Biomedicine & Pharmacotherapy 82 (2016) 595–605

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

The effect of quercetin nanoparticle on cervical cancer progression by inducing apoptosis, autophagy and anti-proliferation via JAK2 suppression

Cheng-lin Luo[a], Yu-qiong Liu[b], Peng Wang[c], Chun-hua Song[c], Kai-juan Wang[c], Li-ping Dai[c], Jian-ying Zhang[c,*], Hua Ye[c,*]

[a] Department of Biological Sciences, University of Texas at El Paso, 500 West University Avenue, El Paso, TX 79968, USA
[b] Department of Pathology, The First Affiliated Hospital of Zhengzhou University, NO.1 JiansheDong Road, Zhengzhou, Henan 450052, PR China
[c] College of Public Health, Zhengzhou University, No.100 Kexue Avenue, Zhengzhou, Henan 450001, PR China

ARTICLE INFO

Article history:
Received 12 April 2016
Received in revised form 19 May 2016
Accepted 19 May 2016

Keywords:
Cervical cancer
JAK2
Quercetin nanoparticles
Cell death
Proliferation

ABSTRACT

Cervical cancer is a cause of cancer death, making it as the one of the most common cause for death among women globally. Though many studies before have explored a lot for cervical cancer prevention and treatment, there are still a lot far from to know based on the molecular mechanisms. Janus kinase 2 (JAK2) has been reported to play an essential role in the progression of apoptosis, autophagy and proliferation for cells. We loaded gold-quercetin into poly (DL-lactide-*co*-glycolide) nanoparticles to cervical cancer cells due to the propertities of quercetin in ameliorating cellular processes and the easier absorbance of nanoparticles. Here, in our study, quercetin nanoparticles (NQ) were administrated to cells to investigate the underlying mechanism by which the cervical cancer was regulated. First, JAK2-inhibited carvical cancer cell lines were involved for our experiments in vitro and in vivo. Western blotting, quantitative RT-PCR (qRT-PCR), ELISA, Immunohistochemistry, and flow-cytometric analysis were used to determine the key signaling pathway regulated by JAK2 for cervical cancer progression. And the role of quercetin nanoparticles was determined during the process. Data here indicated that JAK2, indeed, expressed highly in cancer cell lines compared to the normal cervical cells. And apoptosis and autophagy were found in JAK2-inhibited cancer cells through activating Caspase-3, and suppressing Cyclin-D1 and mTOR regulated by Signal Transducer and Activator of Transcription (STAT) 3/5 and phosphatidylinositide 3-kinase/protein kinases (PI3K/AKT) signaling pathway. The cervical cancer cells proliferation was inhibited. Further, tumor size and weight were reduced by inhibition of JAK2 in vivo experiments. Notably, administration with quercetin nanoparticles displayed similar role with JAK2 suppression, which could inhibit cervical cancer cells proliferation, invasion and migration. In addition, autophogy and apoptosis were induced, promoting cervical cancer cell death. To our knowledge, it was the first time to evaluate the role of quercetin nanoparticles in improving cervical cancer from apoptosis, autophagy and proliferation, which could be a potential target for future therapeutic approach clinically.

1. Introduction

Cervical cancer is the fourth most common cancer in women, and the seventh overall, with an estimated 528,000 new cases [1]. There were an estimated 266,000 mortalities from cervical cancer worldwide, accounting for 7.5% of all female cancer mortalities [2]. Around 87% of mortalities from cervical cancer occur in less developed regions. Studies on the molecular mechanisms underlying tumor invasion and metastasis as well as the effective therapeutic strategies are very important for ameliorating tumor development [3].

