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miR-31-modified bone marrow mesenchymal stem cells facilitate autophagic cell death of colorectal cancer via binding to CCR7 to modulate PI3K/Akt/mTOR signals

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Abstract: **Background**: Autophagic cell death plays a complex role in colorectal cancer (CRC) progression. MicroRNA-31 (miR-31) is implicated in tumor regulation, and bone marrow mesenchymal stem cells (BMSCs) show promise as targeted delivery vehicles for cancer therapy. However, the effect and mechanism of miR-31-modified BMSCs on autophagic cell death in CRC cells remain unclear. **Objective**: This study aimed to explore the effect and underlying mechanism of miR-31-modified BMSCs on inducing autophagic cell death in colorectal cancer cells. **Methods**: HT29 colorectal cancer cells were divided into multiple groups: control, miR-31 mimic, BMSCs co-culture, miR-31-modified BMSCs co-culture, CCR7 overexpression, and combined treatment groups. Key indicators were detected using RT-qPCR, Western blot, and flow cytometry to analyze gene expression, protein levels, and autophagic cell death. **Results**: MiR-31-modified BMSCs significantly elevated miR-31 levels and suppressed CCR7 expression in HT29 cells. Compared to the control group, this treatment significantly increased autophagic cell death (22.84% vs. 6.72%), upregulated the autophagy markers LC3-II and Beclin-1, and inhibited phosphorylation of key proteins in the PI3K/Akt/mTOR pathway. Overexpression of CCR7 effectively reversed these pro-autophagic and signaling inhibitory effects. **Conclusion**: MiR-31-modified BMSCs induce autophagic cell death in colorectal cancer cells by delivering miR-31 to target and inhibit CCR7, thereby suppressing the PI3K/Akt/mTOR signaling pathway. This study provides a novel strategic foundation for BMSC-based gene therapy in colorectal cancer.

Keywords: Autophagy; BMSCs; Colorectal cancer; CCR7; miR-31PI3K/Akt/mTOR pathway

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INTRODUCTION

Colorectal cancer (Huang *et al.*, 2020) is a commonly diagnosed malignancy in the digestive tract. Its pathogenesis involves multiple factors, including oncogenes, tumor suppressor genes, and other regulatory elements, resulting from a multi-step dysregulation process (Bi *et al.*, 2021). Uncontrollable proliferation is considered to be the most serious mediator in this disease. Therefore, investigating the mechanisms of apoptosis and malignancy is expected to reveal the mechanism of tumor occurrence and development. During the occurrence of colorectal cancer, the Phosphatidylinositol 3-kinase/Protein kinase B/Mammalian target of rapamycin (PI3K/Akt/mTOR) pathway is still a hot topic in the medical science community (Huang and Qu, 2020). It is engaged in the autophagy inhibition and survival facilitation of different cells. As a regulatory point, this pathway can guide its signal transduction to modulate the autophagy and apoptotic activity of malignant cells, potentially initiating programmed cell death. In addition, it can also restrain the proliferating potential of cells (Chen *et al.*, 2021), which enables it to be a critical mediator and controller during the occurrence and development of colorectal cancer.

In the clinical study of solid tumors, attention has been paid not only to the cancer cells and their composition of tumor entity, but also to the interstitium that is composed of other types of cells. Tumor interstitium, also known as tumor microenvironment, is composed of macrophages, endothelial cells and cancer-associated fibroblasts (Xu *et al.*, 2020). Bone marrow mesenchymal stem cells (BMSCs) can directly contact and co-culture with colon cancer cells and affect the differentiation process of colon cancer cells (HT-29) via intercellular communication (Huang *et al.*, 2021). In recent years, research progress has been achieved in the field of tumor therapy. It was reported that the interaction of chemokines and their corresponding receptors can enhance the proliferative potential, angiogenesis and oriental migration of malignant cells (Ueta *et al.*, 2021). Chemokines exert different functions in the development and differentiation of hematopoietic cells and immune cells, as well as in the processes of inflammation, angiogenesis and tumorigenesis. As a critical part of the immune system, they can regulate the apoptotic activities of various cells through the PI3K/Akt/mTOR pathway, which enables them to become a new target for tumor therapy in recent years (Zhang *et al.*, 2025). The chemokine receptor, C-C Chemokine Receptor

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Type 7 (CCR7), is one receptor of the chemokine subfamily. Under normal circumstances, CCR7 acts as a guide for T lymphocytes and dendritic cells during their migration and homing (Sun *et al.*, 2020). Studies have reported that it was strongly expressed in colon cancer cells compared with healthy cells, which indicates that its expression may be related to the proliferative and metastatic potentials of malignant cells within the tumor environment (Ghafouri-Fard *et al.*, 2021).

In recent years, microRNAs (miRNAs) have attracted much attention since they were discovered in the 1990s. They are of great significance in the biological and molecular aspects of malignancies at different stages. The research on the occurrence of colon cancer has also made great progress in recent years (Morishita *et al.*, 2021). miRNAs influence the cellular processes of proliferation, apoptosis and metastasis through their interaction with intracellular signal networks. Clinical studies have revealed that miRNAs-related binding sites and gene polymorphisms are related to the risk, survival rate and treatment efficiency of colon cancer. Changes in the expression level of miRNAs may be an important cause of colon cancer. Therefore, miRNAs, as biomarkers, can be adopted for the early diagnosis of colon cancer. It is noteworthy that the function of microRNA is highly context-dependent and its ultimate effect may vary significantly due to differences in cell type, microenvironment and the targeted gene network. While some studies have reported that miR-31 is significantly upregulated in colon cancer tissues and can regulate processes such as proliferation, apoptosis, invasion and metastasis (Mu *et al.*, 2018), where on others have found that transfection of miR-31 into colorectal cancer cells can significantly restrain tumor cell growth. This dual role highlights the complexity of its function (Hao *et al.*, 2020). Based on the recent research progress of CCR7 in tumor therapy and the potential role of miR-31 in colorectal cancer, this study focuses on the targeting of miR-31-modified BMSCs to CCR7 and its influence on the colorectal cancer cells through its manipulation of the PI3K/Akt/mTOR signal pathway. We aim to investigate the role of CCR7-targeting miRNA in the development of colorectal cancer and provide a theoretical hint for the treatment of colorectal cancer.

