

Wharton's jelly mesenchymal stem cell-derived conditioned media inhibits colon cancer cells via activating AMPK/mTOR-mediated autophagy

Dian Dayer¹, Zahra Akbari-Jonoush^{1,2}, Roya Mahdavi^{2,3}, Afshin Amari^{1,2}, Amirhesam Keshavarz-Zarjani^{1,4}, Layasadat Khorsandi^{1,4,*}

1) Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

2) Department of Immunology, School of Medicine Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

3) Cellular and Molecular Research Center, Birjand University of Medical Sciences, Birjand, Iran

4) Department of Anatomical Sciences, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

ABSTRACT

Prior studies have shown that conditioned media derived from Wharton's jelly mesenchymal stem cells (WJ-CM) have anti-cancer properties. This research investigated the impact of WJ-CM on HT-29 colorectal adenocarcinoma cells by examining autophagy biomarkers and the AMPK/mTOR pathway. The HT-29 cells were subjected to WJ-CM and an AMPK activator (AICAR). Autophagy and levels of AMPK and mTOR proteins were investigated. WJ-CM increased the expression of phosphorylated AMPK while reducing the level of phosphorylated mTOR in HT-29 cells. WJ-CM treatment elevated the LC3B/LC3A ratio and ATG7, ATG5, and Beclin-1 expression. However, there was a parallel drop in p62 expression, which indicates autophagy induction. AICAR increased the influence of WJ-CM on viability, as well as the levels of biomarkers associated with autophagy, phosphorylated AMPK, and phosphorylated mTOR in the HT-29 cells. WJ-CM inhibits colorectal cancer cell growth via activating AMPK/mTOR-mediated autophagy.

Keywords: Colon Cancer; AMPK; Stem Cell; Autophagy

INTRODUCTION

Colorectal cancer (CRC) is a common malignancy that frequently presents with a poor prognosis. The current therapeutic methods for CRC are accompanied by several side effects [1]. Many researchers have investigated the influence of mesenchymal stem cells (MSCs) on various types of malignancies. However, the results are varied and controversial due to differences in mesenchymal cells, tumor type, and animal models [2-9]. MSCs secrete various cytokines, chemokines, and growth factors into their culture medium [10]. These biomaterials can promote or suppress tumor cell growth [11, 12].

Wharton's jelly (WJ) from the umbilical cord is suitable for obtaining stem cells using non-invasive techniques, which can be readily cultured on a large scale without raising any ethical concerns. WJMSC prevents the progression of several types of malignant cells, such as cervical

*Corresponding Author: Department of Anatomical Sciences, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; Tel/Fax: +98 3613 3336380; Email: Khorsandi-1@ajums.ac.ir AND khorsandi_cmrc@yahoo.com

cancer, leukemia, and CRC cells [13-15]. Cancer cells under oxidative stress, hypoxia, and nutritional deficiency activate AMPK signaling, which inhibits the mTOR, thereby promoting autophagy [16]. mTOR modulates cell viability, autophagy, and apoptosis of cancerous cells [17]. AMPK and mTOR also have a signaling relationship, and the AMPK-mTOR axis regulates autophagy [18].

When AMPK is phosphorylated, tumor progression is prevented by suppressing the mTOR pathway and inducing autophagy in various malignant cells. On the other hand, the inactivation of AMPK encourages tumor development [19]. There has been no report indicating whether the AMPK/mTOR axis is related to the influence of WJMSC on CRC cells. This study investigated the impact of conditioned media (CM) containing substances secreted by stem cells derived from WJ (WJ-CM) on AMPK/mTOR-dependent autophagy in a human CRC cell line (HT-29).

MATERIALS AND METHODS

Experimental design: WJMSCs (PCS-500-010™) and the HT-29 cells (C466) were from the GEN IRAN and Pasteur Institutes (Iran). After characterization, the WJMSCs were grown in HG-DMEM (Sigma, USA) enriched with routine Pen/Strep (Sigma, USA) and fetal bovine serum (FBS) (Gibco, Germany). When the WJMSCs reached 75% confluence (approximately 3 days), CM was collected and stored at -70°C. The cells were maintained in RPMI medium (Sigma, USA) enriched with Pen/Strep and FBS. These cells were incubated in an environment of 5% CO₂, 95% humidity, and a temperature of 37°C for 24 h. Three groups were used in this experiment: Control: only with the medium for 24 h; WJ-CM: WJ-CM for 24 h (see Table S1); WJ-CM + AICAR (an AMPK activator): 100 µM AICAR+WJ-CM for 24 h.