Janus kinase 2, commonly called JAK2, is a non-receptor tyrosine kinase. It is a member of the Janus kinase family and has been implicated in many signaling pathways regulating apoptosis and proliferation in cells [4]. The JAK kinases regulate members of the signal transducers and activators of transcription (STAT) family [5]. Signal transducers and activators of transcription 3 (STAT3) has

* Corresponding authors.
E-mail addresses: zhangjianying450052@139.com (J.-y. Zhang), yehua450052@139.com (H. Ye).

http://dx.doi.org/10.1016/j.biopha.2016.05.029
0753-3322/

been implicated as an oncogene and therapeutic target in a variety of neoplastic diseases, which is associated with JAK2, playing important role in modulating many cancer progression [6]. Activated STAT3 had been indicated to contribute to oncogenesis directly through the up-regulation of genes encoding apoptosis inhibitors and cell-cycle regulators, such as Bcl-xL and Cyclin-D1. In addition, signal transducer and activator of transcription 5 (STAT5) regulates growth, differentiation, and survival of mammary and hematopoietic cells, which is also vital for tumor growth. On the other, JAK2 activation is linked with PI3K activity. The PI3K/AKT signaling pathway is an important part of intracellular signal transduction, cell proliferation, differentiation, apoptosis and migration, which has been implicated in a variety of tumor growth and metastasis. Glycogen synthase kinase (GSK) is an essential protein downstream of Akt that is involved in the pathological process of many tumors [7]. Also, the mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is ubiquitously expressed in mammalian cells [8]. mTOR is activated downstream of multiple distinct growth factor receptors that have been implicated in lung cancer biology. Thus, these reports highlight JAK2 as an essential target for overcoming cervical cancer.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone), an important dietary flavonoid present in red onions, apples, berries, citrus fruits, tea and red wine [9], exhibits antioxidant, anti-inflammatory, anti-obesity and anticancer properties [10]. Quercetin has received increasing attention as a pro-apoptotic flavonoid with specific, and almost exclusive, effects on tumor cells. The size of nanoparticles used in cancer treatment has been known to be effevtive in preventing tumor growth [11]. Quercetin nanpparticles (NQ) are ubiquitous in the environment and are widely used in medical science. Moreover, their effectiveness in cancer treatment has been reported [12]. Due to their unique physicochemical properties, NQ can cross many barriers, such as the blood brain barrier. And exposing cells to NQ can lead to negative effects, as NQ can be toxic and induce cell death, particularly in cancer cells [13]. From previous study, nanoquercetin can significantly improve the solubility and bioavailability of quercetin and can be used as an effective antioxidant for ROS protection within the polar cytoplasm, and the nano-sized quercetin encapsulated by liposomes enhanced the cellular uptake of human cancer cell MCF-7 [14]. Further, nanoquercetin displayed anti-tumor efficacies in hepatocellular carcinoma in vivo and in vitro [15]. Additionally, it also has been reported to inhibit proliferation and cell cycle arrest in Hepatocarcinoma Cells [16]. And nanoquercetin showed inhibitory role in breast cancer progression via apoptosis induction [17]. Therefore, NQ might be useful for cancer therapy, and the ability to induce cell death is advantageous for preventing and controlling cancer proliferation and progression.

However, it was far from to know whether NQ could suppress cervical cancer growth and further study is necessary to underly the possible mechanism by which NQ performs its role in inhibiting cervical cancer development, which might provide effective therapeutic strategy for cervical cancer prevention.

2. Materials and methods

2.1. Cell treatment

Human cervical cancer cell lines, including Caski, Hela and Siha, were obtained from American Type Culture Collection (ATCC, Rockville). As for normal tissue cells from cervical intra epithelial neoplasia (CIN), it was obtained as described as followings. Briefly, Human cervical tissue sections were obtained and digested using type I collagen (0.2% Gibco) in a gas bath thermostatic oscillator (THZ-82B, Jintan Medical Instrument Factory, Jintan, China) at 37 °C and 200 r/min for 40 min in order to dissociate the cells. The cells were seeded in 6-well flat-bottomed culture plastic plates that were coated with rat tail collagen type I (Sigma-Aldrich, USA) and contained modified keratinocyte serum-free medium (K-SFM) supplemented with 5% fetal bovine serum, antibiotics (2.5 μg/ml amphotericin B; 100 IU/ml penicillin; 100 mg/ml streptomycin) at room temperature overnight in a CO_2 incubator. The medium was then replaced with K-SFM on days 5. The unattached cells were recovered. Then all of the cells were grown and maintained in RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin. Further, Caski and Hela cells cells were pretreated with JAK2 inhibitor Cyt387 (JAK2-Inh) for 30 min or treated with low concentration of 10 μg/ml (NQL) and high concentration of 20 μg/ml (NQH) quercetin nanoparticles for 48 h, prepared for the following studies.

