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Hypoxia-induced miR-9 expression promotes ovarian cancer progression via activating PI3K/AKT/mTOR/GSK3β signaling pathway

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Ovarian cancer (OC) is one of the most prevalent malignant tumors affecting women's life and health. Since OC has a poor prognosis due to extensive metastasis, there is a need to explore a new mechanism of OC metastasis. microRNAs (miRs) are single-stranded, non-coding RNAs. miR-9 has been reported to promote cancer and may provide a new strategy for OC diagnosis. The purpose of this study was to examine the function and underlying mechanism of miR-9 in OC. RT-qPCR was used to assess miR-9 expression levels. Transwell assays were used to determine the number of migrating and invading OC cells. The protein expression levels of the PI3K/AKT/mTOR/GSK3β signaling pathway were examined using western blotting. The results informed that, when compared to normal ovarian tissues, miR-9 was remarkably expressed in OC tissues, and hypoxia might lead to overexpression of miR-9-5p while inhibiting miR-9 notably suppressed the migrating and invading cell numbers in OC cells. *In vivo*, miR-9-5p knockdown inhibited tumor growth in a subcutaneous nude mice model of SKOV3 cells. Our findings suggest that miR-9 could be an underlying oncogene in OC, opening up new avenues for OC diagnosis and treatment of OC by targeting miR-9.

Key words: miR-9; hypoxia; ovarian cancer; migration and invasion; PI3K/AKT/mTOR/GSK3β

Ovarian cancer (OC) is global cancer with the highest mortality rate among all gynecologic malignant tumors [1]. Bloating, constipation, and minor pelvic discomfort are atypical early symptoms of OC. Advanced OC was found in 70% of patients with unpleasant symptoms [2, 3]. Cytoreductive surgery plus carboplatin-paclitaxel chemotherapy is the classic treatment for OC [4]. After systemic therapy, 75% of OC patients will experience tumor recurrence and eventually tumor resistance [5]. Exploring the migration and invasion mechanisms of OC is thus crucial for the early diagnosis and effective treatment of OC patients.

miRNA is a kind of endogenous, non-coding single-stranded small molecule RNA with a length of 19–22 nucleotides, found mostly in eukaryotes, and miRNA functions before or after gene transcription [6]. miRNAs participate in the majority of biogenesis and development processes, including inflammation, proliferation, metabolism, apoptosis, and cancer [7, 8]. microRNA-9 (miR-9) was first identified as a neuronal regulatory factor involved in neurogenesis [9]. The discovery and study of miRNAs open up new avenues for human research into cancer progression and therapy. miRNAs are involved in most biological processes, and their dysregulation has been associated with oncogenic processes such as cell proliferation, metabolic disorders, angiogenesis, invasion, and metastasis [10]. miR-9 is a hypoxia-related miRNA whose expression elevates under hypoxic conditions [11]. In many malignancies, miR-9 plays a tumor-promoting or tumor-suppressing character. In acute myeloid leukemia, miR-9 has been shown to exert a tumor suppressor effect. *In vitro*, miR-9 inhibits not only leukemia cell proliferation, but also migration and invasion, as well as arresting the cell cycle, promoting cell apoptosis, and inhibiting leukemia cell tumorigenesis *in vivo* [12]. In addition, miRNAs can regulate endothelial gene expression, endothelial dysfunction, and atherosclerosis, with miR-9-5p observed in patients with myocardial infarction [13]. Meanwhile, the miR-9 expression is downregulated in ovarian endometrioid carcinoma, ovarian clear cell carcinoma, and recurrent ovarian serous papillary carcinoma. miR-9, as a metastasis-related miRNA, is also involved in OC migration and invasion. The role of miR-9 in the incidence, progression, differentiation, and metastasis of various malignant carcinoma, including colon carcinoma, breast carcinoma, and liver carcinoma, whether as a tumor-promoting gene or a tumor suppressor gene, is

significant. Ischemia or hypoxia is a typical microenvironmental hallmark of nearly all solid tumors due to rapid and uncontrolled tumor proliferation that limits oxygen supply [14]. More aggressive and therapy-resistant tumor phenotypes emerge when tumor cells adapt to hypoxia [15]. According to previous studies, hypoxia could induce the expression of miR-9 in pulmonary arterial hypertension [11]. Nevertheless, how hypoxia-related miR-9 functions in OC is unknown.

The PI3K/AKT/mTOR signaling pathway is composed of phosphatidylinositol-3 kinase (PI3K), protein kinase B (AKT), and mammalian target of rapamycin (mTOR), all of which play a key role in cell growth, survival, metabolism, motility, and genomic instability, as well as angiogenesis and inflammation [16–19]. Glycogen synthase kinase-3 (GSK-3) is a protein kinase that phosphorylates and inhibits glycogen synthase. It is one of the major targets of AKT protein. By phosphorylating serine or threonine residues on its target substrates, GSK3β regulates metabolic functions including cellular gene expression, cell proliferation, and apoptosis [20]. The PI3K/AKT/mTOR signaling pathway is crucial in many tumorigenesis and development processes, such as tumor proliferation, survival, angiogenesis, invasion, migration, and development of drug resistance. According to research, the PI3K/AKT/GSK3β signaling pathway is closely related to the proliferation, invasion, metastasis, and drug resistance of epithelial OC [21]. miRNAs affect multiple biological processes in tumorigenesis by interacting with the PI3K/AKT/mTOR signaling pathway.

This study intended to explore how hypoxia-induced miR-9-5p functions in OC and its probable molecular mechanism.

Patients and methods

Patients. Human OC tissues and normal ovarian tissues were obtained from 30 patients treated at the Second Affiliated Hospital of the Anhui Medical University. Written informed consent was obtained from all patients. The present study was approved by the Biomedical Ethics Committee of Anhui Medical University (Ethics approval number: S20200065).

Cell culture. Two OC cell lines (SKOV3 and HO8910) were acquired from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were cultured under normoxia (21% O_2, 5% CO_2, 74% N_2) or hypoxia (1% O_2, 5% CO_2, 94% N_2) at 37 °C. The cells were cultured in the McCoy'5A medium (SKOV3) or the RPMI 1640 medium (HO8910) supplemented with 10% fetal bovine serum (FBS) (Gibco) and penicillin-streptomycin mixture (Servicebio).

Reagents and antibodies. The following antibodies were used for western blotting: monoclonal anti-PI3K (cat. no. 4255; Cell Signaling Technology, Inc.), monoclonal anti-phosphorylated (p)-PI3K (cat. no. 4228; Cell Signaling Technology, Inc.), monoclonal anti-AKT (cat. no. 4691; Cell Signaling Technology, Inc.), monoclonal anti-p-AKT (Ser473; cat. no. 4060; Cell Signaling Technology, Inc.), monoclonal anti-mTOR (cat. no. 2983; Cell Signaling Technology, Inc.), monoclonal anti-p-mTOR (Ser2448; cat. no. 5536; Cell Signaling Technology, Inc.), monoclonal anti-GSK-3β (cat. no. 12456; Cell Signaling Technology, Inc.), monoclonal anti-p-GSK-3β (cat. no. 5558; Cell Signaling Technology, Inc.), monoclonal anti-HIF-1α (cat. no. 36169; Cell Signaling Technology, Inc.), and polyclonal anti-β-actin (cat. no. AF7018; Affinity Biosciences). Horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. ZB-2301) secondary antibodies were purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. LY294002 were purchased from MedChem Express. SP kit and DAB kit were bought from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.

RT-qPCR. RNA of the cells and tissues was isolated with TRIzol (Invitrogen, Carlsbad, CA, USA). The isolated RNA was reverse-transcribed, and then the cDNA obtained by reverse transcription was used in the subsequent RT-qPCR experiments. The reaction conditions were set to preheating at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and extension at 72 °C for 30 s, for a total of 40 cycles. U6 and β-actin served as the normalized reference. The gene expressions were calculated by using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are depicted in Table 1. Each experiment was repeated three times.

Western blotting. Total proteins from the clinical tissue samples and OC cells were extracted on an ice bath with RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with 1% PMSF and 1% phosphatase inhibitor (Beyotime Biotechnology). The supernatant was collected via centrifugation at 13,000×g for 30 min at 4 °C. The proteins were quantified with the BCA protein concentration assay kit (Beyotime Biotechnology) and separated on 6–12% SDS-PAGE gels, followed by a transfer onto a polyvinylidene fluoride (PVDF) membrane. After the transfer, the

Table 1. Primer sequences for qRT-PCR.