MTT Assay: HT-29 cells were grown on 96-well plates, with 10,000 cells per well and subjected to three different treatments: medium only (control), WJ-CM, and WJ-CM combined with AICAR, for 24 h. After exposure, MTT was incubated at 37°C for 4 h at 37°C. The supernatants were replaced with Dimethyl sulfoxide (DMSO), and the optical densities at 570 nm were recorded.

Colony formation: HT-29 cells (500 cells/well) were cultivated and received either medium alone or media enriched with WJ-CM, with or without AICAR. The cells were maintained at 37°C in a 5% CO₂ environment for two weeks, until the untreated HT-29 cells (control group) formed colonies. The colonies containing at least 50 cells were counted.

Gene Expression Analysis via qRT-PCR: RNA extract was prepared using Parstous RNA extraction kit (A101231, Iran) and subsequently converted into cDNA by Parstous cDNA synthesis kit (A101162, Iran). The prepared cDNA was mixed with Master Mix and primers (Table S2). A 45-cycle protocol was employed for PCR amplification utilizing the AB One-Step apparatus from Germany. Gene expression was evaluated by the $2^{-\Delta\Delta CT}$ formula, using GAPDH as a calibrator.

Western Blot Analysis: The treated cells were homogenized, and their proteins were extracted using the Bradford kit. The extracted proteins were subsequently transferred to a polyvinylidene difluoride membrane. The membranes underwent a blocking procedure and were exposed to primary antibodies diluted in Tris buffer containing 5% BSA (Bovine serum albumin) for 18 h at 4°C. The membranes were then exposed to the secondary antibody for 2 h and subsequently developed using an ECL kit (Sigma).

Statistical Analysis: Data were analyzed using the one-way analysis of variance, accompanied by Tukey's or Kruskal-Wallis tests (employing SPSS 21.0 software). *p*-value < 0.05 was considered statistically significant.

RESULTS

The MTT assay indicated that WJ-CM diminished the viability of cancer cells. The cells treated with WJ-CM + AICAR showed an even greater reduction in viability than the cells treated with WJ-CM alone (Fig. 1). Additionally, WJ-CM significantly diminished the colony generation of HT-29 cells. The WJ-CM + AICAR-treated group presented significantly reduced colony numbers in comparison to the group that received WJ-CM treatment alone (Fig. 2).

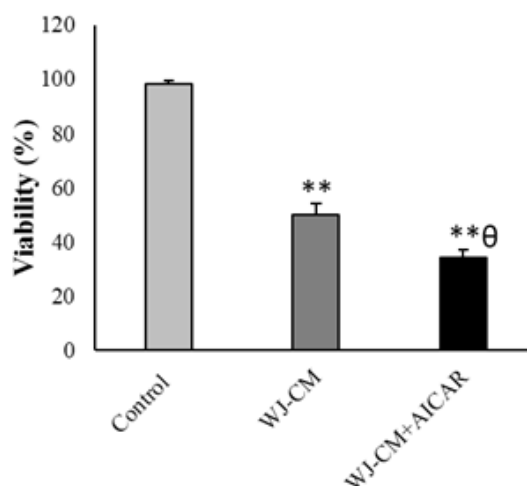


Figure 1: Viability percentage of the HT-29 cells (mean \pm SD; n=6). ** p <0.01, $^{\theta}$ p <0.05, *and $^{\theta}$ show comparison to the control and WJ-CM groups.