2.2. NQ preparation

High performance liquid chromatography (HPLC)-grade quercetin was purchased (>98%, Sigma-Aldrich, USA) in an anhydrous powdered form. Gold nanoparticles (AuNPs) were synthesized by reducing 1 mM gold chloride with a prepared 1 mM quercetin solution (3/7, v/v) in absolute alcohol freshly. The pale-yellow solution turned to deep red as the quercetin nanoparticles were formed. 50 mg Poly (D,L-lactide-*co*-glycolide) (PLGA, Mw 40,000–75,000, Sigma-Aldrich, USA) was added to an aqueous dispersion of AuNPs. Next, we added this mixture drop wise to 20 ml of an aqueous solution with a stabilizer (1% polyoxyethylene-polyoxypropylene; F68). The mixture was stirred at 400 rpm and 4 °C until the organic solvent had evaporated completely. The redundant stabilizer was removed by repeated washing and centrifugation (25,000*g* and 4 °C for 30 min), and the pellet was then resuspended in Milli-Q water. The quercetin nanoparticles were stored at 4 °C for further study.

2.3. Patients

A total of 50 cervical cancer patients from the first affiliated hospital of Zhengzhou University were involved in the present study, between January 2012 and July 2013. Cancer tissues and its adjacent normal cervical tissues were obtained from patients with cervical cancer undergoing surgery. Patients were excluded in our current study if they had received preoperative chemotherapy and/or radiation therapy previously. Peritumoral cervical tissues were obtained from regions >3 cm from the tumor site. Immediately following surgery, tissue samples were fixed in neutral-buffered formalin and embedded in paraffin for following studies. Cervical cancer diagnoses were confirmed via pathological studies and the peritumoral cervical tissue samples were all confirmed to be normal.

2.4. ELISA measurement

The tissue sample was obtained from patients. The concentration of JAK2 was measured using ELISA kits according to the manufacturer's instructions (R&D system, USA).

2.5. Colony formation assays

100 cells per well in 60 mm plates were cultured in 10% FBS DMEM. Cells were treated with Bak of the indicated concentrations for 24 h. After another 7 days of incubation, the cell colonies were washed twice with PBS, fixed with 4% paraformaldehyde for 15 min and then stained by Gimsa for 30 min. Every clones with over 50 cells were evaluated. Clone forming efficiency for cells was calculated based on colonies/number of inoculated cells × 100%.

2.6. Flow cytometry assays

Flow cytometric assay was used to clarify the cell cycle arrest and cells of apoptosis. The cells were collected with trypsinisation and then washed twice with PBS, and fixed in cold 80% ethanol, and finally stored at 4 °C overnight. The cells were washed with PBS twice and RNase A (10 mg/ml) was administrated for analysis. Propidium iodide was then added to tubes at a concentration of 0.05 mg/ml and then incubated for 20 min at 4 °C in the dark. Cell cycle assays were examined with FACSCalibur flow cytometer. FITC-labeled Annexin V/PI staining was applied based on the manufacturer's instructions (Keygen, Nanjing, China). In brief, 1×10^6 cells in each well were suspended with buffer containing FITC-conjugated Annexin V/PI. Samples were then analyzed via flow cytometry.