Gene	Amplicon size (bp)	Forward primer (5'→3')	Reverse primer (5'→3')
Human-β-actin	96	CCCTGGAGAAGAGCTACGAG	GGAAGGAAGGCTGGAAGAGT
Human-GSK3β	180	GGCAGCATGAAAGTTAGCAGA	GGCGACCAGTTCTCCTGAATC
Human-AKT	167	CTTTCGGCAAGGTGATCCTG	GTACTTCAGGGCTGTGAGGA
Human-PI3K	138	TGTGGAGCTCGCTAAAGTCA	CACTCCTGCCCTAAATGGGA
Human-mTOR	178	GGCCAATGACCCAACATCTC	CATGATGCGATGCTCGATGT
Human-HIF-1α	125	ATCCATGTGACCATGAGGAAATG	TCGGCTAGTTAGGGTACACTTC

membranes were blocked with 5% nonfat milk for 2 h, and the membranes were incubated with a primary antibody overnight at 4 °C. The next day, the membranes were washed with TBST buffer and incubated with a secondary antibody for 1 h at room temperature. The protein bands were visualized with an ultrasensitive ECL chemiluminescence kit (Beyotime Biotechnology), and the results were analyzed using ImageJ (National Institutes of Health). Each experiment was repeated three times.

Transwell assay. HO8910 and SKOV3 cells transfected with miR-9 mimics or miR-9 inhibitors were collected and resuspended in RPMI-1640 or McCoy'5A medium without FBS. The cell numbers were adjusted to 4×10^5 cells/ml; 200 µl of the cell suspension was added to the upper chamber (Corning) and 600 µl of RPMI1640 or McCoy'5A medium containing 20% FBS to the lower chamber, followed by incubation under 5% CO_2 at 37 °C for 24 h. Then, 4% paraformaldehyde was added to the chamber for 30 min to fix the cells at the bottom of the chamber, followed by staining with 0.1% crystal violet solution for 10 min. The cells and the crystal violet solution remaining in the upper chamber were wiped off with a cotton swab. The chamber was allowed to dry naturally and then observed under an inverted microscope (Olympus). The images were collected and 5 fields of view were selected for enumeration. In the invasion experiment, Matrigel was diluted at a ratio of 1:3 with the FBS-free medium, and 60 µl of the diluted Matrigel was added to each chamber, spread uniformly on the bottom membrane of the chamber, and placed in a 37 °C incubator to dry; the cell density was then adjusted to 2.5×10^5 cells/ml, following the remaining steps of the migration experiment. Each experiment was repeated three times.

Xenograft tumor. A total of 36 female BALB/c nude mice were randomly assigned into normoxia, hypoxia, lentivirus negative control (shRNA NC), hsa-miR-9-5p interference lentivirus infection (shRNA-miR-9-5p), PI3K inhibitor (LY294002) solvent control (LY294002 NC), and the PI3K inhibitor intervention (LY294002) groups, with 6 mice in each group. The infected shRNA-miR-9-5p lentivirus and its negative control or blank SKOV3 cells were collected and the number of cells was adjusted to 5×10^7 cells/ml. Next, 200 µl of the cell suspension was injected into the right armpit of each mouse. When the tumor volume grew to 100 mm^3, the PI3K inhibitor LY294002 was intraperitoneally injected (75 mg/kg, twice a week for 4 weeks); the solvent control group was treated with the same dose of drug solvent. Meanwhile, except for the OC model control group, the remaining groups were cultured in a hypoxic chamber (10% O_2) for 8 h/day for 4 weeks. The tumor size was measured weekly. At the end of the experiment, the mice were euthanized by inhaling carbon dioxide, dissected to obtain the tumor tissues, weighed, photographed, and then cryopreserved at –80 °C.

The present study was approved by the Ethics Committee of Experimental Animals of Anhui Medical University (Ethics approval number: LISC20190663).

Hematoxylin and eosin (H&E) staining and immunohistochemistry. The tumor tissues were isolated after the mice were sacrificed. Then, 4% paraformaldehyde was used to fix the cells for 24 h, followed by embedding in paraffin. The wax blocks were subsequently cut into 4 µm thick sections for staining. The cut slides were first dewaxed and then hydrated, followed by staining with hematoxylin for the nuclei and with eosin for the cytoplasm and dehydrating. For the immunohistochemical experiments, after dewaxing and hydrating the sections, the sections were heated in a preheated EDTA solution for antigen retrieval, after which an appropriate amount of the endogenous peroxidase blocking agent was used to treat the sections at room temperature for 10 min. The treated section was then incubated with goat serum for 15 min at 37 °C, followed by incubation with anti-p-PI3K (dilution 1:200; cat. no. AF3241; Affinity Biosciences), anti-p-AKT (dilution 1:200; cat. no. AF0016; Affinity Biosciences), anti-p-mTOR (dilution 1:200; cat. no. AF3308; Affinity Biosciences), anti-p-GSK3β (dilution 1:200; cat. no. AF2016; Affinity Biosciences), and anti-HIF-1α (dilution 1:200; cat. no. AF1009; Affinity Biosciences) antibodies to incubate the sections overnight at 4 °C. The slides were then washed with PBS and treated with horseradish enzyme-labeled streptavidin working solution for 15 min at 37 °C. DAB staining and hematoxylin staining were then performed. After rinsing with tap water, the sections were dehydrated and neutral gum was added dropwise to the sections and covered with coverslips. The stained sections were observed under an upright fluorescence microscope (Zeiss GmbH). Each experiment was repeated three times.

Statistical analyses. The GraphPad Prism 8.0 software (GraphPad Software, Inc.) was used for statistical analyses. The data obtained from the experiment were expressed as the mean ± standard deviation. The results were analyzed by Student's t-test or one-way analysis of variance (ANOVA). p-value <0.05 was considered to indicate statistical significance.

Results

miR-9 and HIF-1α were upregulated in OC tissues. The expression levels of miR-9 and HIF-1α mRNA were examined using RT-qPCR in 30 pairs of OC tissues and normal ovarian tissues. The levels of miR-9 and HIF-1α mRNA in the cancer tissues were extraordinarily higher than in the normal tissues (Figure 1A). HIF-1α protein expression was also increased in the OC tissues (Figure 1C). The result indicated that miR-9 and HIF-1α were upregulated in OC tissues.

PI3K/AKT/mTOR/GSK3β signaling pathway was activated in OC tissues. RT-qPCR and western blot assays were performed to explore how PI3K/AKT/mTOR/GSK3β-associated mRNA and proteins altered in OC. Our findings suggest that the phosphorylation levels of PI3K, AKT, mTOR, and GSK3β (Figure 1C) in OC tissues were extremely higher than in normal ovarian tissues, and the

mRNA expression levels were also markedly upregulated (Figure 1B). These results indicated that the PI3K/AKT/mTOR/GSK3β pathway was active in OC tissues.

Hypoxia induced the expression of miR-9 and activated PI3K/AKT/mTOR/GSK3β signaling pathway *in vitro*. Two OC cells, SKOV3 and HO8910, were cultured under hypoxia for 0, 0.5, 1, 2, 4, 8, 16, and 24 h to see if hypoxia affects the expression of miR-9 in OC cells, and how the PI3K/AKT signaling pathway changes under hypoxia. With the prolongation of hypoxia, miR-9, PI3K, AKT, mTOR, GSK3β, and HIF-1α mRNA levels gradually rose, and remained unchanged or decreased (Figures 2A, 2B). The phosphorylation levels of PI3K, AKT, mTOR, GSK3β, and the expression levels of HIF-1α were markedly enhanced in the cells during hypoxia culture, according to western blot data (Figures 3A, 3B). These findings led to the conclusion that hypoxia can elevate miR-9 expression in OC cells and stimulate the PI3K/AKT/mTOR/GSK3β pathway.

