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Aspirin inhibits hypoxia-mediated lung cancer cell stemness and exosome function

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ABSTRACT

Background: Epidemiological studies have illustrated that regular aspirin consumption may decrease the risk of non-small cell lung cancer (NSCLC). The present study aims to investigate the mechanism of aspirin-induced inhibition of NSCLC development during hypoxia.

Methods: A549 cells were pre-treated with the vehicle control or aspirin and then subjected to hypoxic culture. Cell viability was monitored by CCK-8 assay, and flow cytometry was performed to detect cell cycle distributions, apoptosis, and proportion of cancer stem cells (CSCs). Flow cytometric cell sorting was used to separate CSCs. Quantitative reverse transcription–polymerase chain reaction and Western blot were used to detect the mRNA and protein levels of stem cell markers and the related signaling molecules. The abundance of prostaglandin E2 was detected by enzyme-linked immunosorbent assay. Exosomes in the cell culture medium were isolated using ExoQuick, and the number of exosomes was quantified by the EXOCET exosome quantification assay kit. Cell migration and angiogenesis were monitored by transwell migration assay and in vitro angiogenesis experiments.

Results: Aspirin inhibited cell proliferation and induced G2/M cell cycle arrest in hypoxic A549 cells; it also inhibited hypoxia-enhanced stemness in both A549 and $ALDH^+$ cells. The drug reduced hypoxia-enhanced numbers of exosomes in A549 cells and exerted negative effects on the hypoxia-mediated up-regulation of exosomal HIF-1α/COX-2 and expression of exosomal miR-135b and miR-210. While hypoxic-induced exosomes can promote the proliferation, migration, and angiogenesis of other A549 cells, aspirin can weaken this promotion by reducing the amount of exosome secreted and changing exosome contents.

Conclusions: Aspirin inhibits the hypoxia-induced stemness, hypoxic-mediated exosome release, and malignant paracrine effects of A549 cells.

1. Introduction

Lung cancer is a very common cancer and the leading cause of cancer-related deaths worldwide [1,2]. Despite recent advances in clinical practice, including target therapies, however, the 5-year survival rate of lung cancer remains at approximately 16%. One of the main reasons behind the poor prognosis of this cancer type is its resistance to various treatments [2]. A subpopulation of cancer cells with treatment-resistant potential is characterized by the abilities of self-renewal, differentiation, and metastasis, and cells of this subpopulation are called cancer stem cells (CSCs) [3]. Developing specific therapies targeting CSCs is important to improve the clinical outcomes of lung cancer.

The tumor microenvironment (TME) of a solid tumor is composed of cancer cells, stromal cells, blood vessels, and immune cells [4]; it has been implicated in the regulation of cell proliferation, invasion, and metastasis and contributes to the outcome of cancer therapy. Solid tumors feature hypoxic microenvironments due to their rapid expansion and irregular blood flow. Accumulating evidence suggests that hypoxia is associated with the invasive and metastatic potential of cancer cells, as well as their resistance to radio- and chemotherapies, all of which lead to poor clinical outcomes [4,5]. Hypoxia up-regulates the expression of hypoxia-inducible factor-1α (HIF-1α), which, in turn, regulates various biological signals, including stem cell markers (e.g., SOX2 and OCT4) [6]. Hypoxia has consistently been reported to promote cancer cell stemness and drug resistance in lung cancer [7]. HIF-1α also up-regulates cyclooxygenase-2 (COX-2) during hypoxia, thereby leading to the increased abundance of its enzymatic product prostaglandin E2

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(PGE2). Previous studies have reported that COX-2/PGE2 signaling plays crucial roles in cancer cell proliferation, apoptosis, angiogenesis, and stemness [8,9].

Emerging evidence has revealed that cancer-derived exosomes participate in the biological mechanism of the TME [10]. Exosomes, which are small vesicles with diameters measuring 30–100 nm, have been shown to carry microRNAs (miRNAs), mRNAs, DNA fragments, and proteins [10,11]. A recent study demonstrated that exosomes secreted under hypoxia enhance the invasiveness and stemness of cancer cells and induce TME recruitment and reprogramming in prostate cancer [12]. However, the role of exosomes in non-small cell lung cancer (NSCLC) cells during hypoxia remains unclear.

Aspirin, a non-selective COX inhibitor, has been widely used as a nonsteroidal anti-inflammatory drug (NSAID) for over 100 years. Besides its well-known anti-inflammatory effects, aspirin has been reported to inhibit tumorigenesis in various tissues [13]. Epidemiological and clinical studies have illustrated that regular aspirin consumption decreases the risk of NSCLC [14–17], which indicates that aspirin could be a promising agent in the treatment of this cancer. However, the underlying mechanism by which aspirin exerts its anti-tumor role in NSCLC has yet to be determined. The priming effect of aspirin on lymphoma was found to be associated with the altered constitution of TME [18]. We thus hypothesize that aspirin may inhibit tumorigenesis in NSCLC by inducing TME changes under hypoxic conditions.

In this study, we demonstrate that aspirin inhibits the stemness of NSCLC A549 cells and weakens the malignant function of exosomes under hypoxic conditions. These effects could be responsible for the anti-tumor effect of the drug.

2. Materials and methods

2.1. Cell culture and treatment

Human lung adenocarcinoma cell line (A549) and human umbilical vein endothelial cells (HUVEC) were purchased from Cell Resource Center, Peking Union Medical College. Cells were cultured in RMPI-1640 supplemented with 10% Fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA), 100U/ml penicillin and 100 μg/ml streptomycin. Cell culture was maintained at 37°C in a humidified atmosphere with 5% CO_2. For treatments, cells pre-treated with vehicle or different doses of aspirin (Sigma-Aldrich, St Louis, MO, USA) for five min, followed by a hypoxic process with 5% CO_2, 2% O_2, and 93% N_2 in a 37°C incubator.

2.2. Cell proliferation assay

A549 cells were seeded in 96-well plates at a density of 5×10^3 cells per well for24 h before treatment. After 12 h, 24 h, 48 h and 72 h of cultivation, cell proliferation was measured by Cell Counting Kit-8 (CCK-8) system (Beyotime, Haimen, China) according to the manufacturer's instruction. In brief, 10 μL of CCK-8 solution was added to each well and incubated at 37°C for 1 h. The absorbance was measured at 450 nm with a microplate spectrophotometer (BioTek, USA). There were triplicates for each group, and the experiments were repeated at least three times.