2.7. Western blot assays

Briefly, cells were washed with cold PBS and lysed in Laemmli buffer for 5 min. Cell lysates were analyzed via SDS/PAGE and transferred to polyvinylidene difluoride membrane electrophoretically. The blots were dealt with specific antibodies with a secondary detection. The proteins were finally revealed by ECL kit. The primary antibodies were shown as follows (1:1000): JAK2, p-JAK2, STAT3, p-STAT3, STAT5, p-STAT5, Bcl-2, Bax, Bad, Cyto-c, Apaf-1, Caspase-3, AKT, p-AKT, PI3K, GSK, p-GSK, Cyclin-D1, mTOR, p-mTOR, 4EBP, eIF4E, p70S6K, S6RP and GAPDH (Abcam and CST).

2.8. RT-qPCR assays

RNA isolated from cells was reversely transcribed and amplified by ONE-STEP reverse transcription-PCR System (Fermentas). Primer sequences applied in the study were indicated in Table 1. Briefly, after heating 1 min at 95 °C, PCRs were exposed to 30 cycles (GAPDH, 25 cycles) of 95 °C for 25 s, 58 °C for 25 s, and 72 °C for 20 s with a final extension at 68 °C for 10 min. The integrated density value of each band was determined by Gel-Pro Image Analyzer (Bio-Rad, Hercules, Calif), and the ratios were calculated.

2.9. Immunofluorescence assays

After induction by conditioned culture medium, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS containing 0.5% BSA (PBS-BSA) for 30 min. The cells were subsequently incubated with ROS for 30 min, followed by labeling with Alexa Fluor 488-conjugated rabbit anti-mouse or goat anti-rabbit IgG antibody. The cells were viewed under a fluorescentmicroscope.

2.10. Establishment of xenograft tumor models

The mouse experiments were conducted in the Animal Laboratory Center. Caski cells (1×10^7cells) treated with or without Cyt387 and 10 μg/ml (NQL) and 20 μg/ml (NQH) NQ were suspended in 100 μl serum free medium and injected subcutaneously into the left flank of 4- to 6-week old male BALB/c nu/nu nude mice. Tumor size was measured with digital caliper and calculated as V = LS2/2 (L is the longest diameter and S is the shortest diameter). Tumor volume and animal weight were measured twice every seven days and at the end of about 5 weeks after treatment, mice were sacrificed. Tumors were excised, weighted, fixed in 10% neutral formalin, and embedded in paraffin for histological analysis.

Table 1
Primer sequences for RT-PCR analysis.

Gene	Forward primers (5′-3′)	Reverse primers (5′-3′)
Bax	CTGTGATCTTTGCTTGTCGC	GAGATGGTTTGGGGCAACTT
Bad	GAGCGCTACTTGCATAAGAGGA	GTATGAGTCACATCGTTCTGCC
Cyto-c	ACCAGAAGAAGAGGTGCCT	CGCGTCAGAGTTTGATTGTG
Apaf-1	CCTGTACGCTTCTGGCTGCGT	GAGCAGCGTGAGTGGCATGG
Cyclin-D1	CACCTCCGCTGCCCTCATCA	GACACAGCAGGCAGGCCAAG
4EBP	AAGCGTCCTTCTTCAAGTGGAG	ATCCTTTGCTCACGTCTTCTCG
eIF4E	TCCGTCTCCTCGCGCATGCAT	AAGCCATAGGCCATCTGCTCTGT
JAK2	CTAGAGCATCAGTGTGAGCGG	GGCCTGTCTCTATGTGCAGGT
GAPDH	CAAGTTCAACGGCACAGTCAAGG	ACATACTCAGCACCAGCATCACC

2.11. Immunohistochemical assays

The xenograft tumors were performed for hematoxylin and eosin staining. In brief, fresh tissues were fixed in paraffin. For the immunohistochemistry, the fresh tumor tissues were fixed in formalin for 48 h. Then the tissue block was put in paraffin and next cut into the desired thickness with a microtome, and was then fixed into a slide. After washing, the sections were prepared for blocking and incubating with antibodies, including KI-67, p-STAT3, Cyclin-D1 and Caspase-3, which were diluted 1:100 in 5% horse serum with PBS at 4 °C overnight. Sections were then incubated with diluted streptavidin-peroxidase HRP conjugates at room temperature by a staining kit, based on the manufacturer's instructions. The sections were then stained with hematoxylin for 3 min and mounted and analyzed under a phase-contrast microscope.