The upregulation of miR-9 promoted OC cell migration and invasion. To demonstrate the functions of miR-9 in OC migration and invasion, transwell experiments with miR-9 inhibitor or miR-9 mimic were performed, and the results showed that the migrated and invaded cell numbers of the miR-9 inhibitor group were significantly lower than the

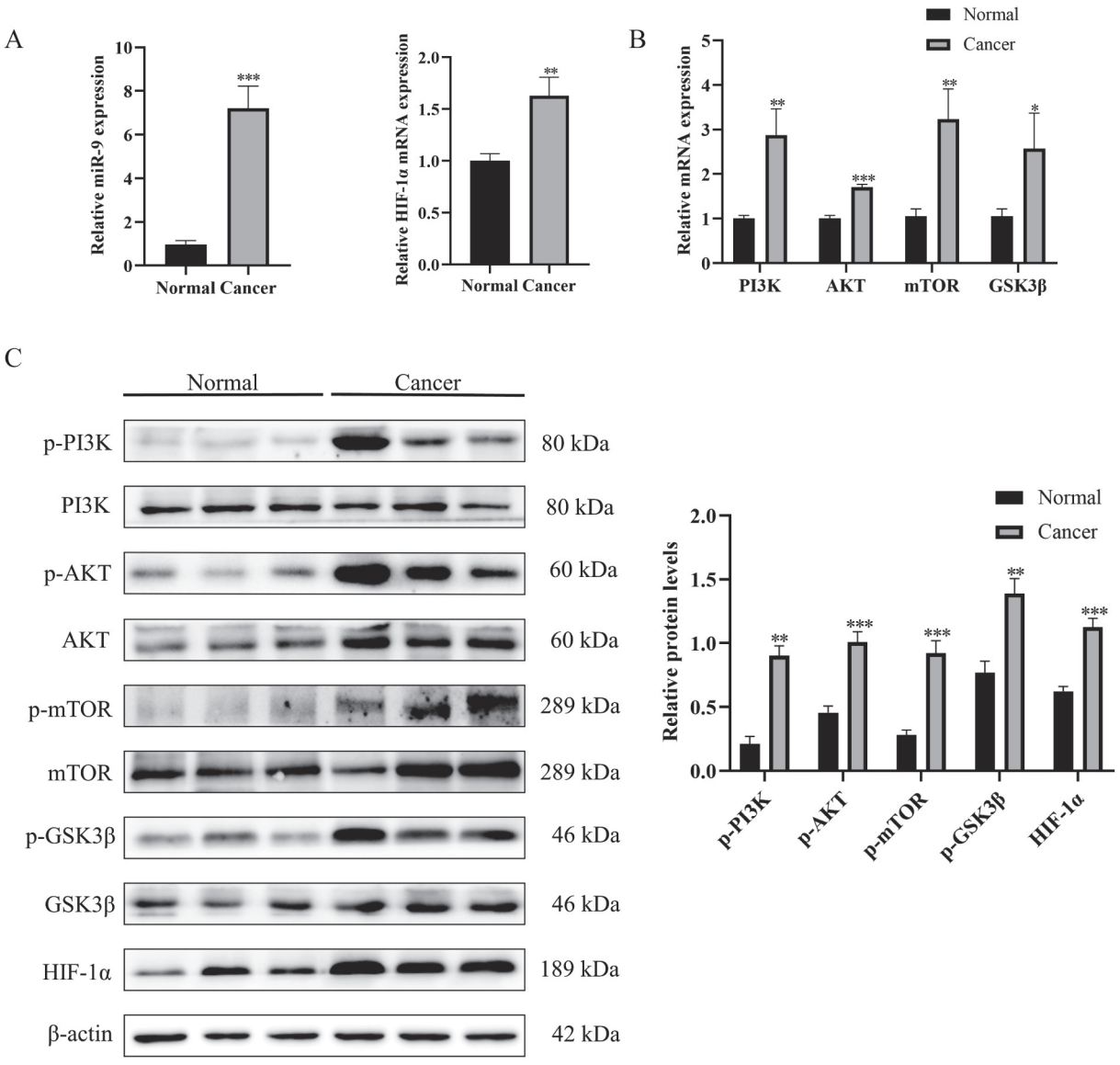

Figure 1. miR-9 and HIF-1α were upregulated in ovarian cancer tissues and PI3K/AKT/mTOR/GSK3β signaling pathway was activated in ovarian cancer. A) The expression levels of miR-9 in ovarian cancer tissues and normal ovarian tissues were confirmed by qRT-PCR. B) The expression levels of PI3K, AKT, mTOR, GSK3β, HIF-1α mRNA in ovarian cancer tissues and normal ovarian tissues were confirmed by qRT-PCR. C) The expression levels of p-PI3K, p-AKT, p-mTOR, p-GSK3β, HIF-1α in ovarian cancer tissues and normal ovarian tissues were confirmed by western blot analysis. Data are expressed as the mean ± standard deviation (n=3). *p<0.05, **p<0.01, ***p<0.001 vs. normal group

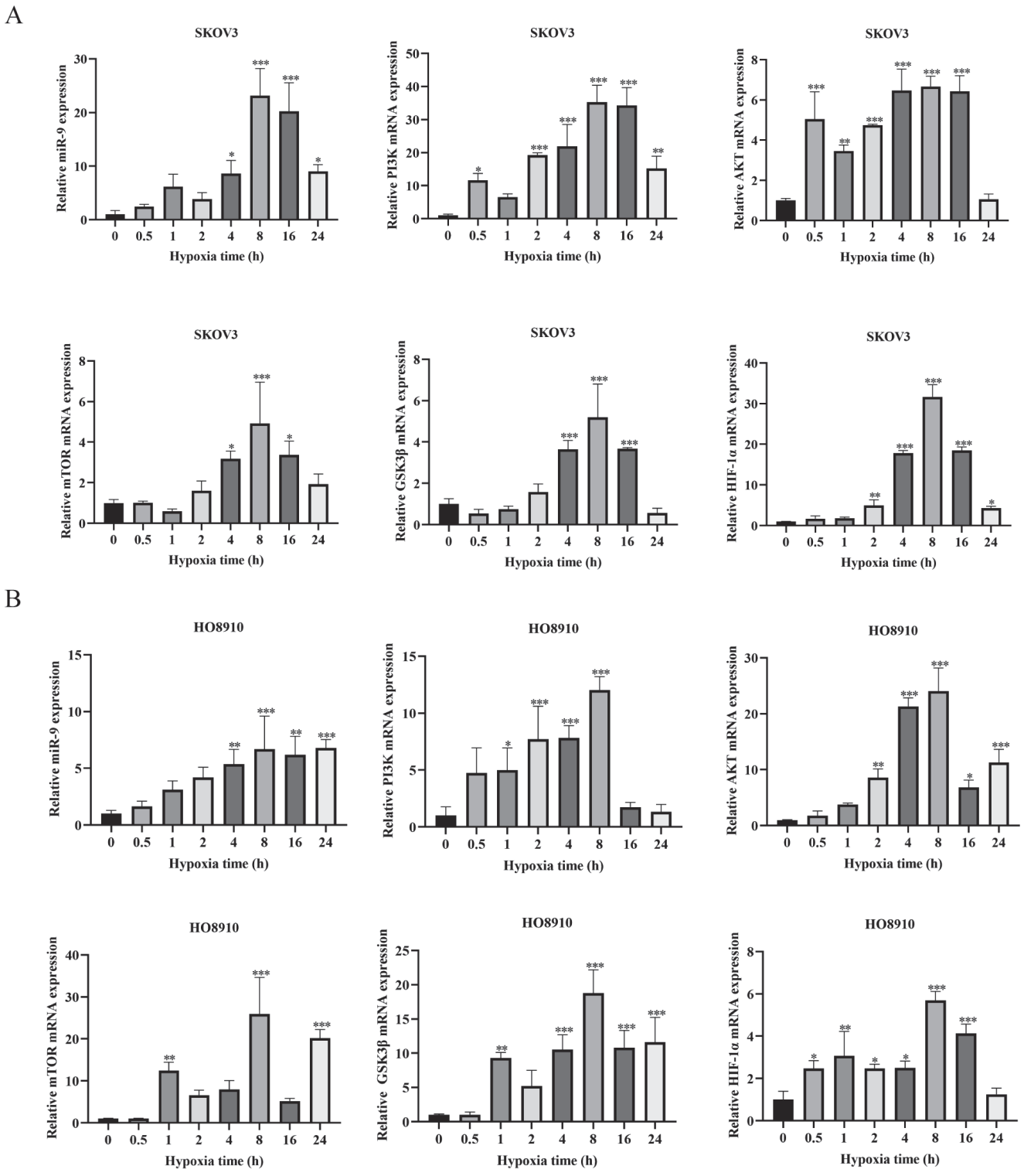

Figure 2. Hypoxia induces high expression of miR-9 and can activate PI3K/AKT/mTOR/GSK3β signaling pathway. A, B) The expression levels of PI3K, AKT, mTOR, GSK3β, and HIF-1α mRNA were detected by RT-qPCR after SKOV3 and HO8910 cells were cultured under hypoxia for different time (0, 0.5, 1, 2, 4, 8, 16, 24 h).