2.3. Cell cycle assay

Cell cycle was analyzed using a Cell Cycle Detection Kit (Keygentec, Nanjing, China). A549 cells were harvested and washed twice with PBS at 24 h after treatments. Cells were then fixed in 1 ml 70% pre-cooled ethanol at 4 °C overnight, followed by incubation with 100 mg/l RNaseA at 37°C for 30 min. Cells were stained with 50 mg/l propidium iodide (PI) at 4 °C for 30 min in darkness, and cells were detected on FACScalibur flow cytometry (BD Biosciences, San Jose, CA, USA) with the excitation wavelength at 488 nm. Data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

2.4. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from A549 cells using TRIzol reagent (Thermo Fisher Scientific) and reverse transcribed with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). RT products were used as templates for qRT-PCR. The mRNA levels of the target genes were analyzed by SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific) according to the manufacturer's instruction (n = 3, each in triplicate). GAPDH was used as an internal control for normalization. The specificity of the fluorescence signal was confirmed by both melting curve and agarose gel electrophoresis. The mRNA levels of target genes were determined by $2^{-\Delta\Delta C}_{T}$ method. The primers were synthesized by SanGon (China). Primers for RT-qPCR sequences are as follows (from 5′ to 3′):

SOX2-F, GGAGAGTAAGAAACAGCATGGA; SOX2-R, GTGGATGGG ATTGGTGTTCT; 4-Oct-F, CAGGAGATATGCAAAGCAGAAAC; 4-Oct-R, GGCACTGCAGGAACAAATTC; ALDH1-F, GTCAAACCAGCAGAGCA AAC; ALDH1-R, GGCCCATAACCAGGAACAATA; COX-2-F, CTATGTGC TAGCCCACAAAGA; COX-2-R, GCATCCACAGATCCCTCAAA; HIF-1a-F, GTCTGCAACATGGAAGGTATTG; HIF-1a-R, GCAGGTCATAGGTGGTT TCT; GAPDH-F, GGTGTGAACCATGAGAAGTATGA; GAPDH-R, GAGTC CTTCCACGATACCAAAG.

2.5. Western blotting

Protein extracts were prepared in RIPA lysis buffer. Total protein concentration was determined by BCA Protein Assay Kit (KeyGEN Biotech, Nanjing, China). An equal amount of protein extracts were resolved by SDS-PAGE and transferred onto the PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk, followed by incubation with corresponding primary and secondary antibodies. Primary antibodies against SOX2 (AF5140), ALDH1 (DF6225), COX-2 (AF7003), HIF-1α (AF1009) were obtained from Affinity Biosciences (Cincinnati, OH, USA). Anti-OCT4 (ab181557), anti-calnexin (ab133615) and anti-GAPDH (ab8245) were purchased from Abcam (Cambridge, MA, USA). Anti-CD63 (Ap5333b) and anti-CD81 (Am8557b) were from Abgent (San Diego, CA, USA). ECL Western blotting detection reagents (Beyotime) were used for protein detection. The X-ray films were scanned, and bands were analyzed by ImageQuant™ LAS 500 (GE Healthcare, Piscataway, NJ, USA).

2.6. Enzyme-linked immunosorbent assay (ELISA)

Specific ELISA kit for human prostaglandin E2 (PGE2, Bio-Swamp, Shanghai, China) was used to quantify the level of PGE2 in cell culture supernatant according to the manufacturer's instructions.

2.7. Aldefluor assay and separation of the ALDH subpopulations by FACS

The Aldefluor reagent (Stem Cell Technologies, Vancouver, Canada) was used to isolate subpopulations with high aldehyde dehydrogenase (ALDH) activity. In brief, A549 cells were collected and resuspended in ALDEFLUOR assay buffer containing ALDH substrate (BAAA, 1 μmol/l per 1×10^6 cells), followed by incubation at 37°C for 50 min in darkness. For each sample, an aliquot of cells was treated with 50 mmol/l diethylamino benzaldehyde (DEAB), an ALDH specific inhibitor, as a negative control. The sorting gates were established using Aldefluor-stained negative controls. Labeled cells were sorted with MoFlo XDP cell sorter (Beckman Coulter, Miami, FL, USA). After sorting, $ALDH1^+$ cells and $ALDH1^-$ cells were cultured in DMEM/F12 (Hyclone, Logan, UT, USA) supplemented with 20 mg/l EGF, 20 mg/l bFGF, 1% B27, 0.1% BSA, 5 mmol/l HEPES, 4 mmol/l Glutamine, 100 U/l penicillin and 100 mg/l streptomycin.