MATERIALS AND METHODS

Experimental reagents and equipments

Dulbecco's modified Eagle medium (DMEM) culture medium and fetal bovine serum were purchased from Hyclone (USA). BMSCs were purchased from Beijing ZEPING Bioscience & Technology Co., Ltd. (China). Penicillin, Trizol reagent, Radio-Immunoprecipitation Assay (RIPA) lysis buffer, Bicinchoninic Acid (BCA) kit, ECL color developing solution were all purchased from Beijing Solarbio Science & Technology Co., Ltd. (China). Lipofectamine 2000 and pcDNA3.1 were purchased from Invitrogen. miR-31-mimic plasmid and miR-31-modified BMSCs were provided by Shanghai GenePharma Co., Ltd. (China). Anti-CCR7 and anti-β-actin (ab8226) antibodies were purchased from Abcam. The primers used in the project were designed via Primer3 Input and synthesized by Sangon Biotech (Shanghai) Co., Ltd. QuantNova SYBR Green PCR kit (Qiagen, Germany), dual-luciferase reporter gene detection system (Promega, USA).

Culture and identification of BMSCs

Culture: The frozen primary BMSCs were thawed, resuspended and cultured

The cryopreserved primary BMSCs were thawed and seeded at a density of 1×10^4 cells/cm² in culture dishes. They were cultured in α-MEM medium supplemented with 10% fetal bovine serum, maintained at 37°C in a saturated humidity incubator with 5% CO_2. The culture medium was replaced with fresh medium every three days. On day 5 of culture, when cell confluence reached approximately 80%-90%, the first passage was performed and these cells were designated as passage 1 (P1). When the cells reached 90% confluence again, they were digested and passaged using trypsin, followed by reseeding at a 1:3 ratio. The experiments utilized stable third-passage (P3) cells with consistent growth status and their morphological observations are shown in fig. 1.

Identification of BMSC Surface Markers by Western Blot

Total protein was extracted from third-generation BMSCs. After determining the protein concentration using the BCA method, 30 μg of protein samples were subjected to SDS-PAGE electrophoresis and transferred onto a PVDF membrane. The membrane was blocked with 5% skimmed milk and then incubated separately with primary antibodies against CD90, CD44, CD45 and the internal reference GAPDH at 4°C overnight. The following day, after washing with TBST, the membrane was incubated with corresponding horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 hours. Finally, the blots were visualized using an ECL chemiluminescence kit and images were captured with a gel imaging system.

Cell culture

The human colon cancer cell line HT29 was purchased from American Type Culture Collection (ATCC) (USA) and cultured in a DMEM medium containing 10% fetal bovine serum and 100 $U\cdot mL^{-1}$ penicillin in an incubator under 5% CO_2, 37ºC.

Cell grouping and treatment

The cells were randomized into 6 groups as follows: control group, miR-31-mimic group, BMSCs group, miR-31-modified BMSCs group, pc-CCR7 group and pc-CCR7 + miR-31 group. The HT29 cells were cultured in a DMEM medium that was supplemented with fetal bovine serum and placed inside an incubator (37ºC, 5% CO_2). The growth of cells was monitored. Afterwards, cells at the

logarithmic phase were seeded after concentration adjustment and received a 24-hour culture. When reaching an appropriate confluence, cells were given the following treatments: transfection of miR-31-mimic, BMSCs, miR-31-modified BMSCs, transfection of pcDNA linked to the CCR7 gene and co-culture with miR-31-modified BMSCs after the transfection of pc-CCR7, respectively. The transfection was performed according to the protocol of the Lipofectamine^{TM}2000 kit. After 24 hours of transfection, samples were obtained for the subsequent experiments.

Detection of miR-31, CCR7 mRNA expression levels via RT-PCR (Fan et al., 2021)

After the grouping and treatment as mentioned in Section 1.3, Trizol was utilized to extract RNA, which was then processed to reverse transcription for cDNA synthesis and PCR reaction. The conditions of Quantitative Real-time Polymerase Chain Reaction (RT-PCR) was set as follows: 2 minutes of pre-denaturation at 95ºC, 5 seconds of denaturation at 95ºC, 10 seconds of annealing at 60ºC orderly for 40 cycles. Afterwards, the levels of CCR7 mRNA and miR-31 were measured according to the instructions of the QuantNova SYBR Green PCR kit. The relative expression level was calculated with the $2^{-\triangle\triangle Ct}$ method. The primer sequences were shown in table 1.

Protein level measurement via Western-blotting (Toden et al., 2019)

After the grouping and treatment as mentioned in Section 1.3, RIPA was utilized to extract protein from cells. Then the protein samples were quantified using a BCA kit. 30μg protein per sample was added with loading buffer, the mixture of which was then boiled for 10 minutes before loading. An equal amount of protein from each sample was separately loaded on the SDS-PAGE gel for electrophoresis. After that, the separated protein samples were transferred to a nitrocellulose membrane. After blocking with 5% skimmed milk solution, different primary antibodies were added separately (anti-LC3 antibody and anti-Beclin-1 antibody were both diluted in 1:500). After overnight incubation at 4℃, the membrane was washed with TBST and added with horseradish peroxidase-labeled secondary antibody (diluted in 1:3000) for 2-hour incubation at room temperature. Afterwards, the membrane was washed with TBST buffer, developed with ECL luminescence and imaged via the gel imaging system. β-actin was employed as an internal control in this study. In the Western Blot experiments of this study, β-actin was used as an internal reference. It is a stable, constitutively expressed housekeeping protein present in all cells. Its primary role is to standardize and normalize protein expression levels. By comparing and calculating the signal intensity of target proteins (such as p-PI3K, p-Akt and p-mTOR) against the β-actin signal intensity in the corresponding samples, technical variations caused by factors like loading amount and transfer efficiency can be eliminated. This ensures that the observed differences in protein expression levels (such as the upregulation in the pc-CCR7 group or downregulation in other groups) reflect genuine biological effects rather than experimental artifacts, thereby guaranteeing the reliability and comparability of the data.