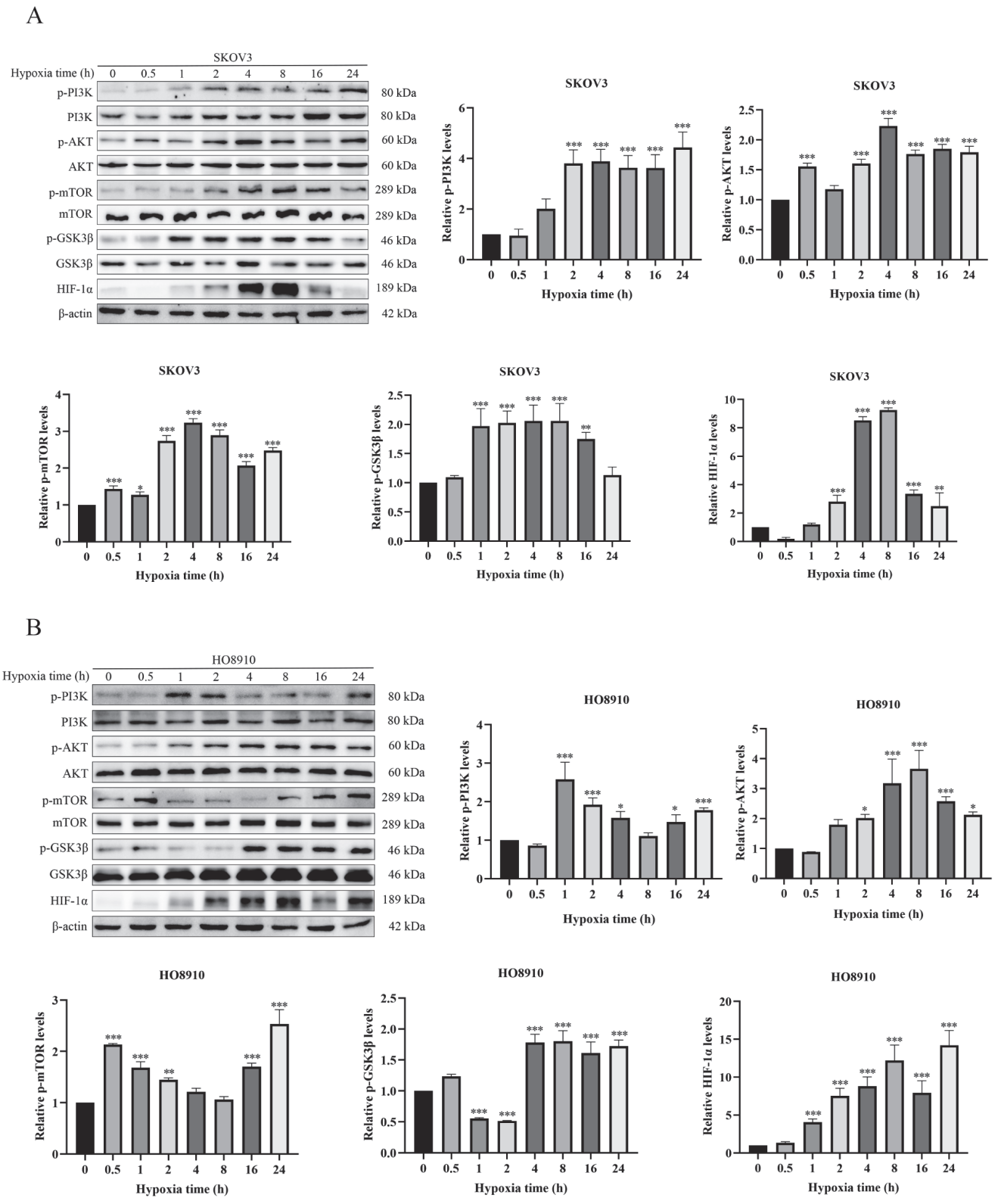

Figure 3. Hypoxia can activate PI3K/AKT/mTOR/GSK3ß pathway. A, B After culturing SKOV3 and HO8910 cells under hypoxia for different time (0, 0.5, 1, 2, 4, 8, 16, 24 h), western blot was used to detect p-PI3K, p-AKT, p-mTOR, p-GSK3β, and HIF- 1α expression level. Data are expressed as the mean ± standard deviation (n=3). *p<0.05, **p<0.01, ***p<0.001 vs. 0 h

miR-9 inhibitor NC group. Cell migration and invasion in the miR-9 mimic group were significantly higher than in the NC group (Figures 4A, 4B). These results implied that miR-9 can increase OC cell migration and invasion. In contrast, the addition of LY294002, an AKT signaling pathway inhibitor, inhibited OC cell migration and invasion when compared to the use of miR-9 mimics alone (Figures 4C, 4D). The results proved that blocking the PI3K/AKT/mTOR/GSK3β pathway might reverse miR-9-induced OC cell migration and invasion.

PI3K/AKT/mTOR/GSK3β pathway was activated *in vitro* by miR-9. Previous research demonstrated that some miRNAs in cancer cells exert oncogenic effects via the PI3K/AKT/mTOR pathway [22]. Our findings showed that the PI3K/AKT/mTOR/GSK3β pathway was active in OC tissues and that hypoxia might potentially activate this pathway. To verify the relationship between miR-9's promoting effect in OC and the PI3K/AKT/mTOR/GSK3β pathway, both the mRNA levels and the phosphorylation levels of the PI3K/AKT/mTOR/GSK3β pathway were investigated. In OC cells, miR-9 overexpression elevated the mRNA levels and the phosphorylation levels of this pathway, but miR-9 inhibition decreased it (Figures 5A–5C). These discoveries suggested that the PI3K/AKT/mTOR/GSK3β pathway is involved in miR-9-induced OC cell migration and invasion. LY2942002 treatment could curb the increased phosphorylation levels of the PI3K/AKT pathway induced by miR-9 overexpression, suggesting that the effect of miR-9 can be inhibited by blocking PI3K/AKT pathway (Figures 5D). These consequences suggested that the PI3K/AKT/mTOR/GSK3β pathway might be activated by upregulating miR-9 expression, thereby verifying that miR-9 promotes cancer via the PI3K/AKT/mTOR/GSK3β pathway.

Knockdown of miR-9 suppressed tumor growth *in vivo*. After confirming that miR-9 promotes tumorigenesis in OC cells via the PI3K/AKT/mTOR/GSK3β signaling pathway. We designed and carried out subcutaneous xenograft tumor experiments with different treated SKOV3 cells to investigate how miR-9 functions in OC progression *in vivo*. When compared to the hypoxia miR-9(–) group, the size and weight of xenograft tumors were considerably smaller in the hypoxia miR-9(–) NC group (Figures 6A, 6B). And the changes in tumor volume of the hypoxia miR-9(–) group following tumor cell injection were significantly slower than in the hypoxia miR-9(–) NC group (Figure 6C). The results of the hypoxia LY294002 group are the same as those of the hypoxia DMSO group. HE staining indicated that the cell counts in the hypoxia miR-9(–) NC group were lower than in the NC group. Immunohistochemical staining revealed that the phosphorylation levels of PI3K, AKT, mTOR, and GSK3β were lower in the hypoxia miR-9(–) group compared to the hypoxia miR-9(–) NC group, as was the expression level of HIF-1α protein (Figures 6D). Overall, hypoxia can overexpress miR-9 and promote tumor growth, miR-9 knockdown inhibits tumorigenicity of OC cells, and the results using LY294002 suggest that miR-9 promotes tumor growth through the PI3K/AKT/mTOR/GSK3β pathway.

Discussion

OC is a common gynecological malignancy. This malignancy has the highest mortality rate of any gynecological tumor, and extensive metastasis of advanced OC is an important cause of death in OC [23]. Because there are no clear distinctive symptoms in the early stages of OC, most OC patients are not diagnosed until they are advanced and have extensive metastases. It is critical to continue looking for new invasion and migratory mechanisms, as well as OC-related targets, to contribute new strategies for OC diagnosis and treatment.