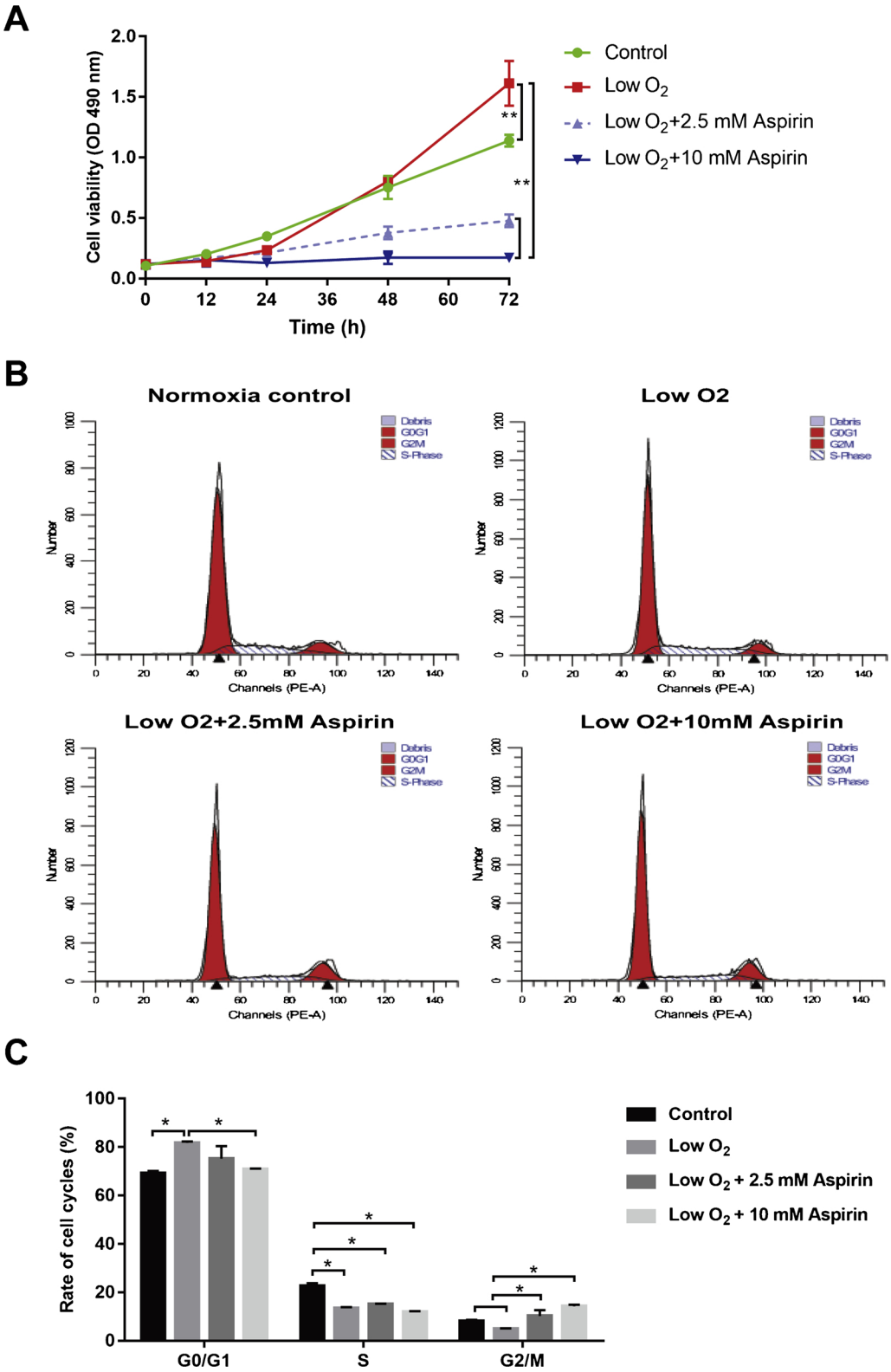

Fig. 1. Effects of aspirin on A549 cell proliferation and cell cycle. (A) Cell viability was detected after 0, 12, 24, 48, or 72 h hypoxic culture by CCK-8 assay. (B) Cell cycle was analyzed by FACS after 24 h of hypoxic culture. The data reported are the representative results of three independent experiments. (C) Quantitative analysis of the cell cycle. The data reported are the representative results of three independent experiments; * indicates $P < 0.05$, ** indicates $P < 0.01$.

2.8. Isolation and analysis of exosomes

A549 cells were cultured in RMPI-1640 supplemented with 10% exosome-depleted FBS for 48 h. Exosomes were extracted using the ExoQuick-TC kit (System Biosciences, Mountain View, CA, USA) according to the manufacturer's protocol. The culture medium was centrifuged at 3000 × *g* for 15 min to remove cells and debris. Then, the supernatants were transferred to new tubes, and the reagents were added. After overnight incubation, the samples were centrifuged at 10,000 × g for 30 min. The exosomes were pelleted and resuspended in

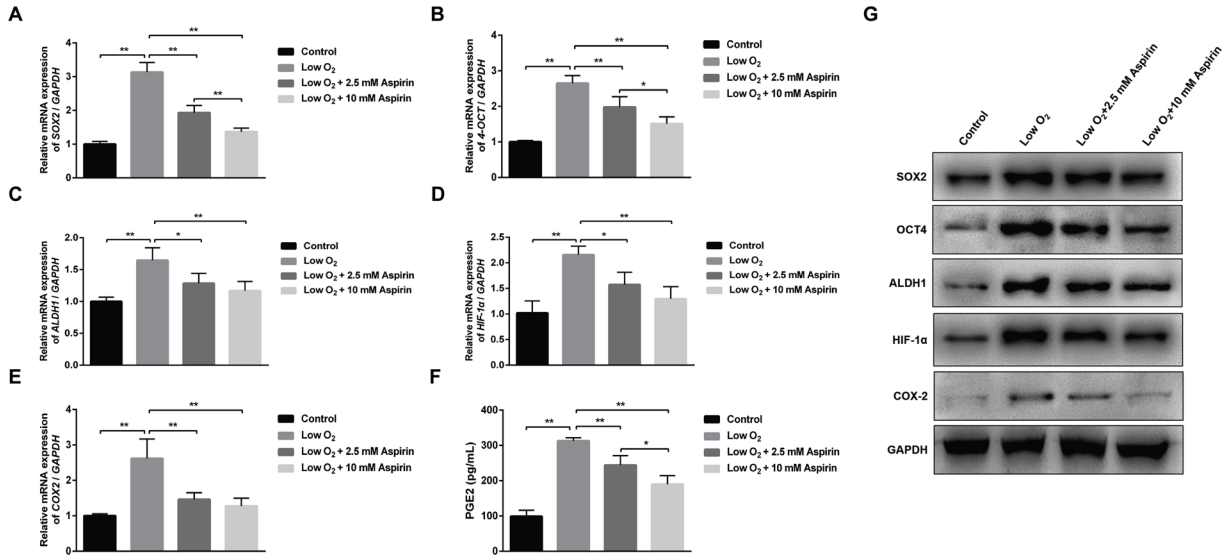

Fig. 2. Aspirin inhibits the hypoxia-enhanced stemness of A549 cells. (A–C) mRNA levels of SOX-2, OCT4, and ALDH1 were determined by qRT–PCR using specific primers, with GAPDH serving as the internal control. (D, E) mRNA levels of HIF-1α and COX-2 were determined by qRT–PCR using specific primers, with GAPDH serving as the internal control. (F) The concentration of PGE2 secreted by A549 cells was determined by ELISA. Each bar indicates the mean ± SD of $n = 3$ experiments. * indicates $P < 0.05$, ** indicates $P < 0.01$. (G) SOX-2, OCT4, ALDH1, HIF-1α, and COX-2 protein levels were determined by Western blot, with GAPDH serving as the loading control.

PBS. The total protein concentration in exosomes was assessed using BCA Protein Assay Kit (KeyGEN Biotech). The number of exosomes from each sample was quantified by the EXOCET exosomes quantification assay kit (System Biosciences). The colorimetric assay at 405 nm was performed along with the standard curve for quantification.

2.9. Transmission electron microscopy (TEM)

Exosomes were isolated and resuspended in 100 µl PBS, and 50 µl of the suspension was placed onto formvar carbon-coated copper grids at room temperature for 20 min. The excess suspension was removed using filter paper. Exosomes were stained with 2% phosphotungstic acid (PTA) at room temperature for 1 min. The grids were fixed with 2% glutaraldehyde at room temperature for 5 min. Images were obtained with a transmission electron microscope (JEM-1230, Jeol Ltd, Japan).

2.10. Transwell migration assay

A549 cells were treated with PBS or different exosomes for 24 h. Cells were collected after 24 h incubation with serum-free medium. 5×10^4 cells were resuspended in serum-free medium and placed in inserts. These inserts were then placed in wells with the serum-containing medium. Cells were cultured for an additional 12 h, and the top cells were removed using a cotton wool swab. The cells on the bottom surface were then fixed with 4% formaldehyde and stained with crystal violet for 30 min. The cells were captured and counted under an inverted microscope (Leica, Germany).

2.11. In vitro angiogenesis experiment

Matrigel (BD Biosciences) was thawed at 4°C overnight, was diluted and layered in a 24-well plate, followed by incubation at 37°C for 2 h to allow polymerization. HUVEC cells (2×10^4 cells/well) were plated onto the Matrigel layer and incubated with exosomes for 24 h. The tube-like structure was visualized and imaged under an inverted microscope (Leica). The number of branches and tube length were quantified by ImageJ software.

2.12. Annexin-V-FITC/PI assay

After aspirin or GW4689 (Sigma-Aldrich, USA) treatment, A549 cells were treated with vehicle or 2 µg/ml cisplatin (DDP) for 24 h. Annexin-V-FITC/PI staining was performed using Annexin-V-FITC/PI Apoptosis Detection Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction. Briefly, cells were harvested and washed twice with PBS and then resuspended in the binding buffer. The 100 µl cell solution (1×10^5 cells) was incubated with 5 µl Annexin-V-FITC for 15 min in darkness, followed by incubation with 5 µl PI for 15 min. The samples were analyzed by flow cytometry (BD Biosciences).

