolated from Cur-medium weakened RKO cell proliferation and migration but had no effect on cell apoptosis. Additionally, Cur suppressed the expression of nuclear factor  $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) p65 in the EVs. These findings suggest Cur may exert anti-tumor effects by changing the functional molecules in the EVs derived from tumors. This study provides some data that may help to fully elucidate the anti-tumor mechanism of Cur. We present this article in accordance with the MDAR reporting checklist (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-24-98/rc>).

## Methods

### *Cell viability assays*

The colorectal cancer cell line RKO (CX0083, Boster) was cultured in Dulbecco's Modified Eagle Medium (DMEM; C11995500, Gibco, ThermoFisher, Beijing, China) supplemented with 10% fetal bovine serum (FBS; PYG0001, Boster, Wuhan, China) and 100 U/mL of penicillin/streptomycin (JY1000, Biotopped, Beijing, China) at 37 °C with 5% carbon dioxide. Cur was purchased from MedChemExpress (HY-N0005, Shanghai, China). Cell viability was examined using the Cell Counting Kit-8 (CCK-8) assay kit (C6005M, UELandy, Suzhou, China). An equal number of cells were seeded into a 96-well plate per well and cultured overnight to enable them to fully recover their morphology. The medium was discarded, and then 0, 1.25, 2.5, 5, 10, and 20  $\mu\text{M}$  of Cur-containing media were added to each group, respectively. After 48 h of cultivation, the culture medium of each well was replaced with 10  $\mu\text{L}$  of CCK-8 (C6005M, UELandy, Suzhou, China) solution and 90  $\mu\text{L}$  of DMEM (C11995500, Gibco, ThermoFisher, Beijing, China). The plates were incubated for 1 h at 37 °C. The optical density (OD) value of each well was measured by a microplate reader (Synergy H1, BioTek Instruments, Inc., Vermont, USA) at 450 nm.

### *Isolation of EVs*

The RKO cells were cultured in 0, 1.25, and 10  $\mu\text{M}$  of Cur-containing 10% exosome-depleted-FBS complete medium (Cur-medium) for 48 h, respectively. The Cur-medium was collected, and the Exosome Extraction and Purification Kit (UR52121, Umibio, Shanghai, China)

### Highlight box

#### Key findings

- Extracellular vesicles (EVs) isolated from curcumin (Cur)-medium weakened RKO cell proliferation and migration.

#### What is known, and what is new?

- It is known that Cur inhibits the proliferation and migration of carcinoma cells.
- This study revealed that Cur exerts anti-tumor effects by suppressing nuclear factor  $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) p65 in EVs to weaken RKO cell proliferation and migration.

#### What is the implication, and what should change now?

- The packaging of Cur into EVs is expected to become an indispensable treatment of colorectal cancer in the future.

was used to precipitate the EVs as per the manufacturer's instructions. The EVs were re-suspended and purified. The Bicinchoninic Acid (BCA) Protein Assay Kit (GK10009, GLPBIO, Shanghai, China) was used to quantify the EVs. The EVs were stored at  $-80^{\circ}\text{C}$  awaiting subsequent use.

### *EV labeling and uptake*

PKH67 (UR52303, Umibio, Shanghai, China) was used to label the EVs with green fluorescence. To investigate whether the RKO cells could uptake the EVs, the cells and PKH67-labeled EVs were added. After 24 h co-culturing, the cells were fixed with 4% paraformaldehyde. Subsequently, anti-fade mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (P0131, Beyotime, Shanghai, China) was used. The uptake of EVs was visualized by fluorescence microscopy (Model BX3-CBH, Olympus Corporation, Tokyo, Japan).

### *Cell proliferation assay*

A CCK-8 assay kit (C6005M, Uelandy, Suzhou, China) was used to test the effect of the EVs isolated from the Cur-medium on RKO cell proliferation. The RKO cells were equally seeded into a 96-well plate after 48 h of cultivation, 10  $\mu\text{L}$  of CCK-8 solution was added to the wells and incubated for 1 h at  $37^{\circ}\text{C}$ . The OD value of each well was measured at 450 nm by a microplate reader (Synergy H1, BioTek Instruments, Inc., Vermont, USA).