Autophagosomal LC3 fluorescence to measure cell apoptosis (Shojaei et al., 2025)

After the grouping and treatment as mentioned in Section 1.3, cells were washed with PBS and processed to 30-minute fixation utilizing 4% paraformaldehyde. After that, cells were given 60-minute blocking with PBS solution containing 0.3% Triton X-100 and 1% bovine serum albumin. Afterwards, the anti-LC3 antibody was added to the samples for overnight incubation at 4°C. Then the FITC-labeled goat anti-rabbit lgG antibody was added for 2-hour incubation at room temperature (protected from light). Eventually, an appropriate amount of Hoechst33342 solution was utilized for 10-minute staining. After mounting the slides, we then observed and took images of the samples under a fluorescence microscope.

Flow cytometry to detect apoptosis after autophagy inhibition (Huang et al., 2021)

After the grouping and treatment as mentioned in Section 1.3, the collected HT29 cells were washed with PBS once and then resuspended in a 500μl binding solution containing 5μl AnnexinV-FITC staining solution that was provided in the human AnnexinV-PI cell apoptosis detection kit. After 15-minute incubation in the dark at room temperature, the samples were proceeded to flow cytometry.

Verification of autophagy-dependent cell death (Shojaei et al., 2025)

To confirm that the miR-31-induced cell death is autophagy-dependent, we conducted a rescue experiment using an autophagy inhibitor. While co-culturing HT-29 cells with miR-31-modified BMSCs (or transfecting with miR-31 mimic), the late-stage autophagy inhibitor chloroquine was added at a final concentration of 50 μM. Chloroquine blocks the complete autophagic flux by inhibiting the fusion of autophagosomes with lysosomes and lysosomal acidification. After 48 hours of co-culture, cells were collected and cell death rates were detected via flow cytometry (Annexin V-FITC/PI double staining). If the miR-31-induced cell death rate is significantly reversed in the presence of chloroquine, it proves that this death process depends on the completion of autophagic flux.

Statistical analysis

The statistical software SPSS 26.0 was adopted for the quantitative analysis of the data in this study. All data were in accordance with the feature of normal distribution. The t-test was applied for comparing two groups, while one-way analysis of variance (ANOVA) for multi-groups comparison. The measurement data were denoted as ($X \pm S$), while the count data were denoted as (n%). In this study, the difference was reported statistically significant when its P-value was less than 0.05 under the α level at 0.05. F/P

represents the F-statistic and its corresponding P-value from one-way ANOVA.

RESULTS

Morphology identification of BMSCs

(1) On the 5th day of culture, BMSCs exhibited a polygonal morphology and intended to grow like colonies, as shown in fig. 1-A. BMSCs at the third passage displayed a whirlpool and radial growth pattern, as shown in fig. 1-B.

(2) Detection of surface markers on BMSCs

The characteristic surface markers of third-generation BMSCs were identified by Western blot analysis. As shown in fig. 2, at the protein level, the cultured cells clearly expressed the mesenchymal stem cell markers CD90, while barely expressing the hematopoietic cell marker CD44 and CD45. The internal reference GAPDH indicated consistent loading amounts. This protein expression profile aligns with the typical characteristics of BMSCs, confirming the correct identity of the cultured cells and their suitability for subsequent experiments. (Fig. 2).

Successful establishment of miR-31-modified BMSCs

Compared with the control group (1.00 ± 0.06), the miR-31 expression level in HT-29 cells of the miR-31 mimic group was significantly increased (1.59 ± 0.12, $P < 0.05$), indicating successful transfection of the miR-31 mimic. The miR-31 expression in the BMSCs co-culture group (2.34 ± 0.30, $P < 0.05$) and the miR-31-modified BMSCs group (4.12 ± 0.13, $P < 0.05$) was also significantly higher than that in the control group, with the most pronounced increase observed in the miR-31-modified BMSCs group, indicating that BMSCs can enhance and deliver miR-31. The miR-31 level in the pc-CCR7 + miR-31 group (3.04 ± 0.15) was also significantly higher than that in the control group ($P < 0.05$), while the miR-31 expression in the pc-CCR7 group (1.49 ± 0.25) showed no significant difference compared to the control group ($P > 0.05$). One-way ANOVA analysis among the groups showed a statistic of $F = 116.4$ with $P < 0.001$, indicating that the differences between the groups were statistically significant. (Table 2 and Fig. 3).

miR-31 facilitated the autophagic cell death of colorectal cancer cells

In comparison with the control cells, those cells in the miR-31-mimic group exhibited a significantly increased rate of autophagic cell death (18.67%, $P<0.05$), which suggested that transfection of miR-31-mimic can lead to enhanced autophagic cell death. Furthermore, cells in the BMSCs group also displayed a significant elevation of autophagic cell death rate (18.63%, $P<0.05$), which suggested that co-culture with BMSCs can lead to a significant elevation in autophagic cell death. Cells in the miR-31-modified BMSCs group exhibited a significantly increased rate of autophagic cell death (24.09%, $P<0.05$), which suggested that co-culture with miR-31-transfected BMSCs can also lead to a significant increase in autophagic cell death. However, the autophagic cell death rate in cells from the pc-CCR7 group was significantly reduced (4.86%, $P<0.05$), which implied that the overexpressed CCR7 can restrain the autophagic cell death. Moreover, cells in the pc-CCR7+miR-31 group displayed a significantly increased rate of autophagic cell death (23.55%, $P<0.05$), which implied that the overexpressed miR-31 can facilitate the autophagic cell death in colorectal cancer cells. (Table 3, Table 4, Fig. 4, Fig. 5 and Fig. 6).

miR-31 facilitated the autophagic cell death of colorectal cancer cells through its association with CCR7 and PI3K/Akt/mTOR signaling pathway

The expression of the CCR7 gene was significantly elevated in cells from the pc-CCR7 group (1.89 ± 0.26, $P < 0.05$) in comparison with those from the control group. Its expression levels in different groups were denoted as follows: miR-31-mimic group (0.54 ± 0.05), BMSCs group (0.55 ± 0.08), miR-31-modified BMSCs group (0.55 ± 0.08) and pc-CCR7+miR-31 group (0.29 ± 0.10), all of which were significantly decreased (all $P < 0.05$) in comparison with the control group. (Table 5, Table 6, Fig. 7).