2.13. Statistical analysis

All experiments were repeated at least three times, and data were presented as mean ± SEM. The data were analyzed by one-way analysis of variance (ANOVA). Statistical comparisons between two groups were estimated using Student's t-test. $P < 0.05$ was considered statistically significant. All statistical analyses were calculated by using SPSS 19.0 statistics software (IBM).

3. Results

3.1. Aspirin inhibits tumorigenesis in A549 cells under hypoxic conditions

To evaluate the effects of aspirin on the tumorigenic properties of cells under hypoxic conditions, cell proliferation was monitored by CCK-8 assay. As shown in Fig. 1A, hypoxia-induced transient cell growth arrest within 24 h of treatment followed by rapid cell proliferation in A549 cells. Aspirin significantly inhibited cell growth in a dose-dependent manner compared with that observed in the corresponding control cells (Fig. 1A). Further, we analyzed the cell cycle by flow cytometry (FACS). After 24 h of culture, aspirin remarkably increased the percentage of G2/M phase cells ($P < 0.05$) in comparison with the hypoxia group (Fig. 1B, C). These findings indicate that aspirin inhibits cell growth and induces cell cycle arrest at the G2/M phase in hypoxic A549 cells.

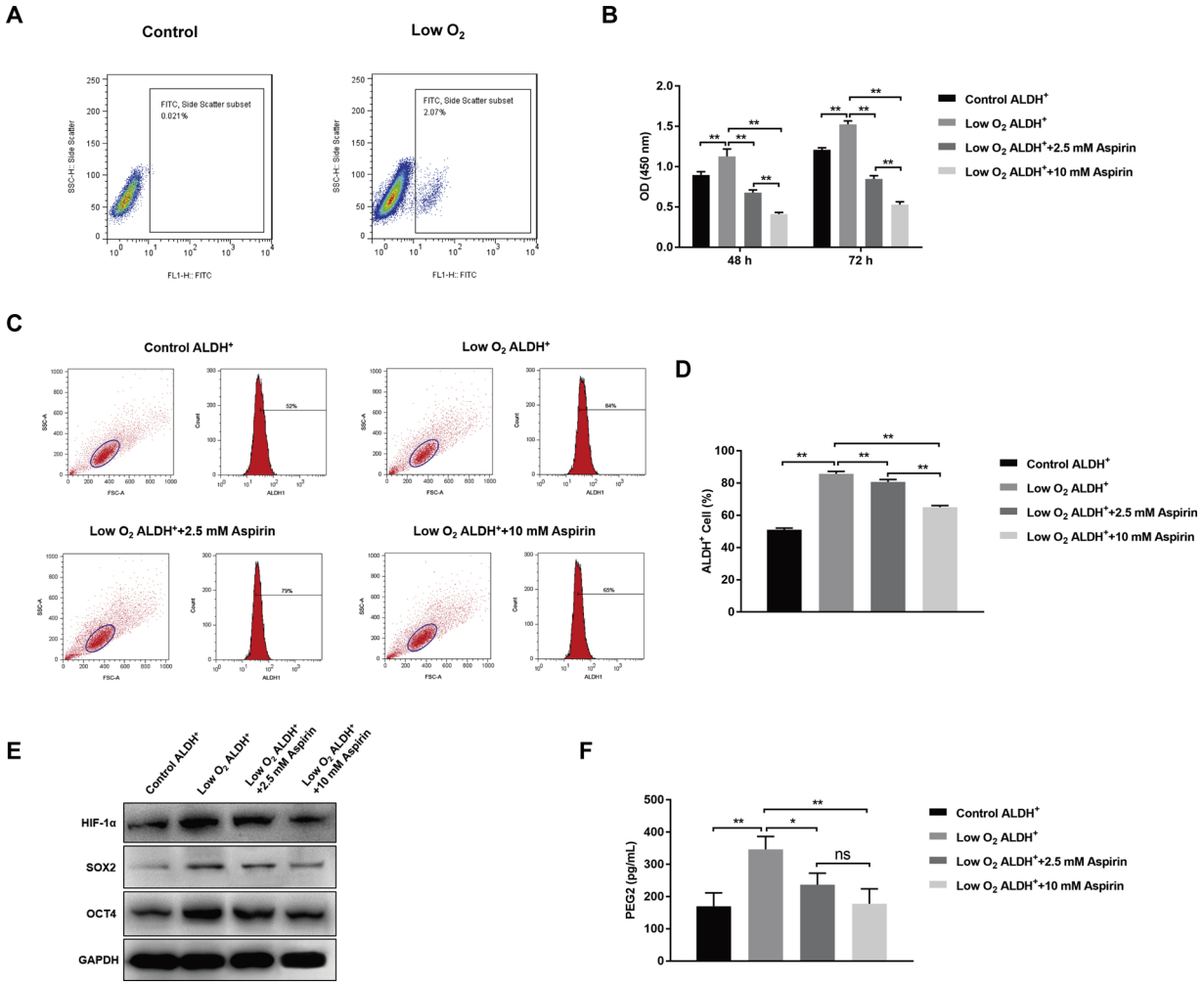

Fig. 3. Aspirin inhibits the hypoxia-mediated stemness of ALDH-positive cells. (A) Representative flow cytometric plots of the analysis of ALDH1 enzymatic activity in A549 cells under normoxic or hypoxic conditions. (B) The second generation of ALDH1$^+$ cells was treated with different conditions of stimulation for 48 or 72 h, and cell viability was detected by CCK-8 assay. (C, D) The proportion of ALDH1$^+$ cells in cell cultures treated under different conditions of stimulation was analyzed by FACS. Representative examples are displayed. (E) OCT4, HIF-1α, and SOX-2 protein levels were determined by Western blot, with GAPDH serving as the loading control. (F) The concentration of PGE2 secreted by ALDH$^+$ cells was determined by ELISA. Each bar indicates the mean ± SD of $n = 3$ experiments; ns indicates non-significant, * indicates $P < 0.05$, ** indicates $P < 0.01$.

3.2. Aspirin inhibits hypoxia-mediated lung cancer cell stemness

Hypoxia is well known to induce the stemness phenotype of various cancer cells, including pancreatic cancer, glioblastoma, and lung cancer [6,19,20]. To investigate the effect of aspirin on the stemness phenotype, we evaluated the levels of stemness-related markers in human non-small-cell lung cancer cell line A549 during hypoxic conditions. As shown in Fig. 2A–C, mRNA levels of SOX2, OCT4, and aldehyde dehydrogenase1 (ALDH1) significantly increased under hypoxia and decreased under pre-treatment with aspirin. Similar results were observed in the protein levels of the corresponding genes, as determined by Western blot (Fig. 2G). To explore the signaling pathway involved in these changes, we examined the expression levels of HIF-1α and COX-2; among these factors, the latter is a known target of aspirin. Hypoxia up-regulated HIF-1α and COX-2 mRNA ($P <$ 0.01) and protein levels when compared with the corresponding levels observed in the control group (Fig. 2D, E, and G). Treatment with aspirin resulted in decreased expression of HIF-1α and COX-2 under hypoxic conditions (Fig. 2D, E, and G). The hypoxia-enhanced secretion of PGE2 into the culture medium was also decreased by aspirin (Fig. 2F). Taken together, the results indicate that aspirin may inhibit hypoxia-mediated stemness via inactivation of HIF-1α/COX-2/PGE2 signaling.