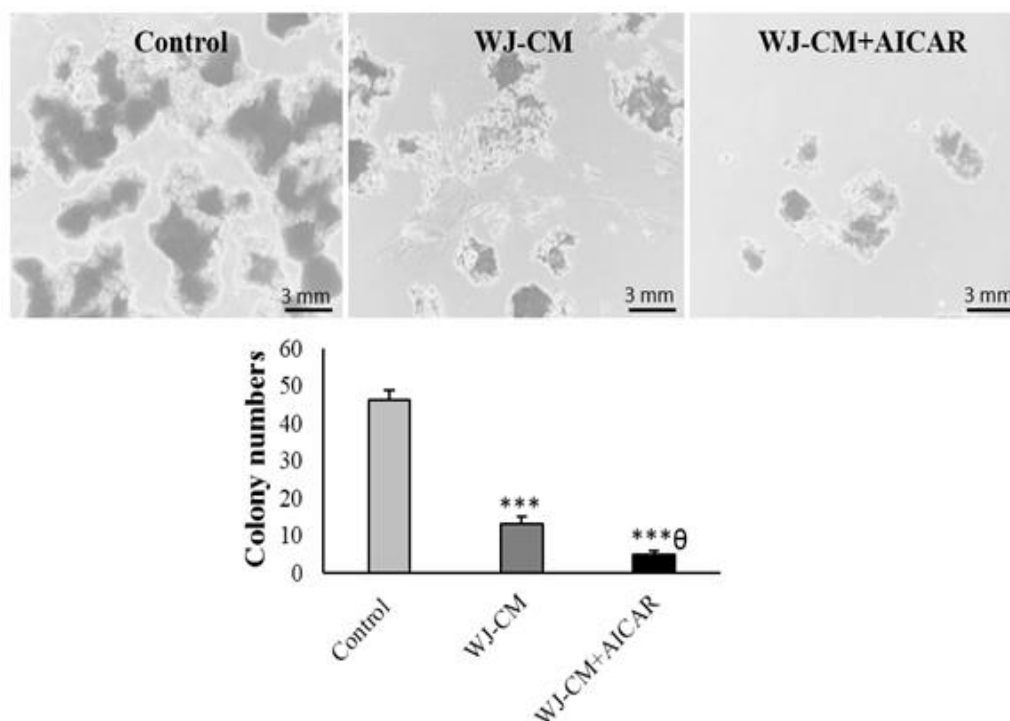


Figure 2: Colony number of the HT-29 cells (mean \pm SD; n=3). *** p <0.001, $^{\theta}$ p <0.05; *and $^{\theta}$ display comparison to the control and WJ-CM treatment.

WJ-CM significantly increased the p-AMPK (phosphorylated AMPK)/AMPK ratio in HT-29 cells. WJ-CM, on the other hand, reduced the HT-29 cells' p-mTOR (phosphorylated mTOR)

to mTOR protein ratio. WJ-CM and AICAR integration increased p-AMPK protein levels while concurrently decreasing p-mTOR levels in comparison to the WJ-CM group (Fig. 3).