For hallmark factors related to the PI3K/Akt/mTOR signal transduction pathway, p-PI3K (1.31 ± 0.03), p-Akt (1.28 ± 0.06) and p-mTOR (1.30 ± 0.04) in the pc-CCR7 group were significantly elevated compared to the control cells ($P<0.05$). However, the expression quantities of p-PI3K, p-Akt and p-mTOR in other groups were significantly reduced compared to the control cells ($P<0.05$). (Table 6).

DISCUSSION

Among patients with colorectal cancer, most were diagnosed with an advanced malignancy in the digestive tract. The occurrence of colorectal cancer is not a rapid event, but a pathological process with multi-stages and multi-steps, which is also mediated by multiple factors. The results of our current study showed that in comparison with the miRNA level in the miR-31 mimic group (1.59 ± 0.12), those cells that were co-cultured with BMSCs exhibited an enhanced miRNA expression (2.34±0.30). The significantly increased expression of miR-31 in HT-29 cells was also denoted. The miR-31 expression in the miR-31-modified BMSCs group was significantly increased (4.12±0.13), which corresponded to the previous result. The expression level of miR-31 in the pc-CCR7 + miR-31 group was also significantly increased (3.04±0.15). This finding indicated that miR-31 was overexpressed in the pc-CCR7 + miR-31-modified BMSCs group, while pc-CCR7 may be silenced in the presence of miR-31 overexpression. This finding is consistent with the results reported by Huang *et al*., (2023). In recent years, there have been many reports on the research of a variety of miRNAs in the occurrence of colorectal cancer. miRNAs can trigger or mediate the occurrence of rectal cancer via modulating different signal transduction pathways.

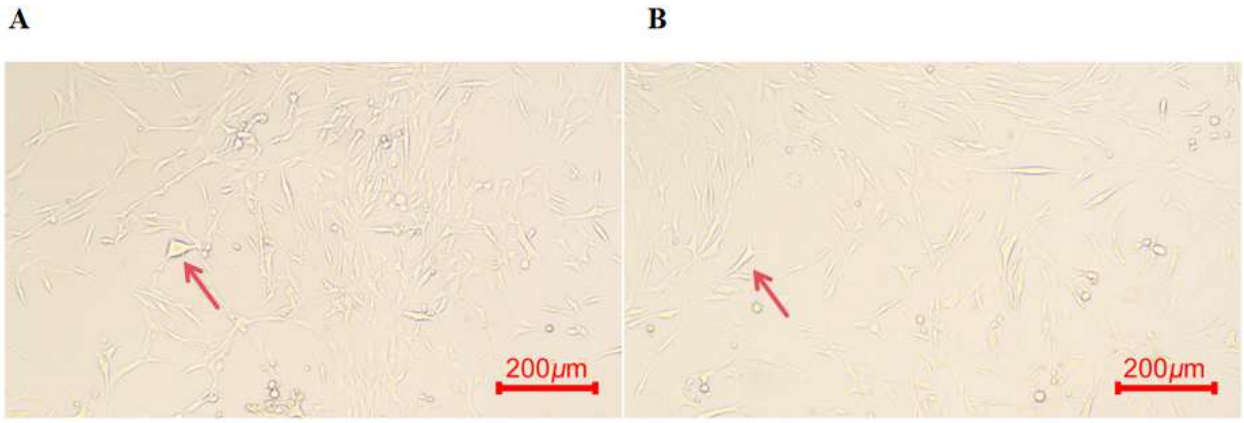

Fig. 1: Morphology identification of BMSCs (×200).
(A) The morphology of BMSCs on the 5th day of culture; (B) The morphology of BMSCs at the third passage.

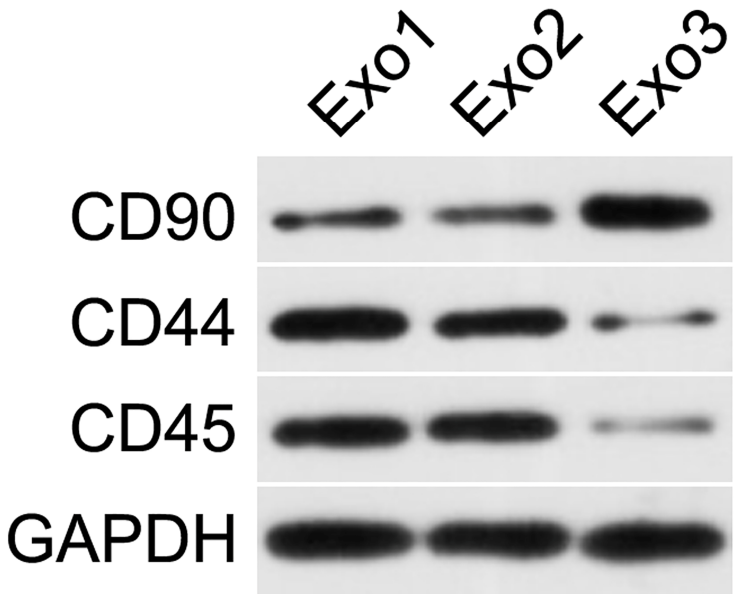

Fig. 2: Detection of surface markers on BMSCs. The protein expressions of CD90, CD44 and CD45 in the third-generation BMSCs were detected by Western blotting. GAPDH serves as an internal reference.