2.12. Transmission electron microscope observation

In order to measure mitochondrial superoxide production, cells were then loaded with 2.5 mM MitoSOX-Red for 20 min in the dark, and washed with Hank's balanced salt solution (HBSS) contained with Ca^{2+} and Mg^{2+}, and then further processed for flow cytometry assays.

2.13. Apoptosis analysis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

Apoptosis assay of samples was determined by TUNEL used an in situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, South San Francisco, CA, USA) according to the manufacturer's protocol. The number of TUNEL-positive cells was counted under a fluorescence microscope. The percentages of apoptotic cells were calculated from the ratio of apoptotic cells to total cells counted. Tissue sections were counter-stained with hematoxylin. Mount and observe sections under light microscopy. The experiment was performed for independently three times.

2.14. Statistical analysis

All data were calculated as means ± standard error of the mean (±SEM) and analyzed by SPSS software (version 17.0, SPSS Inc, Chicago, IL, USA). Groups were compared with Student's *t*-test or one-way analysis of variance (ANOVA), followed by Newman-Keuls post-hoc analysis. A P value of <0.05 was considered statistically significant.

3. Results

3.1. JAK2 was over-expressed in cervical cancer cell lines and was associated with patient survival negatively

In order to analyze the effect of JAK2 on cervical cancer progression, we assessed JAK2 expression in different cell lines. Through western blot assays, we found that JAK2 was highly

activated in cervical cancer cell lines compared to its expression in normal cervical cells of CIN (Fig. 1A and B). And also, RT-qPCR assays were used to explore JAK2 expression from mRNA levels. In consistence with activated JAK2 expression, JAK2 mRNA levels were expressed highly in cervical cancer cell lines (Fig. 1C). In addition, 25 pairs of patients were further involved to measure JAK2 levels and JAK2 expression was significantly higher in the adjacent cervical tissues from the same patient (Fig. 1D). Next, JAK2 protein levels were measured via ELISA kit assays. As it was shown in Fig. 1E, JAK2 expressed highly in cervical cancer tissues compared to the normal adjacent tissue samples. In Fig. 1F, immunochemistry was performed to assess JAK2 expression and JAK2 over-expressed in patient tumor tissues compared to the normal adjacent tissues. In the end, we found that the JAK2 expression in the tumor tissues was negatively related to the patients survival rate with $P = 0.031$ (Fig. 1G). Taken together, theses results indicated that JAK2 over-expression might be needed for cervical cancer tumorigenesis. Therefore, JAK2 high expression was a potential target to be explored as a biomarker for clinical treatment of cervical cancer.

3.2. The effects of JAK2 inhibition on cervical cancer cell migration, invasion and proliferation

Due to the over-expression of JAK2 in cervical cancer based on the data above, then the JAK2 inhibitor was used in cervical cancer cell lines to further confirm the effect of JAK2. First, the colony formation was decreased significantly in Caski and Hela cells with lower JAK2 expression (Fig. 2A–C), suppressing proliferation of cervical cancer cells. Compared to the control group, we found that after the treatment of JAK2 inhibitor, the migrated cells were reduced with great significance in both Caski and Hela cells (Fig. 2D–F). Furthermore, suppression of JAK2 remarkably down-regulated the number of cancer cells, suggesting autophagy could be taken as a survival target to underly the potential molecular mechanisms regulated by JAK2 possibly (Fig. 2G and H).

To indicate the mechanism suggesting the growth inhibition of cervical cancer cells via JAK2 suppression, next we investigated cell cycle profile via flow cytometry (Fig. 3A). We found that JAK2 inhibition led to G2/M arrest, which reached to the peak at 96 h in both Caski (Fig. 3B) and Hela cells (Fig. 3C). Collectively, here we suggested that inhibition of JAK2 might be a potential target for suppressing proliferation, migration as well as inducing autophagy to improve cervical cancer progression.