3.3. Aspirin inhibits the stemness of ALDH-positive cells

Over the past few decades, increasing evidence has supported the role of ALDH1 as a reliable marker for the isolation of CSCs [21,22]. Jiang et al. reported that isolated lung cancer cells with high ALDH1 activity display CSC-like features [23]. As hypoxic conditions are responsible for maintaining the stemness of lung CSCs, we investigated the inhibitory effect of aspirin in ALDH$^+$ cells. Aldefluor assay followed by FACS analysis showed 0.02% ALDH1$^+$ cells in control cells and 2.07% ALDH1 + cells in hypoxic A549 cells (Fig. 3A). Therefore, we separated subpopulations in hypoxic A549 cells according to ALDH1 using FACS cell sorting to enrich in vitro cultures of CSCs. ALDH$^+$ A549 cells may undergo asymmetrical division to generate heterogeneous subpopulations. To eliminate this impact, second- and third-generation ALDH$^+$ cells were used in the subsequent experiments. CCK-8 assays illustrated that hypoxia promotes the proliferation of ALDH$^+$ cells whereas aspirin remarkably inhibits cell growth at 48 and 72 h (Fig. 3B). FACS analysis showed hypoxia-induced ALDH$^+$ cell enrichment and impairment of this enrichment by aspirin in a dose-dependent manner (Fig. 3C,D). Western blot consistently revealed that ALDH$^+$ cells express high levels of the stemness-related markers SOX2 and OCT4 under hypoxic conditions but dose-dependent decreases upon

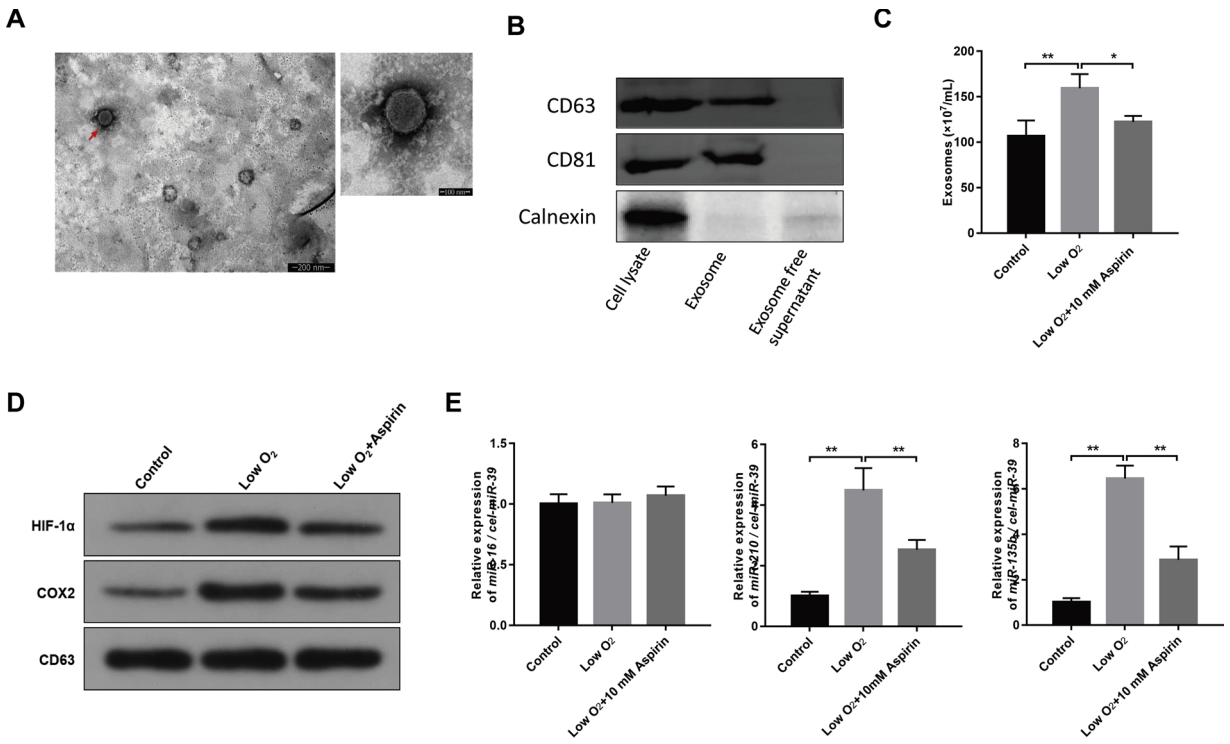

Fig. 4. Aspirin affects the characteristics of exosomes secreted by A549 cells under hypoxic conditions. (A) Transmission electron microscopy of exosomes isolated by the ExoQuick-TC kit. (B) Levels of exosomal proteins CD63 and CD81 and the endoplasmic reticulum marker calnexin were determined by Western blot. The data reported are the representative results of three independent experiments. (C) The number of exosomes from each sample was quantitated by the EXOCET exosome quantification assay kit. (D) Levels of exosomal proteins HIF-1α and COX-2 were determined by Western blot, with CD63 serving as the internal control. The data reported are the representative results of three independent experiments. (E) Exosomal miR-16, miR-135b, and miR-210 were determined by qRT–PCR. Aspirin is used at a concentration of 10 mM. Data were normalized to exogenous cel-miR-39. Each bar represents the mean ± SD of $n = 3$ experiments; ** indicates $P < 0.01$.

aspirin treatment (Fig. 3E). Western blot coupled with ELISA (Fig. 3F) indicated that the hypoxia-mediated activation of HIF-1α/COX-2/PGE2 signaling could be attenuated by aspirin; the second and third generations of $ALDH^+$ cells showed similar results (Supplementary Fig. 1). These findings suggest that aspirin inhibits the hypoxia-mediated maintenance of stemness in $ALDH^+$ cells.

3.4. Aspirin affects the characteristics of exosomes secreted by lung cancer cells under hypoxic conditions

Since cancer-derived exosomes contribute to TME recruitment and reprogramming, we isolated exosomes from the conditioned medium and characterized A549 exosomes by electron microscopy and Western blot. Transmission electron microscopic analysis revealed that the exosomes were smaller than 200 nm (Fig. 4A). CD63 and CD81, which commonly use membrane-bound exosomal markers, were detected in A549 exosomes. To validate the purity of the obtained exosomes, we examined the presence of the endoplasmic reticulum marker calnexin in A549 exosomes. A lack of calnexin expression indicated successful isolation of exosomes from the conditioned medium (Fig. 4B). Exosome quantification analysis showed that hypoxia increased the number of exosomes whereas aspirin reduced exosome numbers in aspirin-treated cells in hypoxia (Fig. 4C).