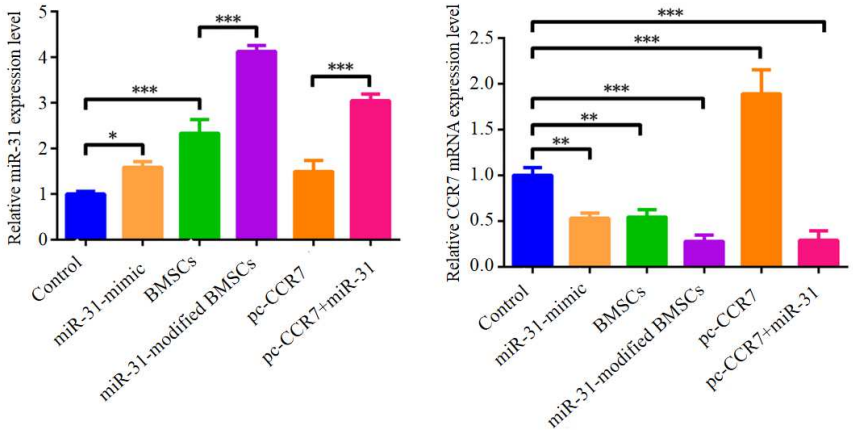

Fig. 3: The relative expression quantification of miR-31 via RT-PCR.

Table 1: Primer sequences.

Gene	Primer sequence		Length of primers (bp)
miR-31	Forward sequence	5′-CAACCUCCUAGAAAGA-3'	22
	Reverse sequence	5′-CAAGATGCAGGCATCG-3'	
CCR7 mRNA	Forward sequence	5'-AAGCGTCACGAGGAAGAGGA-3'	102
	Reverse sequence	5'-GGCTGCTCCTCTTGACCTAT-3'	
β-actin	Forward sequence	5'-GACAACCCATGATGATAAGAG-3'	28
	Reverse sequence	5'-GATGATACGGCTATCGTAATG-3'	

Table 2: The relative expression quantities of miR-31 ($\bar{x} \pm s$).

Groups	Relative miR-31 expression level	P-value vs. control group	P-value for pairwise comparisons
Control Group	1.00 ± 0.06	-	-
miR-31 Mimic Group	1.59 ± 0.12	< 0.05	Control Group vs. This Group: 0.022
BMSCs Group	2.34 ± 0.30	< 0.05	Control Group vs. This Group: 0.000
miR-31-modified BMSCs Group	4.12 ± 0.13	< 0.05	Control Group vs. This Group: 0.000
pc-CCR7 Group	1.49 ± 0.25	> 0.05 (NS)	Control Group vs. This Group: 0.062
pc-CCR7 + miR-31 Group	3.04 ± 0.15	< 0.05	Control Group vs. This Group: 0.000
Statistical Test	F = 116.4, P < 0.001		miR-31 Mimic Group vs. BMSCs Group: 0.003 BMSCs Group vs. miR-31-modified BMSCs Group: 0.000

Table 3: The autophagic cell death rate in each group (n (%).

Group	Autophagic cell death rate
Control group	6.72
miR-31-mimic group	18.67
BMSCs group	18.63
miR-31-modified BMSCs group	24.09
pc-CCR7 group	4.86
pc-CCR7 + miR-31 group	23.55
F/P	89.69/0.000

Table 4: The expression of autophagic cell death-related genes.

Group	LC3	Beclin-1	P53	BCL-2
Control group	1.00±0.05	1.00±0.12	1.00±0.10	1.01±0.13
miR-31-mimic group	2.41±0.40	1.78±0.17	1.54±0.07	0.69±0.07
BMSCs group	5.15±0.43	3.19±0.23	2.45±0.19	0.48±0.04
miR-31-modified BMSCs group	7.08±0.60	4.91±0.34	3.46±0.35	0.28±0.01
pc-CCR7 group	0.42±0.10	0.65±0.14	0.75±0.03	1.10±0.04
pc-CCR7 + miR-31 group	6.85±0.55	6.05±0.46	3.61±0.10	0.37±0.02
F/P	151.8/0.000	188.1/0.000	146.5/0.000	81.60/0.000

Table 5: The relative expression level of CCR7 mRNA ($\bar{x} \pm s$).

Group	Relative mRNA level of CCR7
Control group	1.00±0.08
miR-31-mimic group	0.54±0.05
BMSCs group	0.55±0.08
miR-31-modified BMSCs group	0.28±0.07
pc-CCR7 group	1.89±0.26
pc-CCR7 + miR-31 group	0.29±0.10
F/P	67.63/0.000

Table 6: The expression of proteins related to the PI3K/Akt/mTOR signaling pathway.

Group	p-PI3K	p-Akt	p-mTOR
Control group	1.00±0.11	1.00±0.05	1.00±0.03
miR-31-mimic group	0.82±0.03	0.79±0.02	0.79±0.05
BMSCs group	0.74±0.07	0.79±0.04	0.78±0.08
miR-31-modified BMSCs group	0.82±0.02	0.78±0.05	0.80±0.05
pc-CCR7 group	1.31±0.03	1.28±0.06	1.30±0.04
pc-CCR7 + miR-31 group	0.50±0.06	0.48±0.09	0.51±0.06
F/P	57.20/0.000	66.51/0.000	74.61/0.000