The discovery and study of miRNAs provide new ideas for human research on cancer progression and strategies for diagnosis and treatment. Most biological processes involve miRNAs, and their dysregulation has been associated with oncogenic processes such as cell proliferation, metabolic disorders, angiogenesis, invasion, and metastasis [24]. miR-9 is a hypoxia-related miRNA whose expression elevates under hypoxic conditions [11]. miR-9 is overexpressed in cervical tumor tissues and can promote the growth of human cervical cancer cells SiHA. *In vitro* and *in vivo* experiments confirmed that miR-9 can stimulate the proliferation and migration of SiHA cells. Conversely, inhibition of miR-9 can induce apoptosis of SiHA cells, and downregulation of miR-9 expression may be a promising new therapeutic strategy for cervical cancer [25]. Meanwhile, miR-9 has been demonstrated to be downregulated in ovarian endometrioid carcinoma, ovarian clear cell carcinoma, and recurrent ovarian serous papillary carcinoma. miR-9, as a metastasis-related miRNA, is also a crucial factor in the metastasis of OC. miR-9 contributes greatly to the occurrence, differentiation, and metastasis of these cancers such as colon carcinoma, breast carcinoma, and liver carcinoma, whether as a tumor-promoting gene or a tumor suppressor gene.

miR-9 was found to be downregulated in endometrioid and clear cell OCs, as well as recurrent serous papillary ovarian adenocarcinomas, as compared to primary tumors. Upregulating miR-9 improves prognosis in OC, extends patient survival, as well as enhances the sensitivity of platinum chemotherapy drugs [26–28]. Other studies, however, have found that the expression of miR-9 in metastatic serous OC tissues is remarkably higher than in primary cancer tissues. miR-9 stimulation can enhance OC cell migration and invasion [2]. miR-9 downregulation is related to OC growth, metastasis, and poor prognosis. Patients with high miR-9 levels, on the other hand, responded more effectively to chemotherapy and had longer survival. In OC, miR-9 can also inhibit DNA damage repair, implying that miR-9 activation could be an effective treatment for OC [29]. As the evidence grows that miR-9 is critical in the pathogenesis of OC. In the previous

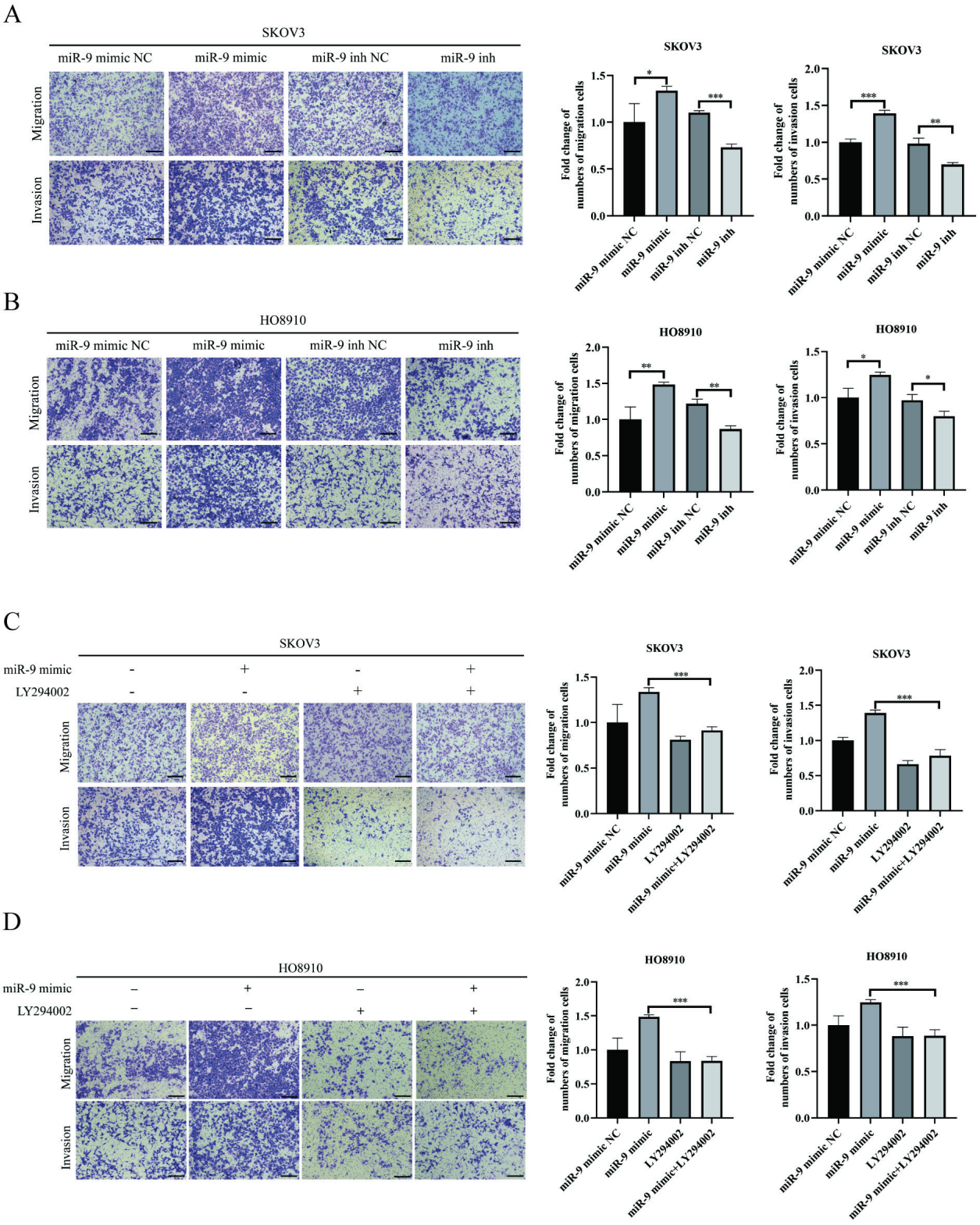

Figure 4. The upregulation of miR-9 promotes ovarian cancer migration and invasion. A, B) SKOV3 and HO8910 cells were transfected with miR-9 mimic or miR-9 inhibitor for 24 h, and their effects on cell migration and invasion were detected by Transwell assay. C, D) The promotion of migration and invasion of ovarian cancer cells by overexpression of miR-9 can be reversed when 20 μM of LY294002 were added. Scale bar: 100 μm, magnification: 100×. *p<0.05, **p<0.01, ***p<0.001