### *TUNEL staining*

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed using the YF<sup>®488</sup> TUNEL apoptosis detection kit (T6013S, Uelandy, Suzhou, China). The TUNEL reaction mixture, including TdT enzyme and TUNEL reaction buffer, was incubated for 1 h at  $37^{\circ}\text{C}$ . The sample was processed with anti-fade mounting medium with DAPI (P0131, Beyotime, Shanghai, China). Next, the sample was visualized by fluorescence microscopy (Model Bx3-Cbh, Olympus Corporation, Tokyo, Japan).

### *Transwell assay*

Equal numbers of cells were cultured in 1% FBS medium. Cell suspension (200  $\mu\text{L}$ ) was added to the upper chamber of the Transwell (04122024, Corning, Shanghai, China). Medium (500  $\mu\text{L}$ ), including 30% FBS, was added to the

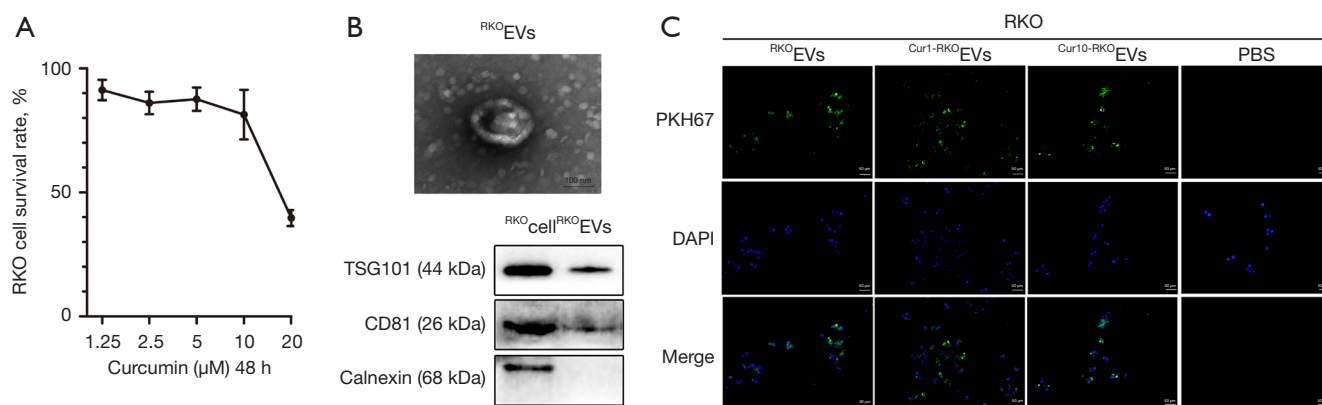
lower chamber. After 24 h, 4% paraformaldehyde was used to fix the cells for 20 min. The cells were then dyed with 0.1% crystal violet (C0121, Beyotime, Shanghai, China) for 8 min. Finally, the cells were counted under a microscope (Model Bx3-Cbh, Olympus Corporation, Tokyo, Japan).

### *Western blotting analysis*

The total protein of the RKO cells was extracted with RIPA Lysis Buffer (AR0102, Boster, Wuhan, China), and the total protein of the EVs was extracted with the special purpose lysate (UR33101, Umibio, Shanghai, China), separated by SDS-PAGE, and transferred onto PVDF membranes (0000202622, Millipore, Boston, USA). The membranes were blocked with 5% skim milk powder at room temperature for 2 h and were then incubated with primary antibodies overnight at  $4^{\circ}\text{C}$  and incubated with a secondary antibody at room temperature for 1 h. The bands were detected using an enhanced chemiluminescence gel imaging system (10017142, Bio-Rad, Shanghai, China). The blots were analyzed with image J software. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or  $\beta$ -actin was used as the control. Polyclonal antibodies against GAPDH (10494-1-AP, 1:4,000), TSG101 (28283-1-AP, 1:4,000), vimentin (10366-1-AP, 1:2,000), E-cadherin (60335-1-Ig, 1:4,000), and monoclonal antibodies against CD81 (66866-1-Ig, 1:1,000) were obtained from Proteintech<sup>®</sup>, Wuhan, China. Polyclonal antibodies against  $\beta$ -actin (AC026, 1:20,000) were obtained from ABclonal, Wuhan, China. Polyclonal antibodies against proliferating cell nuclear antigen (PCNA; HY-P80268, 1:4,000), Bcl-2-related X protein (Bax) (HY-P80028, 1:4,000), NF- $\kappa\text{B}$  p65 (HY-P80765, 1:1,000), and Calnexin (HY-P80578, 1:1,000) were obtained from MedChemExpress, Shanghai, China. HRP Conjugated AffiniPure Goat Anti-mouse IgG (H+L) (BA1050; 1:10,000) and HRP Conjugated AffiniPure Goat Anti-rabbit IgG (H+L) (BA1054; 1:10,000) were obtained from Boster, Wuhan, China.