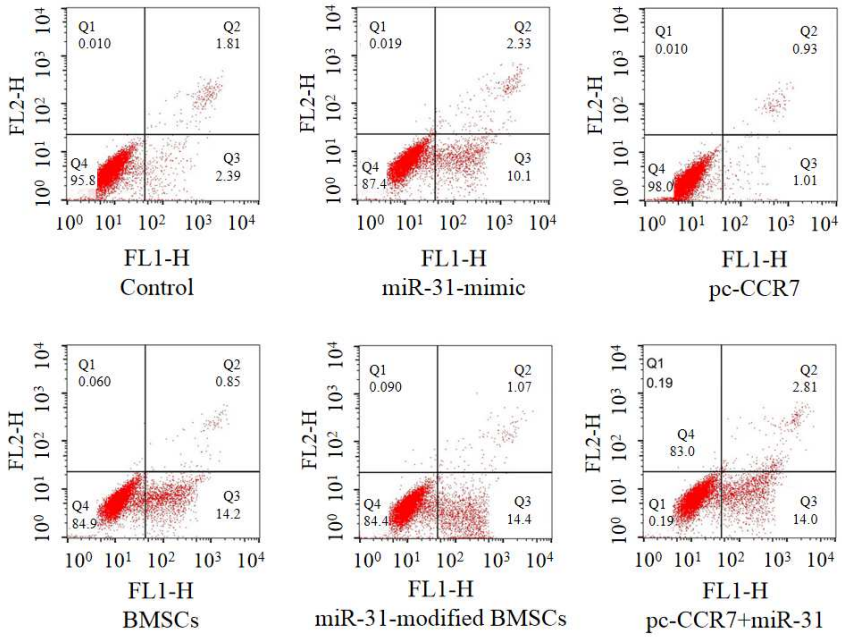

Fig. 4: Detection of cellular apoptosis via flow cytometry.

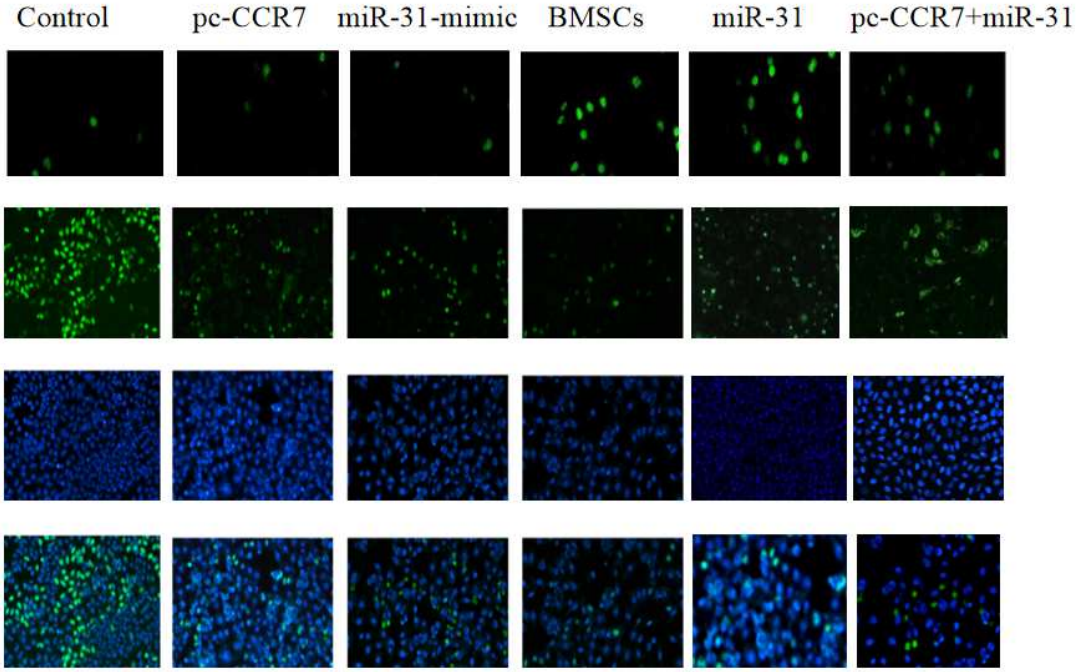

Fig. 5: Detection of cellular apoptosis via fluorescence method.