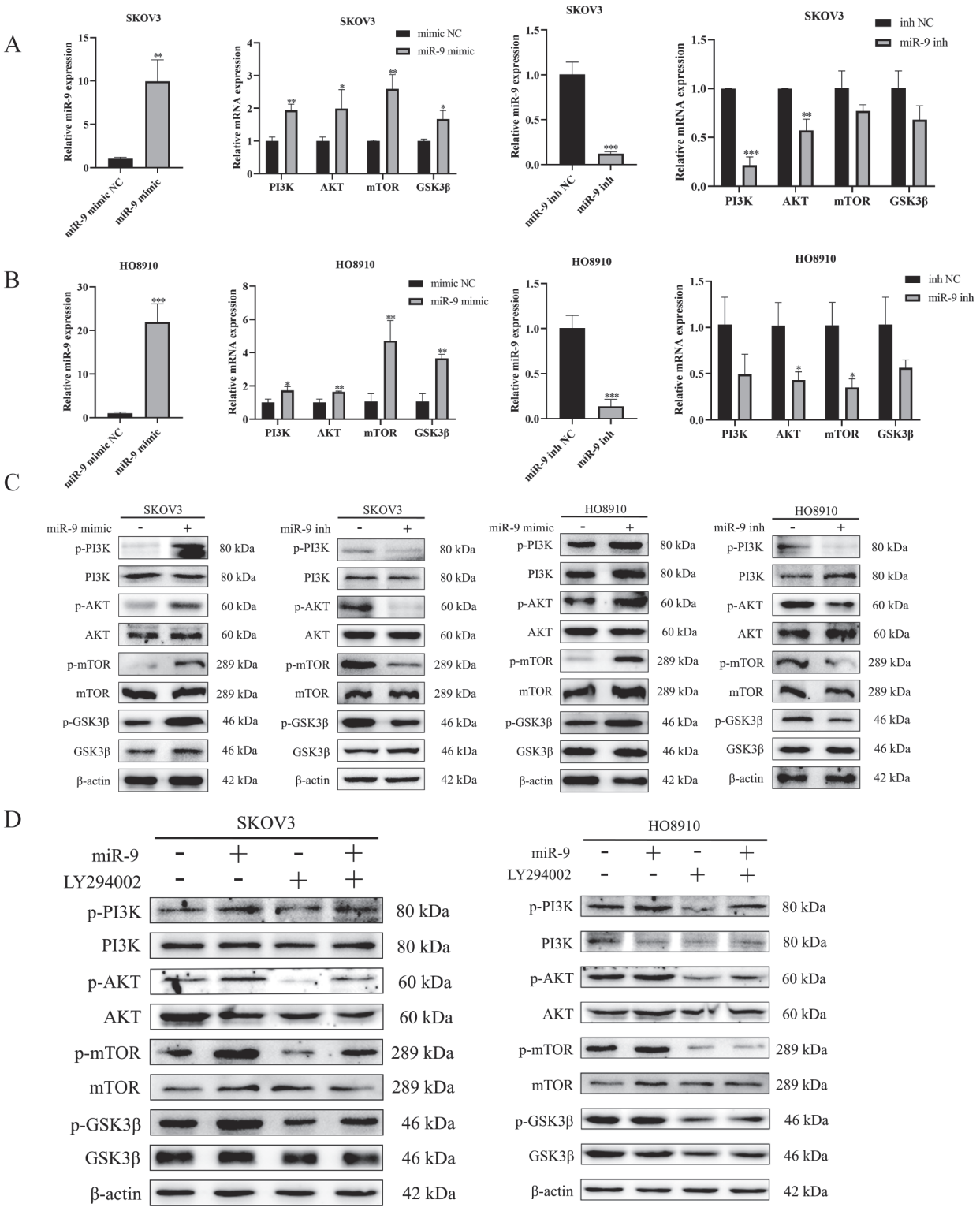

Figure 5. Overexpression of miR-9 activates the PI3K/AKT/mTOR/GSK3β signaling pathway. A, B) SKOV3 and HO8910 cells were transfected with miR-9 mimic or miR-9 inhibitor for 24 h, and then the mRNA levels of PI3K, AKT, mTOR, and GSK3β were detected by RT-qPCR. C) SKOV3 and HO8910 cells were transfected with miR-9 mimic or miR-9 inhibitor for 24 h, the expression of p-PI3K, p-AKT, p-mTOR, and p-GSK3β was detected by western blotting. D) SKOV3 and HO8910 cells were transfected with miR-9 mimic NC or miR-9 mimic for 24 h, and 20 μM LY294002 was added. Western blotting was used to detect the expression of p-PI3K, p-AKT, p-mTOR, and p-GSK3β. *p<0.05, **p<0.01, ***p<0.001 vs. control group

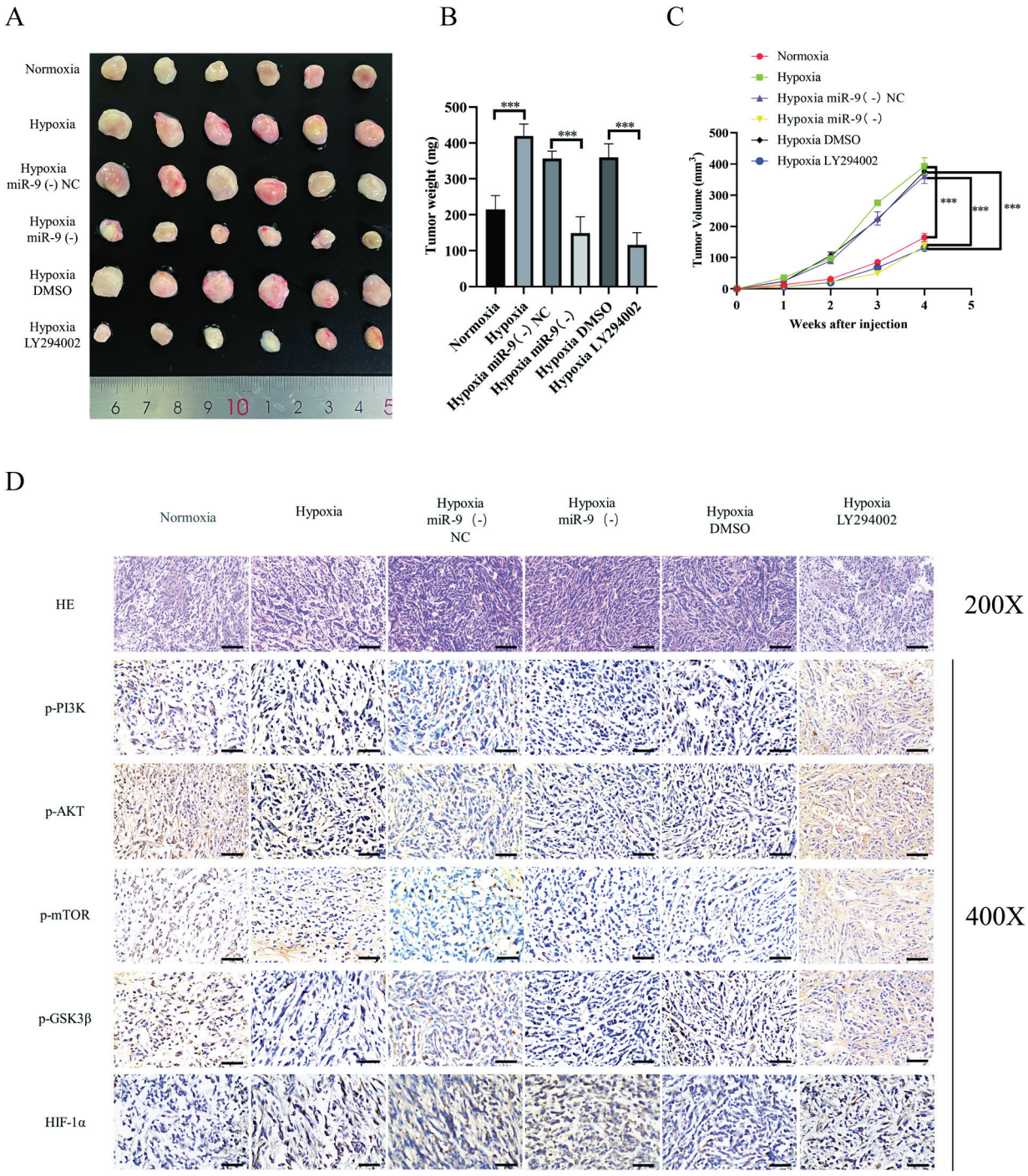

Figure 6. Knockdown of miR-9 inhibits the growth of ovarian cancer xenografts in nude mice. A) Determining the size of tumors formed by different cells. B) Quantification of tumor weights. C) Quantification of tumor volumes. D) H&E staining was used to analyze the number of tumor cells in tumor tissue sections. Scale bar: 100 μm, magnification: 200×. The expression of p-PI3K, p-AKT, p-mTOR, p-GSK3β, and HIF-1α in tumor tissue sections were detected by immunohistochemistry. Scale bar: 50 μm, magnification: 400×. ***p<0.001 vs. each control group

stage, we used RT-qPCR to detect the expression of a series of miRNAs and screened out the most upregulated miRNA-9 in OC cells induced by hypoxia. It was found that miRNA-9 was significantly differentially expressed in an anoxic environment. No studies have been reported on the signaling pathways and factors acting in the phenotypic transformation of ovarian cancer cells due to miRNA-9 overexpression.