### *Statistical analysis*

The statistical analyses were performed using SPSS 26.0 and GraphPad Prism 8.0 software. Statistically significant differences were calculated using a Student's *t*-test and an analysis of variance (ANOVA), followed by LSD's test. In the western blotting analysis,  $\beta$ -actin or GAPDH was used as the control, and the control group was homogenized.



**Figure 1** RKO cells took up the EVs from the surrounding environment. (A) Viability of the RKO cells after treatment with Cur for 48 h. The data are presented as mean  $\pm$  SD. (B) The EVs were analyzed by TEM (magnification,  $\times 60$  k; scale bar: 100 nm) and a western blotting analysis of the typical biomarkers of EVs. (C) The RKO cells were co-cultured with <sup>RKO</sup>EVs, <sup>Cur1-RKO</sup>EVs, <sup>Cur10-RKO</sup>EVs, and PBS, respectively, and the green fluorescence signals in cells were detected by fluorescence microscopy (magnification,  $\times 200$ ). EVs, extracellular vesicles; Cur, curcumin; SD, standard deviation; TEM, transmission electron microscopy; PBS, phosphate buffered saline.

The data in this study are all presented as the mean  $\pm$  standard deviation (SD) from the experiments. The experiments were performed in triplicate. Significance was defined as a P value  $< 0.05$ .

## Results

### RKO cells took up EVs from surrounding environment

After the treatment with 1.25, 2.5, 5, 10, or 20  $\mu$ M Cur for 48 h, the survival rates of the RKO cells, measured by CCK-8, were 91.23%  $\pm$  4.13%, 86.00%  $\pm$  4.53%, 87.53%  $\pm$  4.67%, 81.35%  $\pm$  9.99%, and 39.64%  $\pm$  3.26%, respectively (Figure 1A). Cur elicited a dose-dependent decrease in the RKO cell viability. Based on the sensitivity to the chemical treatment, we selected Cur at a dose of 1.25 and 10  $\mu$ M for our subsequent experiments. In a previous study, RKO cells were cultured for 48 h in 10% exosome-depleted-FBS conditioned medium, the medium from the RKO cells was collected, and the EVs were isolated from it using an extraction and purification kit (<sup>RKO</sup>EVs). The <sup>RKO</sup>EVs had already been identified (Figure 1B). Next, 1.25 or 10  $\mu$ M of Cur were added to treat the RKO cells for 48 h, and the EVs were isolated from the Cur-medium, which were named the <sup>Cur1-RKO</sup>EVs and <sup>Cur10-RKO</sup>EVs, respectively. The PKH67-labeled <sup>RKO</sup>EVs, <sup>Cur1-RKO</sup>EVs, and <sup>Cur10-RKO</sup>EVs were co-cultured with the RKO cells, respectively, and the fluorescently labeled EVs were detected in the RKO cells

(Figure 1C), suggesting that the RKO cells took up the EVs from the surrounding environment.