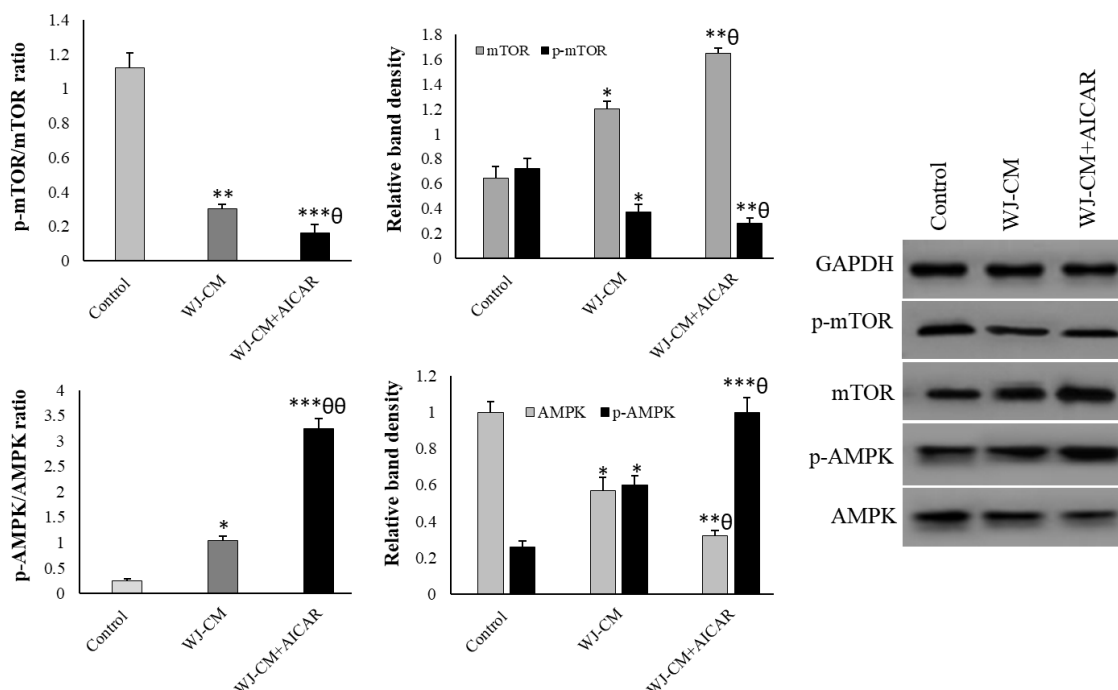


Figure 3: Western blotting results of relative expression of AMPK and mTOR proteins (mean \pm SD; n=3). * p <0.05, ** p <0.01, *** p <0.001, ^θ p <0.05, ^{θθ} p <0.01; * and ^θ display comparison to the control and WJ-CM treatment.

The administration of WJ-CM increased the expression of the Beclin-1, *ATG5*, and *ATG7* genes, and decreased the expression of the *p62* gene. Results from Western blotting indicated that WJ-CM significantly enhanced the LC3B/LC3A ratio. AICAR plus WJ-CM significantly enhanced the *ATG5*, *ATG7*, Beclin-1, and *LC3B*, and decreased *p62* expression (Fig. 4 and 5).

DISCUSSION

WJ-CM suppressed cell proliferation, induced autophagy, elevated p-AMPK levels, and reduced p-mTOR levels in HT-29 cells. Consistent with our findings, Wan et al. (2023) observed that CM of umbilical cord MSCs suppressed proliferation and survival of a granulosa tumor cell line [19]. Widowati et al. found that WJ-CM decreased the survival rate and induced apoptosis in MCF7 and T47D breast cancer cells [20].

The reduced viability of the WJ-CM may be caused by apoptosis in the HT-29 cells. Although apoptosis was not assessed in this study, previous studies have shown the apoptotic impacts of WJMSCs-derived secretome or MSC-derived CM on CRC cells [15, 21]. CM isolated from MSCs of umbilical cord and adipose tissue encourages apoptosis in the human glioma cells [22].

In our study, decreased viability was aligned with decreased p-mTOR and increased p-AMPK proteins in the WJ-CM-exposed cells. Increasing p-mTOR leads to the overexpression of proteins that promote CRC, and targeting mTOR can induce cancer cell death [23]. The mTOR signaling pathways facilitate the proliferation of gastric cancer cells [24]. MSCs derived from the umbilical cord prevented growth and diminished the level of mTOR in melanoma cells [6]. AMPK directly enhances autophagy by phosphorylating proteins associated with autophagy [25]. AMPK activation affects autophagy, apoptosis, and the cell cycle checkpoints [26].