HIF-1α may be involved in hypoxic enhancement of exosome release in breast cancer cells [26]. We examined the expression of exosomal HIF-1α and its downstream effector COX-2 after aspirin treatment under hypoxic conditions. In Fig. 4D, hypoxia resulted in a significant induction of exosomal HIF-1α and COX-2, and this hypoxia-mediated up-regulation was abrogated by aspirin. To study the effect of aspirin on exosomal miRNAs, miR-135b and miR-210 were selected as potential candidates by their ability to mediate the hypoxic effect in exosomes [24–26]; miR-16 was also chosen as a stable endogenous reference miRNA [24,27]. While levels of exosomal miR-135b and miR-210 were elevated under hypoxic conditions, aspirin remarkably inhibited the expression of these two exosomal miRNAs (Fig. 4E). These findings indicate that aspirin not only reduces hypoxia-enhanced exosome numbers but also changes the content of exosomes from A549 cells.

3.5. Aspirin inhibits the paracrine effect of hypoxic exosomes

Since the cancer-promoting factors (HIF-1a, COX2, miR-135b, and miR-210) are abnormally elevated in hypoxic exosomes, we compared the exocrine effects of exosomes on other A549 cells under different conditions. As shown in Fig. 5A, compared with that in PBS, co-culture with hypoxic exosomes significantly increased cell proliferation after 48 or 72 h of treatment. A decrease in proliferation-promoting effect was observed in A549 cells co-cultured with exosomes secreted by aspirin-treated cells (Fig. 5A). Transwell migration assay showed that normoxic exosomes slightly promote the migration of A549 cells while the presence of hypoxic exosomes significantly increases A549 cell migration. By contrast, the migration-promoting ability of exosomes from aspirin-treated cells was significantly down-regulated (Fig. 5B,C). In vitro angiogenesis experiments showed similar changes in the angiogenesis capacity of HUVEC cells (Fig. 5D–F). Taken together, the results indicate that aspirin attenuates the paracrine-promoting effects of hypoxic exosomes on the proliferation, migration, and angiogenesis of other cells.

To further establish the relationship between proliferation and stemness of A549 cells and exosomes under hypoxic conditions, we used the exosome secretion inhibitor GW4869 for validation experiments. Currently, GW4869, a neutral sphingomyelinase inhibitor, is the most widely used pharmacological agent for blocking exosome generation [28–30]. As shown in Fig. 6A, compared with that in DMSO, co-

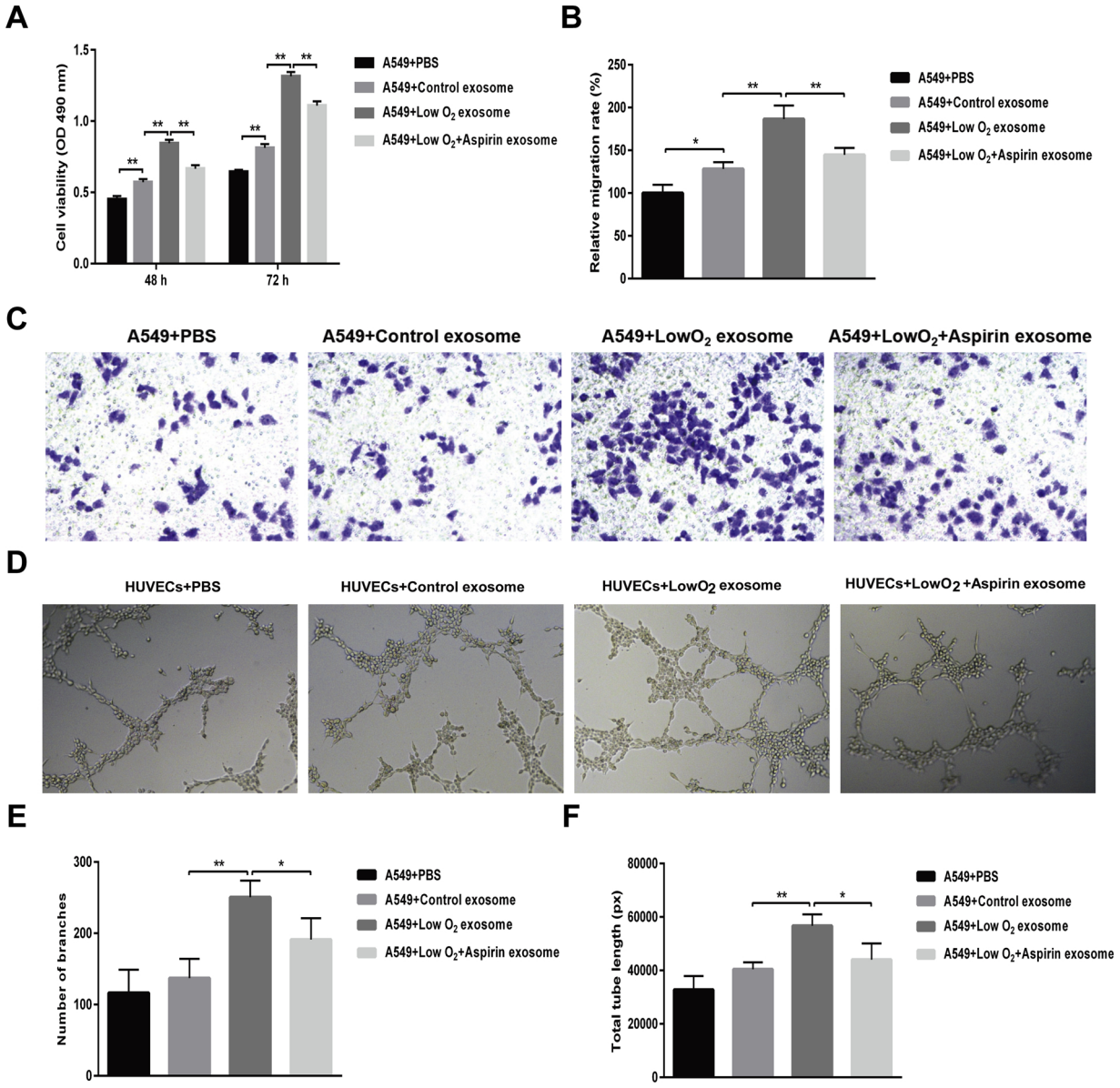

Fig. 5. Exosomes secreted by aspirin-treated cells under hypoxia enhance the proliferation, migration, and angiogenesis of receipt cells. Aspirin is used at a concentration of 10 mM. (A) A549 cells were treated with different exosomes for 48 or 72 h, and cell viability was detected by CCK-8 assay. (B, C) Transwell migration assay to evaluate the effect of exosomes secreted by aspirin-treated cells on A549 cell migration. (D) The effect of different exosomes on tube formation by HUVECs was determined by an in vitro angiogenesis experiment. The data reported are the representative results of three independent experiments. (E, F) Quantitative analysis of the in vitro angiogenesis experiment. Number of branches and tube length were quantified by ImageJ software. Each bar indicates the mean ± SD of $n = 3$ experiments; * indicates $P < 0.05$, ** indicates $P < 0.01$.

culture with GW4869 or Aspirin significantly decreased cell proliferation after 72 h of treatment. However, the combined use of aspirin and GW4869 did not demonstrate additive effects, suggesting that they have a similar mechanism for cell proliferation inhibition. Similarly, GW4869 was also able to reduce the ratio of ALDH + cells in A549 cells under hypoxic conditions (Fig. 6B), suggesting that GW4869 inhibits the hypoxia-induced stemness of A549 cells.