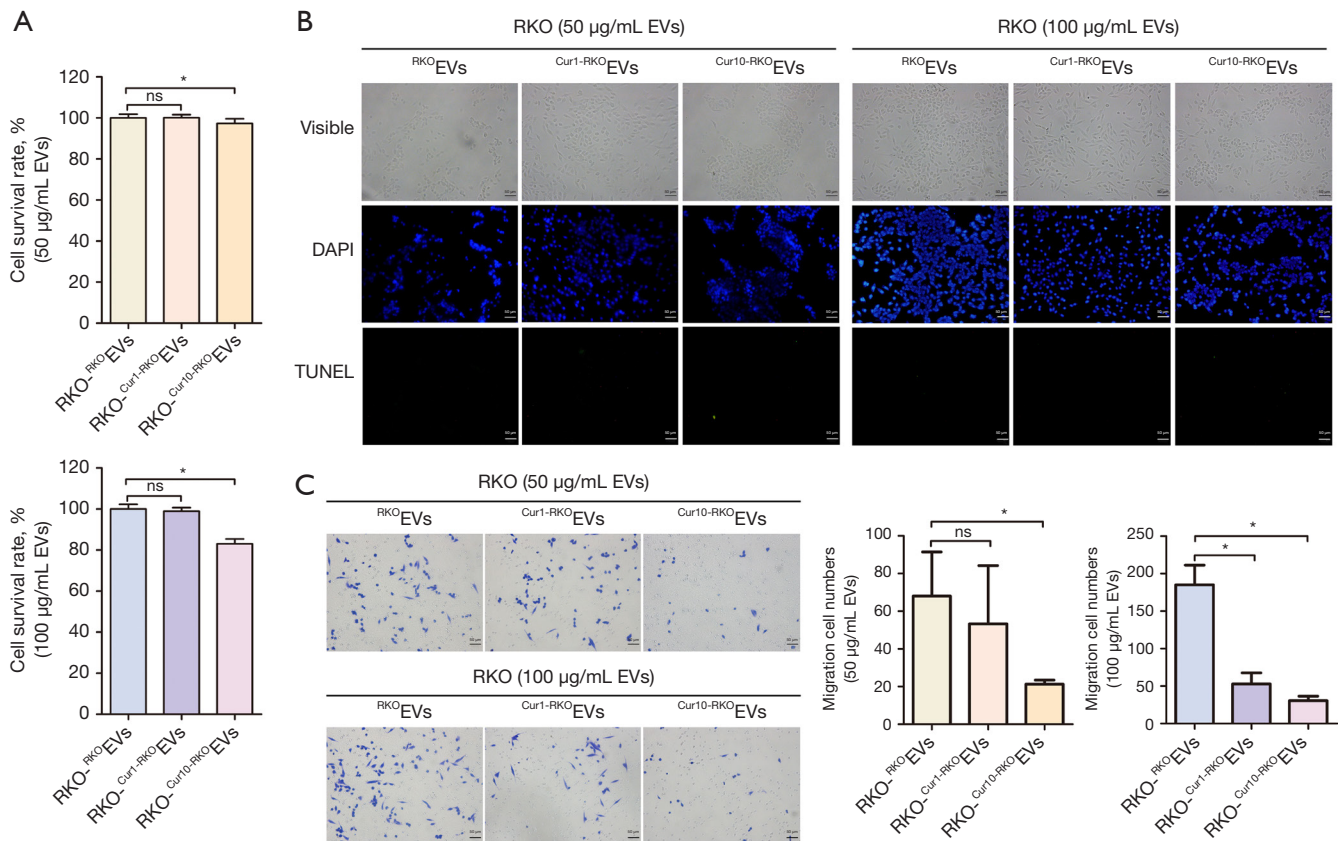
### EVs isolated from Cur-medium inhibited RKO cell proliferation