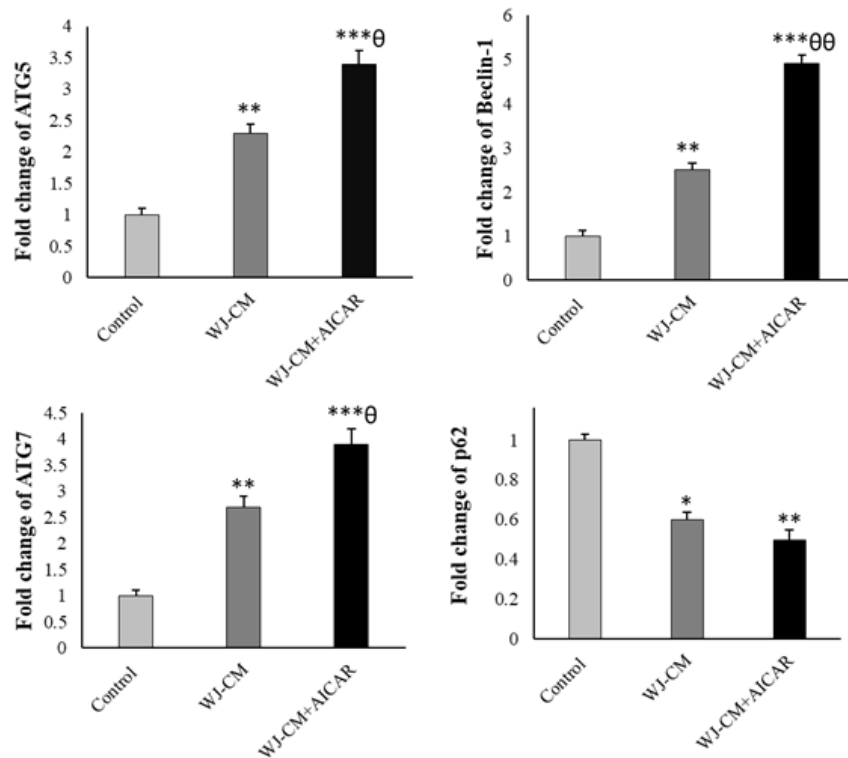


Figure 4: mRNA expression of autophagy-related genes (mean \pm SD; n=3). * p <0.05, ** p <0.01, *** p <0.001, ^θ p <0.05, ^{θθ} p <0.01; *and ^θdisplay comparison to the control and WJ-CM treatment.

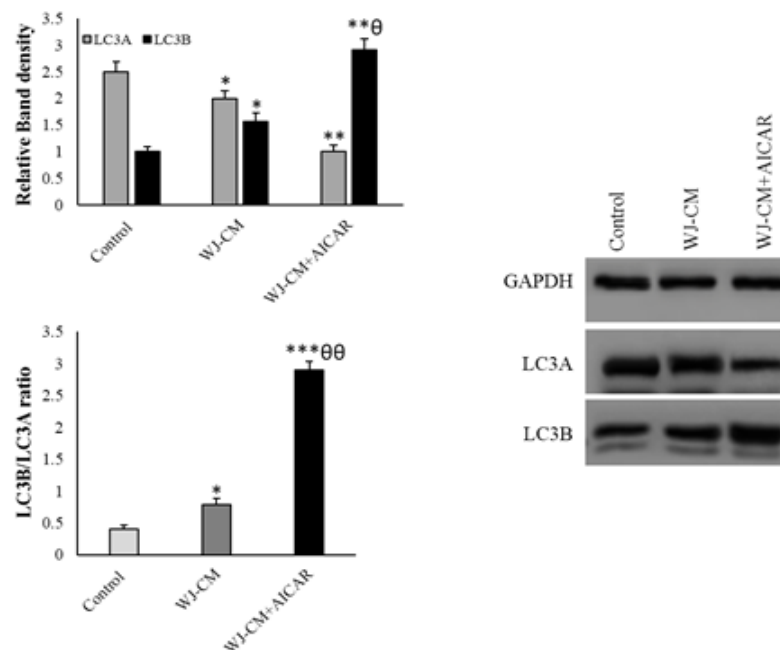


Figure 5: Autophagy assessments in the HT-29 cells (mean \pm SD; n=6). * p <0.05, ** p <0.01, *** p <0.001, ^θ p <0.05, ^{θθ} p <0.01, *and ^θdisplay comparison to the control and WJ-CM groups.

This research utilized AICAR to confirm the contribution of AMPK in the WJ-CM-triggered HT-29 cells. The results demonstrate that AICAR, when used in conjunction with WJ-CM, significantly prevented the growth of HT-29 cells. WJ-CM, with or without AICAR, reduced the p-mTOR expression and enhanced the p-AMPK level in the HT-29 cells.

Therefore, WJ-CM has the potential to function as an activator of AMPK and autophagy in the CRC cells. Safari and colleagues have reported that the secretome derived from amniotic MSCs promotes autophagy-dependent cell death in pancreatic cancerous cells [27]. To determine autophagy, the expression of autophagy-related biomarkers was analyzed. WJ-CM enhanced the LC3B to LC3A ratio and boosted ATG7, ATG5, and Beclin-1 expression while concurrently reducing the expression of p62, thereby inducing autophagy. The results suggest that WJ-CM is capable of effectively promoting autophagy in HT-29 cells. WJ-CM may trigger autophagy in CRC cells by modifying the AMPK/mTOR axis. Other research has also reported that AMPK/mTOR-dependent autophagy inhibits CRC growth [28-31]. Kong et al. have reported that Larotrectinib markedly inhibits CRC cell growth via activating AMPK/mTOR-mediated autophagy [32].

In conclusion, the higher p-AMPK and lower p-mTOR protein levels suggest that WJ-CM stimulates autophagy through AMPK/mTOR axis modulation. WJ-CM may be a therapeutic option for managing CRC.

Acknowledgements: This article is supported by the research council of Ahvaz Jundishapur University of Medical Sciences with grant number: CMRC-0242

Conflict of Interest: We declare that there are no conflicts of interests associated to this manuscript.