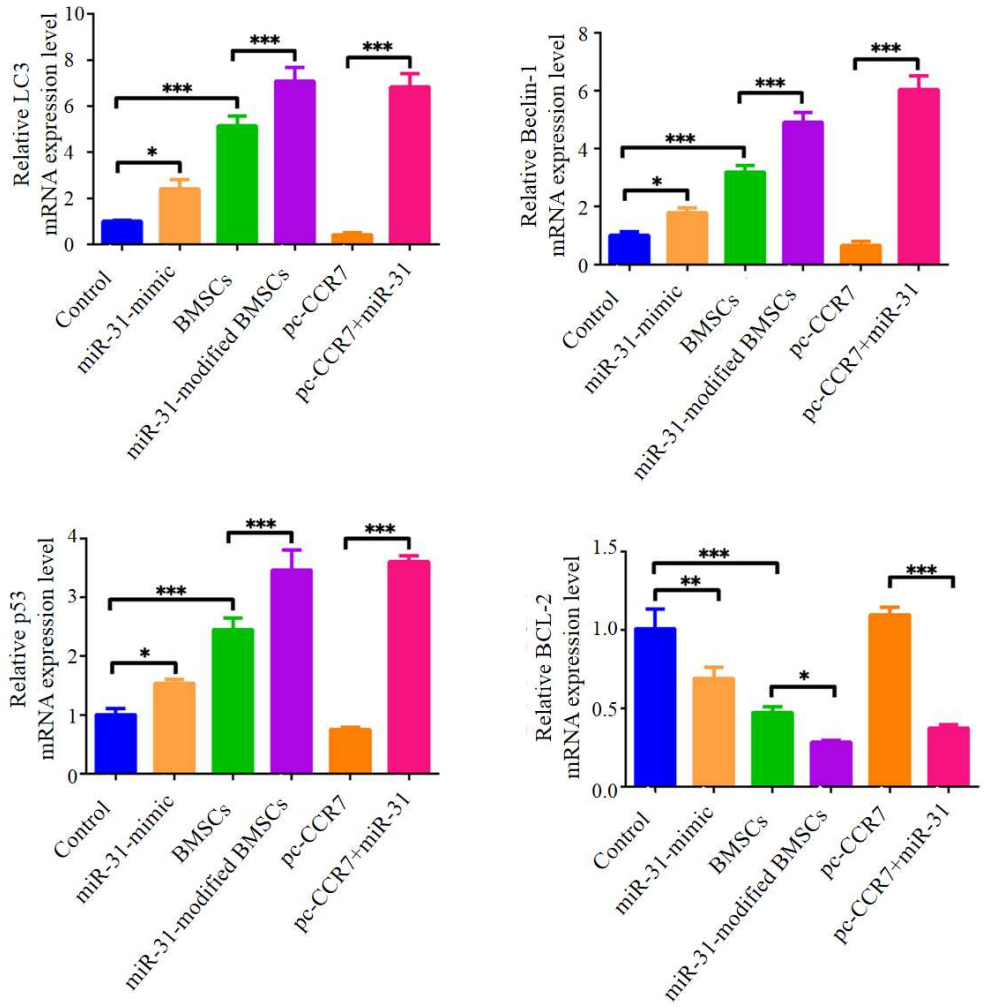

Fig. 6: The expression of autophagic cell death-related genes via RT-PCR.

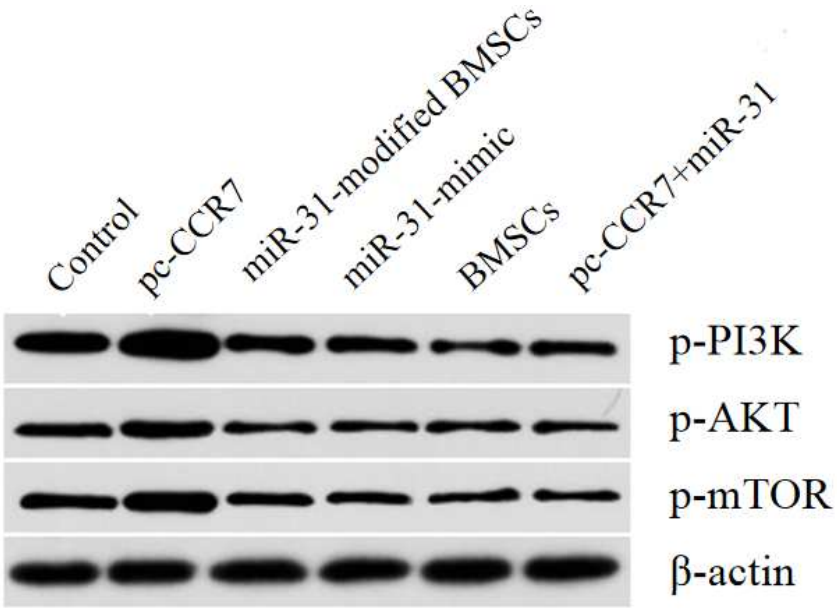

Fig. 7: The expression measurement of proteins related to the PI3K/Akt/mTOR signaling pathway via Western blot.

miRNAs are endogenous microRNAs (miRNAs, miRs) with a length of approximately 22 nucleotides, which are non-coding single-stranded small RNAs that can specifically promote the degradation of certain mRNAs, thereby retarding the translation of these mRNAs. Therefore, miRNAs can modulate their target genes and participate in multiple cellular processes, including cell growth, differentiation and apoptosis (Torii *et al.*, 2021). The results of several clinical studies have disclosed that miR-31 expression in specimens from patients with aggressive colorectal cancer and poorer prognosis was significantly higher than that in other patients (Ashoori *et al.*, 2022). Moreover, the more advanced TNM (Tumor Node Metastasis) stage, the higher the expression level of miR-31. Given the essential influence of uncontrolled cell proliferation in cancer deterioration, our current study focuses on the mechanism of miR-31-modified BMSCs in facilitating the autophagic cell death of colorectal malignant cells via targeting to CCR7 to modulate the PI3K/Akt/mTOR signals.

The results of flow cytometry in our current study exhibited that transfection of miR-31-mimic can lead to an elevation of autophagic cell death in cancer cells. Cells in the BMSCs group also displayed a significant increase in autophagic cell death rate (18.63%, $P<0.05$), which suggested that co-culture with BMSCs can lead to a significant elevation in autophagic cell death. Moreover, cells in the miR-31-modified BMSCs group exhibited a significantly increased rate of autophagic cell death (24.09%, $P<0.05$), which suggested that co-culture with miR-31-transfected BMSCs can further result in a significant increase in autophagic cell death. However, the autophagic cell death rate in cells from the pc-CCR7 group was significantly reduced (4.86%, $P<0.05$), which implied that the overexpressed CCR7 can restrain the autophagic cell death. Moreover, cells in the pc-CCR7+miR-31 group displayed a significantly increased rate of autophagic cell death (23.55%, $P<0.05$), which further implied that overexpressed miR-31 and miR-31-modified BMSCs can facilitate autophagic cell death in colorectal cancer cells. The experimental design of this study included both "miR-31 mimic" transfection and co-culture with "miR-31-modified BMSCs" to systematically elucidate the mechanism of action of miR-31 and its potential clinical application pathways. First, by directly transfecting miR-31 mimics into HT-29 cells, we demonstrated that overexpression of miR-31 within the cancer cells alone can effectively induce autophagic cell death. This provides the most direct evidence for the core tumor-suppressive function of miR-31 and establishes the theoretical foundation for subsequent research. However, directly delivering nucleic acid drugs to tumor cells in vivo faces numerous challenges, such as low efficiency, poor stability and off-target effects. Therefore, we further established a co-culture system with miR-31-modified BMSCs. This experimental group not only simulates the physiological process of intercellular communication within the tumor microenvironment but, more importantly, its results confirm that BMSCs can serve as efficient "living carriers" to successfully deliver functional miR-31 to target cancer cells via paracrine signaling or exosomes, thereby eliciting a stronger anti-tumor effect. These two sets of experiments complement each other, forming a complete chain of evidence from "proof of principle" to "delivery strategy exploration." This not only deepens our understanding of the biological functions of miR-31 but also provides critical experimental support for the future development of mesenchymal stem cell-mediated gene therapy for colorectal cancer.