3.3. Quercetin nanoparticle inhibited cervical cancer cell proliferation, migration and invasion

Quercetin has been known as an effective regent extracted from natural palnts, displaying roles in many cellular processes, including inflammation, oxidative stress, and apoptosis [18]. Nanoparticles of quercetin is more easier to be absored by cells

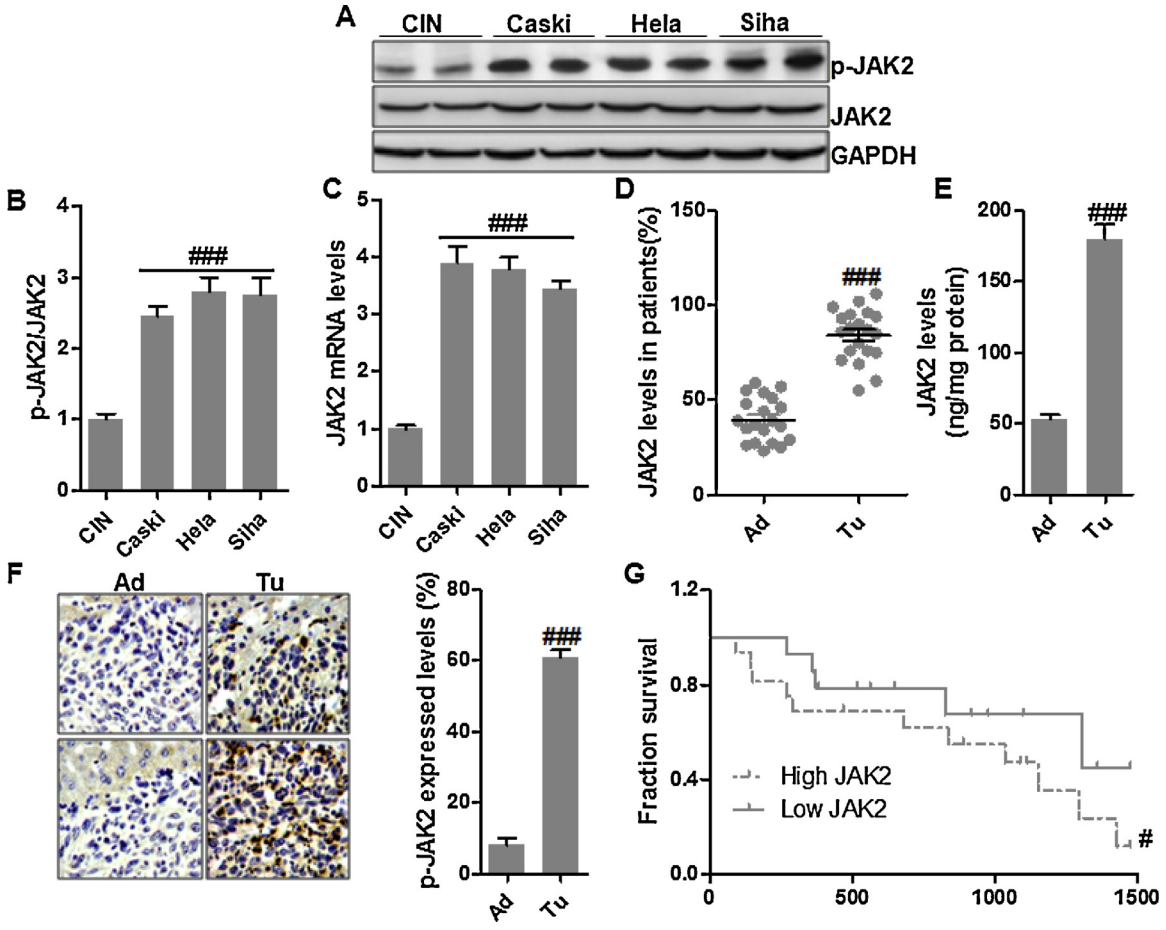

Fig. 1. JAK2 was over-expressed in cervical cancer cell lines and was associated with patient survival negatively. (A) Relative protein levels in different cervical cancer cell lines of JAK2 and p-JAK2 via western blot assays; (B) The evaluation of JAK2 protein expressed levels based on western blot assays; (C) Relative mRNA expression of JAK2 in in different cervical cancer cell lines via RT-PCR; (D) Quantification of JAK2 expression in cervical cancer tissues and adjacent cervical tissues of patients; (E) JAK2 expressed levels in serum of 15 pairs of