The RKO cells were treated with equal numbers of EVs (50  $\mu$ g/mL) for 48 h. Compared with the RKO cells treated with <sup>RKO</sup>EVs, the survival rates were 100.13%  $\pm$  1.48% and 97.07%  $\pm$  1.28% when the cells were treated with <sup>Cur1-RKO</sup>EVs and <sup>Cur10-RKO</sup>EVs, respectively but the PCNA expression of the cells did not change significantly in that time (Figure 2,3), which might be because the cells all had a higher survival rate. Further, the RKO cells were treated with 100  $\mu$ g/mL of EVs for 48 h. The survival rate of the RKO cells (81.76%  $\pm$  1.84%) and the expression of the PCNA were significantly inhibited when the cells were treated with the <sup>Cur10-RKO</sup>EVs compared to the <sup>RKO</sup>EVs, but the cell survival rate and PCNA expression did not change significantly when the RKO cells were treated with the <sup>Cur1-RKO</sup>EVs (98.85%  $\pm$  1.51%) (Figures 2,3). The data suggested that the EVs isolated from the Cur-medium inhibited RKO cell proliferation.

### EVs isolated from the Cur-medium had no effect on RKO cell apoptosis

Generally, the early apoptosis rate of the normal cells was  $< 5\%$ . The apoptosis ability of the RKO cells was tested





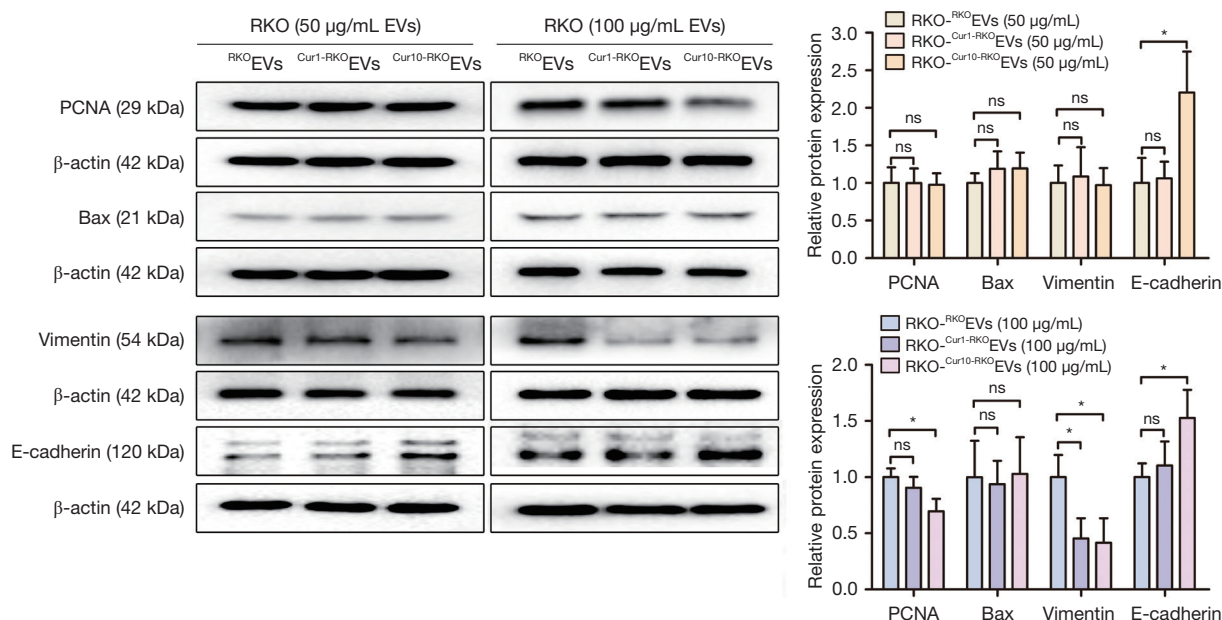
**Figure 2** The effects of EVs isolated from the curcumin-medium on RKO cell proliferation, apoptosis, and migration. (A) RKO cell proliferation was analyzed by CCK-8. (B) RKO cell apoptosis was detected by TUNEL assay. TUNEL staining was performed using the YF<sup>®488</sup> TUNEL apoptosis detection kit (magnification,  $\times 200$ ). (C) RKO cell migration was analyzed by Transwell assay. The cells were stained by 0.1% crystal violet. The data are presented as mean  $\pm$  SD (\*,  $P < 0.05$ ). EVs, extracellular vesicles; DAPI, 4',6-diamidino-2-phenylindole; Cur, curcumin; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; ns, no significance; CCK-8, Cell Counting Kit-8; SD, standard deviation.

via TUNEL assay. The RKO cells were treated with equal numbers of EVs for 48 h. Compared with those treated with the RKO-EVs, there was only sporadic green fluorescence in the TUNEL staining when the RKO cells were treated with the Cur1-RKO-EVs and Cur10-RKO-EVs. There was no significant apoptosis in either group. Consistent with this result, the expression of the apoptotic protein Bax also showed no significant change (Figures 2,3). The data indicated that the EVs isolated from the Cur-medium had no effect on RKO cell apoptosis.