The PI3K/AKT/mTOR pathway is frequently altered in OC, providing a pathway that can be addressed in OC therapy. The PI3K/AKT/mTOR pathway carcinogenic function in OC is complicated and is important in the occurrence, chemotherapy, and radiotherapy resistance of OC [30]. Some miRNAs have been demonstrated to directly target related molecules on the PI3K/AKT/mTOR pathway, while the PI3K/AKT/mTOR pathway can also function by regulating miRNAs. The two regulate each other and act together on the progression of cancer [31]. miRNAs can not only play their oncogene roles via the PI3K/AKT/mTOR pathway, such as promoting cell proliferation and inhibiting apoptosis, but they can also act as tumor suppressor genes, inhibiting the pathway and tumor progression [20]. Triterpenoids from *Tripterygium wilfordii* can significantly downregulate miR-21 in gastric tumor cells and induce cancer cell apoptosis via PI3K/AKT. Furthermore, miR-21 inhibitors reduced the expression of phosphorylated AKT. Upregulating miR-21, on the other hand, enhanced PI3K/AKT activities and reduced triptolide-induced apoptosis in gastric cancer cells, an effect that could be reversed by PI3K inhibitors [32]. Curcumin was discovered to inhibit the PI3K/AKT pathway by upregulating the expression of miR-192-5p, resulting in the inhibition of non-small cell lung cancer cells proliferation and promotion of apoptosis. Researchers proposed that after treating cancer cells with curcumin, the expression of miR-192-5p upregulated, while the PI3K/AKT pathway was inhibited. Inhibition of miR-192-5p reversed the effects of curcumin [33]. Another study showed that curcumin inhibited the development of prostate cancer by stimulating miR-9 levels via the PDK1/AKT/mTOR pathway [34]. Histone deacetylase inhibitors inhibited the proliferation of Burkitt lymphoma cells by inhibiting the PI3K/AKT pathway and upregulating miR-143, miR-145, and miR-101 [35]. METTL3 regulates the maturation of pre-miR-126, which then modulates the PI3K/Akt/mTOR pathway, and it also plays a role in the occurrence of OC [18, 36]. miRNAs have multiple functions in the progression of OC. miR-21 may block PTEN levels, promoting OC cell proliferation and migration [37].

In this work, we discovered a novel molecular mechanism of OC migration and invasion (Figure 7). In hypoxia, we found that miR-9 is upregulated in OC tissues. Further research discovered that hypoxia increases the phosphorylation levels of PI3K, AKT, mTOR, and GSK3β, indicating that hypoxia can induce the PI3K/AKT/mTOR/GSK3β pathway. The association of miR-9 with the PI3K/AKT/mTOR/GSK3β signaling pathway in OC cell migration and invasion was elucidated. OC cells were treated with miR-9 mimics or inhibitors, and transwell results indicated that miR-9 upregulation promoted OC cells migration and invasion, whereas inhibition had the opposite effect. Further western blot experiments revealed that miR-9 upregulation might activate the PI3K/AKT/mTOR/GSK3β pathway, with opposite inhibitory outcomes. The results of *in vivo* xenograft experiments are congruent with the conclusions of *in vitro* experiments. This conclusion proves our hypothesis, and increased miR-9 expression in a hypoxic environment can activate the PI3K/AKT/mTOR/GSK3β signaling pathway, promoting OC cell migration and invasion.

This study has certain limitations and deficiencies. First, whether hypoxia-inducible factor 1α may regulate miR-9 expression, our findings show that miR-9 overexpression is closely associated with HIF-1α. Hypoxia has been found in studies to induce miR-9 upregulation by stimulating pri-miR-9-1 and pri-miR-9-3 via the HIF-1 pathway [11]. It remains to be seen whether the results are the same in OC. Second, miR-9 elevates OC cell migration and invasion via the PI3K/AKT/mTOR/GSK3β pathway. What is its specific target? GSK3β is a critical target of miR-9-5p [38], and the target relationship between the two needs to be studied further. Our findings present a novel mechanism for studying OC cell migration and invasion.

In summary, our study proposes that miR-9 was upregulated in OC tissues, while hypoxia could induce miR-9 expression. miR-9 upregulation promoted OC cell migration and invasion via the PI3K/AKT/mTOR/GSK3β pathway. Our findings provide new treatment options for OC.

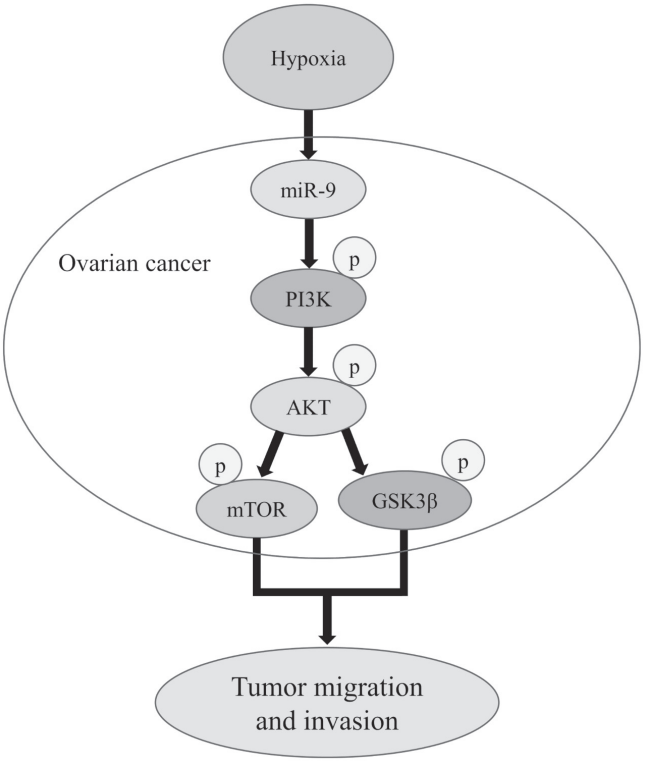

Figure 7. Mechanisms of miR-9-induced ovarian cancer migration and invasion. Hypoxia upregulates the expression of miR-9, promotes the phosphorylation of PI3K, AKT, mTOR, GSK3β, and ultimately promotes the migration and invasion of ovarian cancer.

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References

[1] MILLER KD, NOGUEIRA L, MARIOTTO AB, ROWLAND JH, YABROFF KR et al. Cancer treatment and survivorship statistics. CA Cancer J Clin 2019; 69: 363–385. https://doi.org/10.3322/caac.21565

[2] ZHOU B, XU H, XIA M, QI M, LI D et al. Overexpressed miR-9 promotes tumor metastasis via targeting E-cadherin in serous ovarian cancer. Front Med 2017; 11: 214–222. https://doi.org/10.1007/s11684-017-0518-7

[3] WENG H, FENG X, LAN Y, ZHENG Z. TCP1 regulates PI3K/AKT/mTOR signaling pathway to promote proliferation of ovarian cancer cells. J Ovarian Res 2021; 14: 82. https://doi.org/10.1186/s13048-021-00832-x

[4] GAO Y, LI Y, ZHANG C, HAN J, LIANG H et al. Evaluating the benefits of neoadjuvant chemotherapy for advanced epithelial ovarian cancer: a retrospective study. J Ovarian Res 2019; 12: 85. https://doi.org/10.1186/s13048-019-0562-9

[5] ZHENG L, CUI C, SHI O, LU X, LI Y et al. Incidence and mortality of ovarian cancer at the global, regional, and national levels, 1990–2017. Gynecol Oncol 2020; 159: 239–247. https://doi.org/10.1016/j.ygyno.2020.07.008

[6] NAKAMURA K, SAWADA K, MIYAMOTO M, KINOSE Y, YOSHIMURA A et al. Downregulation of miR-194-5p induces paclitaxel resistance in ovarian cancer cells by altering MDM2 expression. Oncotarget 2019; 10: 673–683. https://doi.org/10.18632/oncotarget.26586

[7] ZHOU W, XI D, SHI Y, WANG L, ZHONG H et al. MicroRNA 1929 3p participates in murine cytomegalovirus induced hypertensive vascular remodeling through Ednra/NLRP3 inflammasome activation. Int J Mol Med 2021; 47: 719–731. https://doi.org/10.3892/ijmm.2020.4829

[8] PINELLI S, ALINOVI R, POLI D, CORRADI M, PELOSI G et al. Overexpression of microRNA 486 affects the proliferation and chemosensitivity of mesothelioma cell lines by targeting PIM1. Int J Mol Med 2021; 47: 117. https://doi.org/10.3892/ijmm.2021.4950

[9] HAN S, SHAO Y, HUANG J, SUN S, LIU Y et al. Prognostic value of microRNA-9 in cancers a systematic review and meta-analysis. Oncotarget 2016; 7: 67020–67032. https://doi.org/10.18632/oncotarget.11466

[10] YOKOI A, MATSUZAKI JAO, YAMAMOTO Y, YONEOKA Y, TAKAHASHI K et al. Integrated extracellular microRNA profiling for ovarian cancer screening. Nat Commun 2018; 9: 4319. https://doi.org/10.1038/s41467-018-06434-4

[11] SHAN F, LI J, HUANG QY. HIF-1 alpha-induced up-regulation of miR-9 contributes to phenotypic modulation in pulmonary artery smooth muscle cells during hypoxia. J Cell Physiol 2014; 229: 1511–1520. https://doi.org/10.1002/jcp.24593

[12] ZHU B, XI X, LIU Q, CHENG Y, YANG H. MiR-9 functions as a tumor suppressor in acute myeloid leukemia by targeting CX chemokine receptor 4. Am J Transl Res 2019; 11: 3384–3397.