Ethics approval: This project is approved by the Ethics committee of Ahvaz Jundishapur University of Medical Sciences (approved number: IR.AJUMS.REC.1402.693).

Authors' Contribution: I.K. designed the study and supervised the experiments, D.D., R.M., and ZAJ contributed in performing experiments and collecting data. A.A. and A.K.Z. made contributions in interpreting data. All these authors have substantial contributions to the final manuscript and approved this submission.

REFERENCES

1. Puzzo M, Santo MD, Morelli C, Leggio A, Catalano S, Pasqua L. Colorectal cancer: current and future therapeutic approaches and related technologies addressing multidrug strategies against multiple-level resistance mechanisms. *Int J Mol Sci* 2025;26:1313.
2. Xiang Z, Hua M, Hao Z, Biao H, Zhu C, Zhai G, Wu J. The roles of mesenchymal stem cells in gastrointestinal cancers. *Front Immunol* 2022;13:844001.
3. Gu JJ, Hoj J, Rouse C, Pendergast AM. Mesenchymal stem cells promote metastasis through activation of an ABL-MMP9 signaling axis in lung cancer cells. *PLoS One* 2020;15: e0241423.
4. Zhang L. The role of mesenchymal stem cells in modulating the breast cancer microenvironment. *Cell Transplant* 2023;32:9636897231220073.
5. Alkhuriji AF, Alsaiani SG, Alomar SY, Alnafjan AA, Alobaid H, El-Khadragy MF. Effect of mesenchymal stem cells on cytochrome-c release and inflammation in colon cancer induced by 1,2-dimethylhydrazine in Wistar albino rats. *Biosci Rep* 2021;26:41:BSR20204356.
6. Wang W, Li L, Chen F, Yang Y. Umbilical cord-derived mesenchymal stem cells can inhibit the biological functions of melanoma A375 cells. *Oncol Rep* 2018;40:511-517.
7. Gan L, Shen H, Li X, Han Z, Jing Y, Yang X, Wu M, Xia Y. Mesenchymal stem cells promote chemoresistance by activating autophagy in intrahepatic cholangiocarcinoma. *Oncol Rep* 2020;45:107-118.
8. Slama Y, Ah-Pine F, Khettab M, Arcambal A, Begue M, Dutheil F, Gasque P. The dual role of mesenchymal stem cells in cancer pathophysiology: Pro-tumorigenic effects versus therapeutic potential. *Int J Mol Sci* 2023;24:13511.