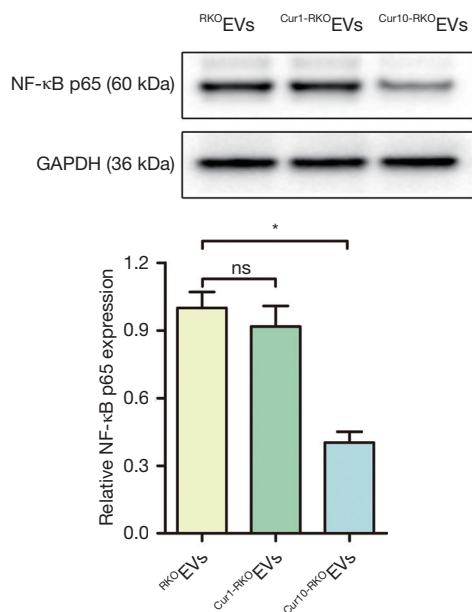
#### ***EVs isolated from Cur-medium weakened the EMT and migration of the RKO cells***

Compared with those treated with equal numbers of RKO-EVs for 48 h, the expression of E-cadherin was

increased when the RKO cells were treated with the Cur10-RKO-EVs. However, E-cadherin expression did not change significantly when the RKO cells were treated with the Cur1-RKO-EVs. Similarly, there was no significant change in the expression of vimentin when the RKO cells were treated with 50 µg/mL of the Cur1-RKO-EVs or the Cur10-RKO-EVs compared to the RKO-EVs, but vimentin expression was decreased when the RKO cells were treated with 100 µg/mL of the Cur1-RKO-EVs or the Cur10-RKO-EVs. Meanwhile, the migration ability of the RKO cells was decreased when the cells were treated with the Cur10-RKO-EVs compared to the RKO-EVs. The migration ability of the RKO cells was also decreased when the cells were treated with 100 µg/mL of the Cur1-RKO-EVs compared to 100 µg/mL of the RKO-EVs, but no significant change was observed



**Figure 3** Western blotting analysis of the proteins. The expression of PCNA, Bax, vimentin, and E-cadherin was analyzed by western blotting.  $\beta$ -actin was used as a control and the control group was homogenized. The data are presented as mean  $\pm$  SD (\*,  $P < 0.05$ ). EVs, extracellular vesicles; PCNA, proliferating cell nuclear antigen; Cur, curcumin; ns, no significance; SD, standard deviation.



**Figure 4** Curcumin suppressed the expression of NF- $\kappa$ B p65 in RKO EVs. The expression of NF- $\kappa$ B p65 in RKO EVs, Cur1-RKO EVs and Cur10-RKO EVs was analyzed by western blotting, respectively. The data are presented as mean  $\pm$  SD (\*,  $P < 0.05$ ). EVs, extracellular vesicles; Cur, curcumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ns, no significance; SD, standard deviation.

when the cells were treated with the 50  $\mu$ g/mL of the Cur1-RKO EVs (Figures 2,3). The data revealed that the EVs isolated from the Cur-medium weakened the EMT and migration ability of the RKO cells.

#### Cur suppressed the expression of NF- $\kappa$ B p65 in RKO EVs

To investigate whether the EVs isolated from the Cur-medium weakened RKO cell proliferation and migration via the inhibition of NF- $\kappa$ B p65, western blotting was used to determine the NF- $\kappa$ B p65 expression levels. The results showed that NF- $\kappa$ B p65 was present in the RKO EVs, Cur1-RKO EVs, and Cur10-RKO EVs. Compared with the RKO EVs, the expression of NF- $\kappa$ B p65 was significantly decreased in the Cur10-RKO EVs, while no significant change was observed in the Cur1-RKO EVs (Figure 4). The data revealed that the EVs isolated from the Cur-medium weakened RKO cell proliferation and migration partly via the inhibition of NF- $\kappa$ B p65.