Xia et al. reported that exosomes released by A549 cells exposed to DDP (cis-diamminedichloroplatinum, cisplatin) could communicate with other cells and influence the resistance of these cells to DDP [31]. Since aspirin could change the secretory characteristics of exosome in A549 cells, we further investigated whether aspirin augments DDP-induced cell apoptosis under hypoxic conditions. As expected, hypoxia reduced DDP sensitivity compared with that of the normoxia group, and an enhanced population of apoptotic cells was found in A549 cells treated with aspirin and DDP in hypoxia (Fig. 7A, B). Inhibition of exosome secretion by GW4869, yielded similar results. In addition, we also used the method of co-incubation of exosomes and other cells to detect the effect of exosomes on the efficacy of cisplatin. The results of cell viability assay showed that hypoxia-induced exosomes significantly attenuated the inhibitory effect of cisplatin on the proliferation of A549 cells, while the inhibition effect of cisplatin was significantly reduced in the aspirin-treated exosomes group (Fig. 7C). These data suggest that aspirin and DDP exert a synergistic effect on apoptosis under hypoxic conditions in A549 cells, at least in part, through the exosome pathway.

4. Discussion

Hypoxia is a common feature of the TME of solid tumors due to their rapid expansion and complex vasculature; it is associated with aggressive tumorigenic properties and poor clinical outcomes [32]. Hypoxic induction of gene expression occurs mainly through HIF-1α

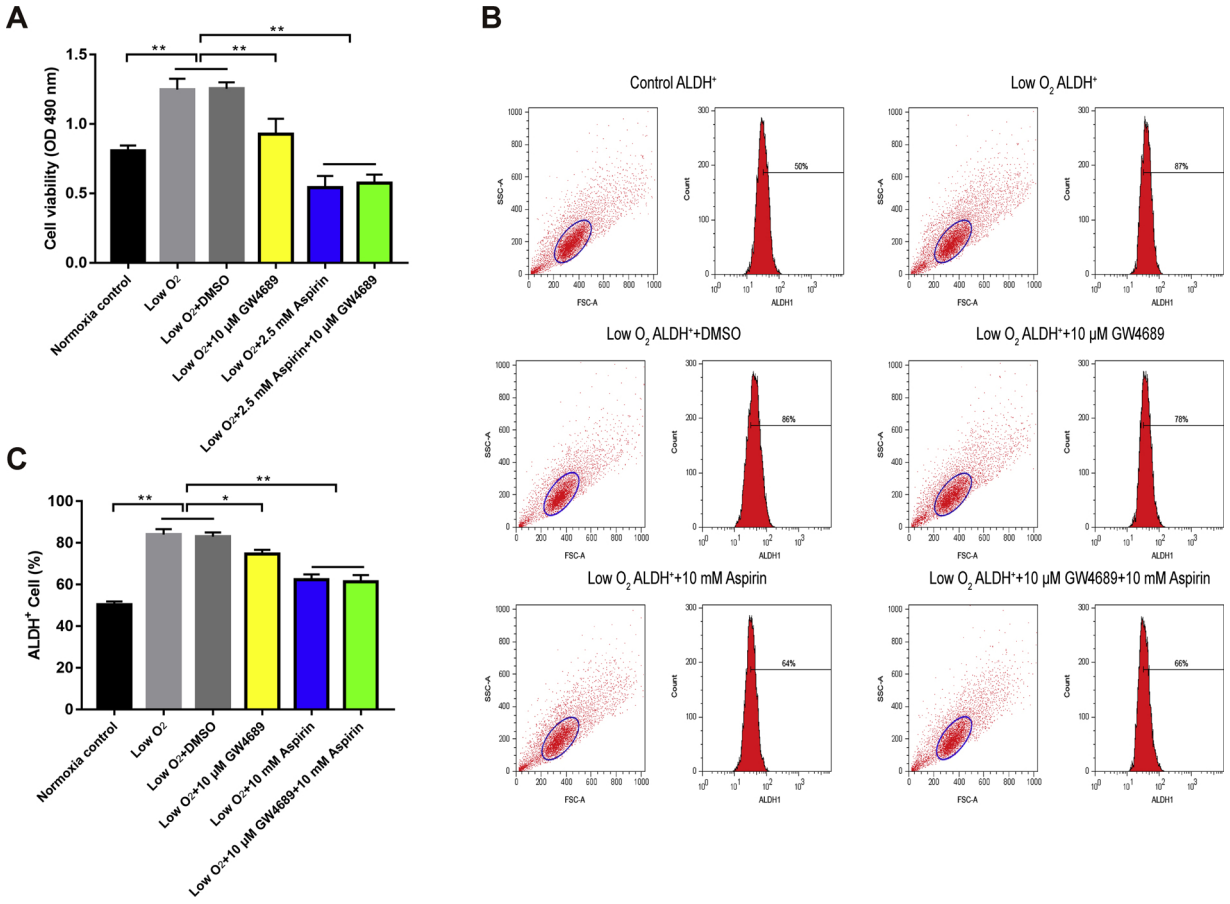

Fig. 6. Correlation between exosome and proliferation and stemness of A549 cells under hypoxia. (A) A549 cells were treated with 10 μM GW4869 or 2.5 mM Aspirinfor 72 h, and cell viability was detected by CCK-8 assay. (B, C) The second generation of ALDH1 + cells was treated with different conditions of stimulation for 72 h, the proportion of ALDH1 + cells in cell cultures was analyzed by FACS. Representative examples are displayed. Each bar indicates the mean ± SD of $n = 3$ experiments; * indicates $P < 0.05$, ** indicates $P < 0.01$.

accumulation. HIF-1α has been reported to induce the transcriptional activation of COX-2 in A549 cells under hypoxic conditions [33,34]. Activation of the COX-2/PGE2 pathway plays an important role in cancer cell survival and tumor angiogenesis [9,34]. Moreover, COX-2 is frequently overexpressed in NSCLC and associated with poor prognosis in NSCLC patients [8]. These findings suggest that COX-2/PGE2 may be an important target of NSCLC therapy.