normal persons and patients; (F) JAK2 positive cells in cervical cancer tissues and normally adjacent cervical tissues; (G) The relationship of JAK2 expression status with patient survival. Data were presented as mean ± S.E.M. with three independent experiments. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$.

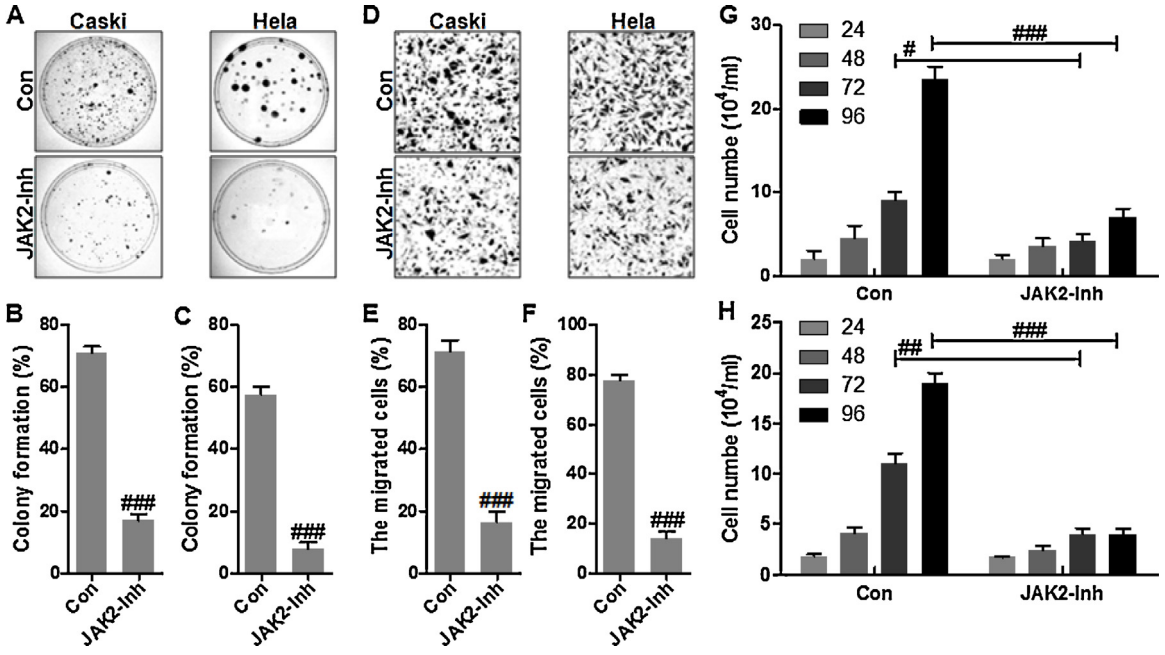

Fig. 2. The effects of JAK2 inhibition on cervical cancer cell migration and invasion. The effect of JAK2-inhibition on colony formation of Caski (A,B) and (A,C) Hela cells respectively; Transwell assay was performed to analyze the migrating cells by examining the number of (D,E) Caski cells and (D,F) Hela cells from the upper chamber to the lower membrane; (G) and (H) were cell proliferation assays of Caski and Hela cells, respectively. Data were presented as mean ± S.E.M. with three independent experiments. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus the control group.