9. Hu W, Wang W, Jiang X, Wang Z, Lin R. Mesenchymal stem cells can prevent or promote the progression of colon cancer based on their timing of administration. *J Transl Med* 2023; 21:227.
10. Rasouli M, Alavi M, D'Angelo A, Sobhani N, Roudi R, Safari F. Exploring the dichotomy of the mesenchymal stem cell secretome: Implications for tumor modulation via cell-signaling pathways. *Int Immunopharmacol* 2024;143:113265.
11. Saadh MJ, Mohamed AH, Almoyad MAA, Allela OQB Amin AH, Malquisto AA, Jin WT, Sârbu I, AlShamsi F, Elsaid FG, Akhavan-Sigari R. Dual role of mesenchymal stem/stromal cells and their cell-free extracellular vesicles in colorectal cancer. *Cell Biochem Funct* 2024; 42: e3962.
12. Ataei A, Azizi M, Hajisadeghi S, Madani M, Khorami M, Hassantash S, Saeidpour Masouleh S, Barati G. The therapeutic effects of mesenchymal stem cells and their secretome on oral squamous cell carcinoma. *Cur Mol Med* 2024;24:1195-1207.
13. Abas BI, Demirbolat GM, Cevik O. Wharton jelly-derived mesenchymal stem cell exosomes induce apoptosis and suppress EMT signaling in cervical cancer cells as an effective drug carrier system of paclitaxel. *PLoS One* 2022;17:e0274607.
14. Süleymanoğlu M, Erol Bozkurt A, Abatay Sel F, Özdemir IA, Oğuz FS, Kuruca DS, Aktaş Z, Karakaş Z, Öncül MO. In vitro anti-leukemic effect of Wharton's jelly derived mesenchymal stem cells. *Mol Biol Rep* 2024;51:595.
15. Rezaei-Tazangi F, Alidadi H, Samimi A, Karimi S, Kahorsandi L. Effects of wharton's jelly mesenchymal stem cells-derived secretome on colon carcinoma HT-29 cells. *Tissue Cell* 2020;67:101413.
16. Penugurti V, Manne RK, Bai L, Kant R, Lin HK. AMPK: The energy sensor at the crossroads of aging and cancer. *Semin Cancer Biol* 2024;106-107:15-27.
17. Huang S. mTOR signaling in metabolism and cancer. *Cells* 2020;9:2278.
18. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* 2011; 13:132-141.
19. Wan W, Miao Y, Niu Y, Zhu K, Ma Y, Pan M, Ma B, Wei Q. Human umbilical cord mesenchymal stem cells conditioned medium exerts anti-tumor effects on KGN cells in a cell density-dependent manner through activation of the Hippo pathway. *Stem Cell Res Ther* 2023;14:46
20. Widowati W, Murti H, Widyastuti H, Laksmiawati DR, Rizal R, Widya Kusuma HS, Sumitro SB, Widodo MA, Bachtar I. Decreased inhibition of proliferation and induction of apoptosis in breast cancer cell lines (T47D and MCF7) from treatment with conditioned medium derived from hypoxia-treated wharton's jelly MSCs compared with normoxia-treated MSCs. *Int J Hematol Oncol Stem Cell Res* 2021;15:77-89.
21. Khamooshi R, Salimi A, Halabian R, Saeedi P. Apoptotic effects of mesenchymal stem cells' conditioned medium on colorectal cancer cell lines. *Tissue Cell* 2023;85:102247.
22. Yang C, Lei D, Ouyang W, Ren J, Li H, Hu J, Huang S. Conditioned media from human adipose tissue-derived mesenchymal stem cells and umbilical cord-derived mesenchymal stem cells efficiently induced the apoptosis and differentiation in human glioma cell lines in vitro. *Biomed Res Int* 2014;2014:109389.
23. Zou Z, Tao T, Li H, Zhu X. mTOR signaling pathway and mTOR inhibitors in cancer: progress and challenges. *Cell Biosci* 2020;10:31.
24. Hibdon ES, Razumilava N, Keeley TM, Wong G, Solanki S, Shah YM, Samuelson LC. Notch and mTOR signaling pathways promote human gastric cancer cell proliferation. *Neoplasia* 2019;21:702-712.
25. Li Y, Chen Y. AMPK and autophagy. *Adv Exp Med Biol* 2019;1206:85-108.
26. Keerthana CK, Rayginia TP, Shifana SC, Anto NP, Kalimuthu K, Isakov N, Anto RJ. The role of AMPK in cancer metabolism and its impact on the immunomodulation of the tumor microenvironment. *Front Immunol* 2023;14:1114582.
27. Safari F, Dadvar F. In vitro evaluation of autophagy and cell death induction in Panc1 pancreatic cancer by secretome of hAMSCs through downregulation of p-AKT/p-mTOR

- and upregulation of p-AMPK/ULK1 signal transduction pathways. *Tissue Cell* 2023;84: 102160.
28. Ni X, Shang FS, Wang TF, Wu DJ, Chen DG, Zhuang B. Ellagic acid induces apoptosis and autophagy in colon cancer through the AMPK/mTOR pathway. *Tissue Cell* 2023;81: 102032.
29. Hu C, Cao Y, Li P, Tang X, Yang M, Gu S, Xiong K, Li T, Xiao T. Oleanolic acid induces autophagy and apoptosis via the AMPK-mTOR signaling pathway in colon cancer. *J Oncol* 2021;2021:8281718.
30. Wang GY, Zhang L, Geng YD, Wang B, Feng XJ, Chen ZL, Wei W, Jiang L. β -Elemene induces apoptosis and autophagy in colorectal cancer cells through regulating the ROS/AMPK/mTOR pathway. *Chin J Nat Med* 2022;20:9-21.
31. Tang S, Cai S, Ji S, Yan X, Zhang W, Xue Qiao, Zhang H, Ye M, Yu S. Isoangustone A induces autophagic cell death in colorectal cancer cells by activating AMPK signaling. *Fitoterapia* 2021; 152:104935.
32. Kong W, Zhu H, Zheng S, Yin G, Yu P, Shan Y, Liu X, Ying R, Zhu H, Ma S. Larotrectinib induces autophagic cell death through AMPK/mTOR signalling in colon cancer. *J Cell Mol Med* 2022;26: 5539-5550.