Aspirin, an NSAID, is also known as a non-selective COX inhibitor [35,36]. The drug was recently reported to inhibit epithelial-mesenchymal transition and migration of highly invasive K-ras-expressing NSCLC cells by targeting Slug [35]. However, the role of aspirin in tumorigenesis under hypoxic conditions remains unknown. Direct inhibition of COX-2 is generally accepted as the main pathway by which aspirin inhibits cancer development [9]. We thus analyzed the function of the COX-2/PGE2 pathway in the anti-tumor effect of aspirin during hypoxia. As expected, aspirin inhibited tumorigenesis and stemness under hypoxic conditions via HIF-1α/COX-2/PGE2 signaling in NSCLC cells. This finding is consistent with a recent study reporting that COX-2 maintains the stemness of nasopharyngeal carcinoma cell lines [37]. Collectively, our findings indicate that aspirin has therapeutic potential for suppressing lung cancer development and stemness.

Emerging evidence indicates that communication within the components of the TME could promote tumorigenesis through the release of exosomes [38,39]. Protein estimation coupled with exosome quantification assay suggests that hypoxia significantly increases exosome protein concentrations and numbers of exosomes in conditioned medium. Hypoxia also resulted in a remarkable increase in exosomal HIF-1α and COX-2 protein levels. These findings are consistent with a recent report demonstrating that hypoxia promotes the release of exosomes in breast cancer cells and that this process may be mediated by HIF-1α [24]. Interestingly, we found that aspirin inhibited the hypoxia-enhanced release of exosomes, which could be partly mediated by the HIF-1α/COX-2 pathway. Exosomes secreted by aspirin-treated cells inhibited hypoxia-enhanced cell proliferation, migration, and angiogenesis in A549 cells in vitro. The data indicate that hypoxic A549 cells release large amounts of exosomes into their TME to promote their own survival, migration, and tube formation and that this process could be partially abrogated by aspirin. Besides increases in exosome number, we also demonstrated that miR-135b and miR-210 are elevated in hypoxic exosomes [24,27] Given the roles of exosomal miR-135b and miR-210 in promoting angiogenesis and metastasis in response to hypoxia [24–26], these exosomal miRNAs may be potential therapeutic targets during hypoxia. Here, we showed that aspirin reduces the expression of exosomal miR-135b and miR-210 under hypoxic conditions. In short, aspirin may inhibit hypoxic enhancement of exosome release via the HIF-1α/COX-2 pathway and target exosomal miR-135b and miR-210 to inhibit cell proliferation, migration, and angiogenesis in NSCLC cells. Our experimental data further suggest that the synergistic effect of aspirin and cisplatin on lung cancer cells may be due to changes in exosome function. These findings provide novel insights into the mechanism of aspirin-inhibited tumorigenesis during hypoxia.

In summary, we provide evidence of the inhibitory effect of aspirin on the stemness of human lung cancer cells and demonstrate that aspirin impedes the exosome secretion and malignant paracrine effect of these cells.

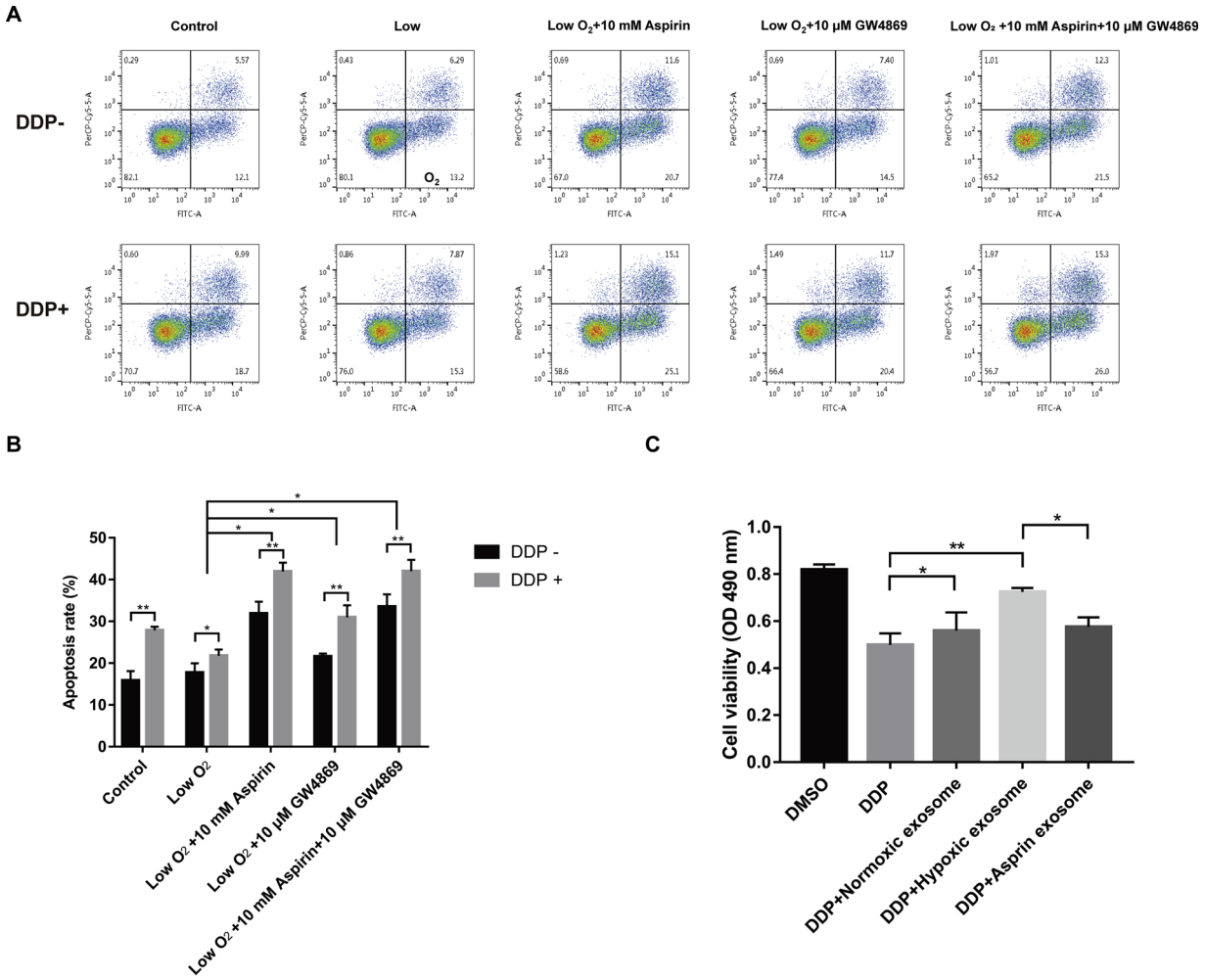

Fig. 7. Synergistic effect of aspirin and cisplatin on lung cancer cells. (A) Apoptosis of A549 cells was quantitatively measured by FACS after 24 h hypoxic culture. Data are representative images of 3 independent experiments. (B) Quantitative analysis of cell apoptotic analysis. Each bar is a mean ± SD of $n = 3$ experiments. (C) A549 cells were treated with cisplatin and different exosomes for 48 h, and cell viability was detected by CCK-8 assay.* indicates $P < 0.05$, ** indicates $P < 0.01$.

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Conflict of Interest

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.03.008.

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