LC3 is a homolog of the yeast Atg8 gene in mammalian cells. It is regarded as a specific indicator of autophagosome formation. It locates in the autophagosome membrane and participates in the process of autophagy (Noh *et al.*, 2020). The result of LC3 green fluorescence staining also proved the above results. It is also consistent with the findings from the previous study., which discovered that miR-31 can impede the malignant process of cancer cells (Salah *et al.*, 2020). Proliferation impediment and apoptosis enhancement of colorectal cancer cells are manipulated by multiple signal transduction pathways. Furthermore, one of the most essential pathways in this regard is the PI3K/Akt/mTOR pathway. It is also one of the pathways that are most closely related to apoptosis (Du *et al.*, 2021, Hannafon *et al.*, 2016, Lei *et al.*, 2020, Yang *et al.*, 2020). CCR7 is a receptor to the members of the chemokine subfamily and exerts a critical mediatory function during the occurrence and development of malignancies. It was reported that CCR7 may enhance the proliferative potential, angiogenesis and oriental migration of malignant cells through its regulation of the PI3K/Akt/mTOR signaling pathway (Yi *et al.*, 2020, Zhang *et al.*, 2025). The findings of our current study exhibited that miR-31 was remarkably expressed in the CCR7-overexpressed HT29 cells when being co-cultured with miR-31-transfected BMSCs. For hallmark factors related to the PI3K/Akt/mTOR signal transduction pathway, p-PI3K (1.31 ± 0.03), p-Akt (1.28 ± 0.06) and p-mTOR (1.30 ± 0.04) in the pc-CCR7 group were significantly elevated compared to the control cells ($P<0.05$). However, the expression quantities of p-PI3K, p-Akt and p-Akt in other groups were significantly reduced compared to the control cells ($P<0.05$). This finding indicated that miR-31-modified BMSCs may target CCR7 to silence its expression, thereby affecting the activity of PI3K/Akt/mTOR signal transduction pathway. As a result of the activated intracellular PI3K and its downstream signal molecule Akt, the mTOR complex was activated, leading to the clearance of those ubiquitinated proteins and the trigger of autophagy. Consequently, the cell cycle can be accelerated, which affected the life process of colorectal cancer cells and induced their autophagic cell death. miRNAs may target and silence the upstream signals of the

PI3K/Akt/mTOR pathway (Zhang *et al*., 2025b) thereby affecting its expression and activity. This finding is consistent with our observation of the cells in the pc-CCR7 + miR-31 group, which exhibited a reduced CCR7 expression as well as decreased levels of proteins that were involved in the PI3K/Akt/mTOR signal pathway.

The results of this study indicate that both transfection of miR-31 mimics and co-culture with BMSCs overexpressing miR-31 can significantly enhance autophagic cell death in HT-29 cells. This finding appears to contradict some studies reporting that miR-31 plays a pro-tumorigenic role in colorectal cancer (Fan *et al*., 2021, Shojaei *et al*., 2025). However, this discrepancy may stem from the "context-dependent" nature of miRNA function. It is speculated that, compared to directly transfecting miR-31 mimics into cancer cells, delivery via BMSCs more closely mimics intercellular communication. This "paracrine" or exosome-mediated delivery method may result in miR-31 acting on HT-29 cells with different dosage, kinetics and subcellular localization, thereby eliciting different biological responses. At the same time, a single miRNA can simultaneously regulate hundreds of mRNAs and the final phenotypic outcome is the net result of the combined effects of all its targets. In the present experimental system, we confirmed that miR-31 can directly or indirectly target CCR7, thereby strongly inhibiting the downstream PI3K/Akt/mTOR signaling pathway. It is well known that PI3K/Akt/mTOR is a central pathway for cell survival and the negative regulation of autophagy. Therefore, in the context of this study, the pro-apoptotic/pro-autophagic effects mediated via the CCR7-PI3K/Akt/mTOR axis may have outweighed the potential pro-proliferative effects of miR-31 observed in other contexts, ultimately dominating the final cell fate. Thus, our findings do not negate the potential promoting role of miR-31 in other aspects of colorectal cancer, but rather reveal a new possibility: under specific cellular microenvironments and delivery methods, miR-31 can exert tumor-suppressive functions by inhibiting the key CCR7/PI3K/Akt/mTOR survival signaling pathway. This discovery underscores the critical importance of fully considering context specificity when evaluating the therapeutic potential of miRNAs.

CONCLUSION

Conclusively, miR-31-modified BMSCs inhibit the PI3K/Akt/mTOR signal transduction pathway in colorectal cancer cells (HT29 cells) via targeting CCR7, thereby influencing the autophagic cell death of colorectal cancer cells. In our current study, BMSCs were transfected with miR-31-mimic plasmid. Potential false-positive transfection effects were not ruled out, which may introduce some bias. Further detailed studies are required in the near future.

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Authors' contributions
Wei Zhang: Investigation, Formal analysis, Data Curation, Writing - Original Draft, Funding acquisition.
Zhiwan Shu: Conceptualization, Supervision, Writing - Review & Editing.
Xinhua Qiang: Methodology, Validation, Writing - Review & Editing, Supervision.
Zhenfa Chen: Conceptualization, Resources, Project administration, Writing - Review & Editing, Supervision.
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Data availability statement
Data are available from the corresponding author upon reasonable request.

Ethical approval
This study was conducted in vitro using established human cell lines. The human colon cancer cell line HT29 was purchased from the American Type Culture Collection (ATCC) and the Bone Marrow Mesenchymal Stem Cells (BMSCs) were obtained from commercial sources. The study did not involve any direct experiments on human participants or animals. Therefore, ethical approval was not required for this work.

Conflicts of interest
There are no conflicts to declare.

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