[13] YI J, GAO ZF. MicroRNA-9-5p promotes angiogenesis but inhibits apoptosis and inflammation of high glucose-induced injury in human umbilical vascular endothelial cells by targeting CXCR4. Int J Biol Macromol 2019; 130: 1–9. https://doi.org/10.1016/j.ijbiomac.2019.02.003

[14] SHAO C, YANG F, MIAO S, LIU W, WANG C et al. Role of hypoxia-induced exosomes in tumor biology. Mol Cancer 2018; 17: 120. https://doi.org/10.1186/s12943-018-0869-y

[15] JING X, YANG F, SHAO C, WEI K, XIE M et al. Role of hypoxia in cancer therapy by regulating the tumor microenvironment. Mol Cancer 2019; 18: 157. https://doi.org/10.1186/s12943-019-1089-9

[16] FATTAHI S, AMJADI-MOHEB F, TABARIPOUR R, ASHRAFI GH, AKHAVAN-NIAKI H. PI3K/AKT/mTOR signaling in gastric cancer: Epigenetics and beyond. Life Sci 2020; 262: 118513. https://doi.org/10.1016/j.lfs.2020.118513

[17] MIRICESCU D, TOTAN A, STANESCU S, II, BADOIU SC, STEFANI C et al. PI3K/AKT/mTOR Signaling Pathway in Breast Cancer: From Molecular Landscape to Clinical Aspects. Int J Mol Sci 2022; 22: 173. https://doi.org/10.3390/ijms22010173

[18] EDIRIWEERA MK, TENNEKOON KH, SAMARAKOON SR. Role of the PI3K/AKT/mTOR signaling pathway in ovarian cancer: Biological and therapeutic significance. Semin Cancer Biol 2019; 59: 147–160. https://doi.org/10.1016/j.semcancer.2019.05.012

[19] MIRZA-AGHAZADEH-ATTARI M, EKRAMI EM, AGHDAS SAM. Targeting PI3K/Akt/mTOR signaling pathway by polyphenols: Implication for cancer therapy. Life Sci 2020; 255: 117481. https://doi.org/10.1016/j.lfs.2020.117481

[20] YAN X, LYU T, JIA N, YU Y, HUA K et al. Huaier aqueous extract inhibits ovarian cancer cell motility via the AKT/GSK3beta/beta-catenin pathway. PLoS One 2013; 8: e63731. https://doi.org/10.1371/journal.pone.0063731

[21] TONG X, ZHAO J, ZHANG Y, MU P, WANG X. Expression levels of MRP1, GST-pi, and GSK3beta in ovarian cancer and the relationship with drug resistance and prognosis of patients. Oncol Lett 2019; 18: 22–28. https://doi.org/10.3892/ol.2019.10315

[22] AKBARZADEH M, MIHANFAR A, AKBARZADEH S, YOUSEFI B, MAJIDINIA M. Crosstalk between miRNA and PI3K/AKT/mTOR signaling pathway in cancer. Life Sci 2021; 285: 119984. https://doi.org/10.1016/j.lfs.2021.119984

[23] LI YT, LEE WL, TSUI KH. Endometrial thickness still presents a best reference to predict endometrial cancer. Taiwan J Obstet Gynecol 2016; 55: 148–149. https://doi.org/10.1016/j.tjog.2015.07.003

[24] YOKOI A, MATSUZAKI JAO, YAMAMOTO Y, YONEOKA Y, TAKAHASHI K et al. Integrated extracellular microRNA profiling for ovarian cancer screening. Nat Commun 2018; 9: 4319. https://doi.org/10.1038/s41467-018-06434-4

[25] ZHANG H, ZHANG Z, WANG S, ZHANG S, BI J. The mechanisms involved in miR-9 regulated apoptosis in cervical cancer by targeting FOXO3. Biomed Pharmacother 2018; 102: 626–632. https://doi.org/10.1016/j.biopha.2018.03.019

[26] IORIO MV, VISONE R, DI LEVA G, DONATI V, PETROCCA F et al. MicroRNA signatures in human ovarian cancer. Cancer Res 2007; 67: 8699–8707. https://doi.org/10.1158/0008-5472.CAN-07-1936

[27] MAVRIKAKI M, ANASTASIADOU E, OZDEMIR RA, POTTER D, HELMHOLZ C et al. Overexpression of miR-9 in the Nucleus Accumbens Increases Oxycodone Self-Administration. Int J Neuropsychopharmacol 2019; 22: 383–393. https://doi.org/10.1093/ijnp/pyz015

[28] LAIOS A, O'TOOLE S, FLAVIN R, MARTIN C, KELLY L et al. Potential role of miR-9 and miR-223 in recurrent ovarian cancer. Mol Cancer 2008; 7: 35. https://doi.org/10.1186/1476-4598-7-35

[29] HUANG X, TENG Y, YANG H, MA J. Propofol inhibits invasion and growth of ovarian cancer cells via regulating miR-9/NF-kappaB signal. Braz J Med Biol Res 2016; 49: e5717. https://doi.org/10.1590/1414-431X20165717

[30] MABUCHI S, KURODA H, TAKAHASHI R, SASANO T. The PI3K/AKT/mTOR pathway as a therapeutic target in ovarian cancer. Gynecol Oncol 2015; 137: 173–179. https://doi.org/10.1016/j.ygyno.2015.02.003

[31] ZHANG Y, HUANG B, WANG HY, CHANG A, ZHENG XFS. Emerging Role of MicroRNAs in mTOR Signaling. Cell Mol Life Sci 2017; 74: 2613–2625. https://doi.org/10.1007/s00018-017-2485-1

[32] SHA M, YE J, ZHANG LX, LUAN ZY, CHEN YB et al. Celastrol induces apoptosis of gastric cancer cells by miR-21 inhibiting PI3K/Akt-NF-κB signaling pathway. Pharmacology 2014; 93: 39–46. https://doi.org/10.1159/000357683

[33] JIN H, QIAO F, WANG Y, XU Y, SHANG Y. Curcumin inhibits cell proliferation and induces apoptosis of human non-small cell lung cancer cells through the upregulation of miR-192-5p and suppression of PI3K/Akt signaling pathway. Oncol Rep 2015; 34: 2782–2789. https://doi.org/10.3892/or.2015.4258

[34] SHENG W, XU W, DING J, LI L, YOU X et al. Curcumol inhibits the malignant progression of prostate cancer and regulates the PDK1/AKT/mTOR pathway by targeting miR-9. Oncol Rep 2021; 46: 246. https://doi.org/10.3892/or.2021.8197

[35] FERREIRA AC, ROBAINA MC, REZENDE LM, SEVERINO P, KLUMB CE. Histone deacetylase inhibitor prevents cell growth in Burkitt's lymphoma by regulating PI3K/Akt pathways and leads to upregulation of miR-143, miR-145, and miR-101. Ann Hematol 2014; 93: 983–993. https://doi.org/10.1007/s00277-014-2021-4

[36] HAN B, CHU C, SU X, LIU F, YUAN J et al. N(6)-methyladenosine-dependent primary microRNA-126 processing activated PI3K-AKT-mTOR pathway drove the development of pulmonary fibrosis induced by nanoscale carbon black particles in rats. Nanotoxicology 2020; 14: 1–20. https://doi.org/10.1080/17435390.2019.1661041

[37] LOU Y, YANG X, WANG F, CUI Z, HUANG Y. MicroRNA-21 promotes the cell proliferation, invasion and migration abilities in ovarian epithelial carcinomas through inhibiting the expression of PTEN protein. Int J Mol Med 2010; 26: 819–827. https://doi.org/10.3892/ijmm_00000530

[38] LIU J, ZUO X, HAN J, DAI Q, XU H et al. MiR-9-5p inhibits mitochondrial damage and oxidative stress in AD cell models by targeting GSK-3β. Biosci Biotechnol Biochem 2020; 84: 2273–2280. https://doi.org/10.1080/09168451.2020.1797469