#### Discussion

Colorectal cancer remains a non-negligible cause of cancer-related death (16-18). Surgical therapy is less effective

in advanced colorectal cancer patients than those early patients (19). Additional therapy is required to delay the progression of colorectal cancer, including cell proliferation suppression, apoptosis facilitation, and cell migration inhibition.

PCNA is a non-histone protein that assists DNA polymerase (20). DNA polymerase is important for cellular replication (21). PCNA expression is used as an indicator of cell proliferation due to its effects on mitotic activity (22,23). In this study, PCNA expression was also used as an indicator of cell proliferation. A CCK-8 assay and western blotting analysis of PCNA showed that the EVs isolated from the Cur-medium inhibited RKO cell proliferation. Bax (also called Bcl-2-related X protein) is a pro-apoptotic factor, leading to caspase activation and resulting in apoptosis (24,25). It has been reported that Cur accelerates cell apoptosis, and inhibits cell proliferation and invasion in colorectal cancer cells (26). However, Bax expression and TUNEL staining showed that the EVs isolated from the Cur-medium had no effect on RKO cell apoptosis in this study. Our results also showed that the EVs isolated from the Cur-medium decreased vimentin expression but increased E-cadherin expression in the colorectal cancer cells. Vimentin and E-cadherin are the protein markers of EMT. Decreased E-cadherin and increased vimentin expression in cells indicates that the cells undergo EMT, facilitating cell migration (27,28). These above results showed the promising role of Cur as a therapeutic agent for colorectal cancer treatment due to its multi-dimensional anti-cancer properties.

NF- $\kappa$ B is a transcription factor that regulates the genes implicated in the progression of carcinoma cells (29). NF- $\kappa$ B p65 is the key protein in the NF- $\kappa$ B signaling pathway. The inhibition of NF- $\kappa$ B p65 represents the classical point responsible for the anti-cancer action of Cur (30). It has been reported that Cur inhibits NF- $\kappa$ B expression in colorectal cancer (31). Our results showed that Cur exerts an anti-tumor effect by suppressing NF- $\kappa$ B p65 in EVs. Combined with the results of this study, it appears that Cur not only suppresses NF- $\kappa$ B p65 expression in colorectal cancer cells but also suppresses NF- $\kappa$ B p65 expression in EVs.

The bioavailability of Cur is limited. In recent years, researchers have established liposomes, micelle, nanoparticles, phospholipid complexes, and other methods to improve the bioavailability of Cur, (32). Osterman *et al.* found that Cur was packaged into EVs isolated from Cur-medium (15). EVs could become the new carrier-mediated

transfer system to improve the bioavailability of Cur and thus its clinical application. However, it was not known whether Cur could change the functional molecules in the EVs derived from tumors. The present study found that Cur suppressed the expression of NF- $\kappa$ B p65 in EVs derived from RKO cells. It also revealed that Cur may exert anti-tumor effects by changing the functional molecules in EVs derived from tumors. This study further elucidated the anti-tumor mechanism of Cur. Importantly, due to the function of EVs, packaging large doses of Cur into EVs for the targeted treatment of cancer may prolong the survival time of patients in the future.

## Conclusions

Collectively, the findings of this study suggest that Cur exerts anti-tumor effects via the suppression of NF- $\kappa$ B p65 in EVs to weaken RKO cell proliferation and migration. The packaging of Cur into EVs is expected to become an indispensable treatment of colorectal cancer in the future.

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## Footnote

*Reporting Checklist:* The authors have completed the MDAR reporting checklist. Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-24-98/rc>

*Data Sharing Statement:* Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-24-98/dss>

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*Conflicts of Interest:* Both authors have completed the ICMJE uniform disclosure form (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-24-98/coif>). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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