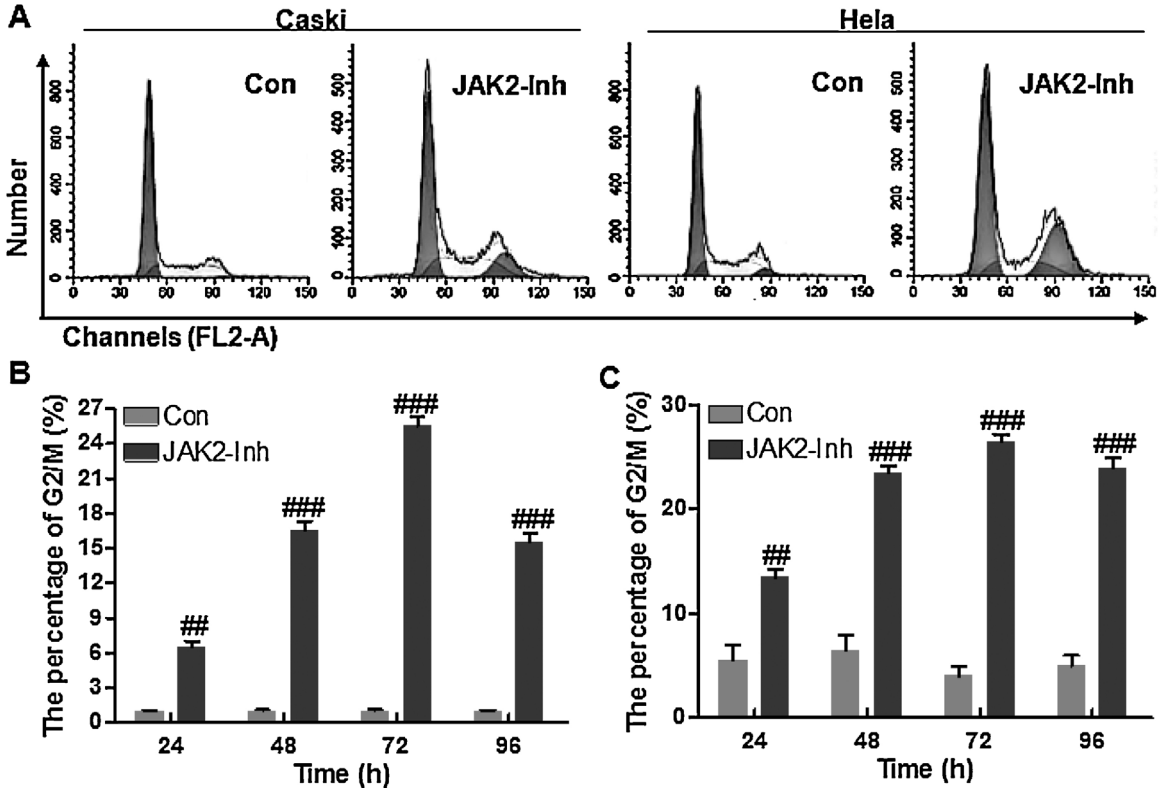

Fig. 3. The effects of JAK2 inhibition on cervical cancer cell proliferation. (A) JAK2-inhibition induced G2/M cell cycle arrest in Caski and Hela cells respectively via assays of PI staining and FASC; (B) and (C) The calculation of G2/M cell cycle arrest based on flow cytometry assays of Caski and Hela cells, respectively. Data were presented as mean ± S.E.M. with three independent experiments. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus the control group.

or tissues [19]. In this part, cervical cancer cell of Caski was conducted to continue our study. Here, in Fig. 4A, we found that apparent double-membrane autophagosome, vacuoles with engulfed bulk cytoplasm as well as cytoplasmic organelles in JAK-Inh group and NQL as well as NQH groups, but not observed in the normal cervical cancer cells, which were hallmark of autophagy. Also, the colony formation was conducted to investigate whether quercetin nanoparticle has any effects on cervical

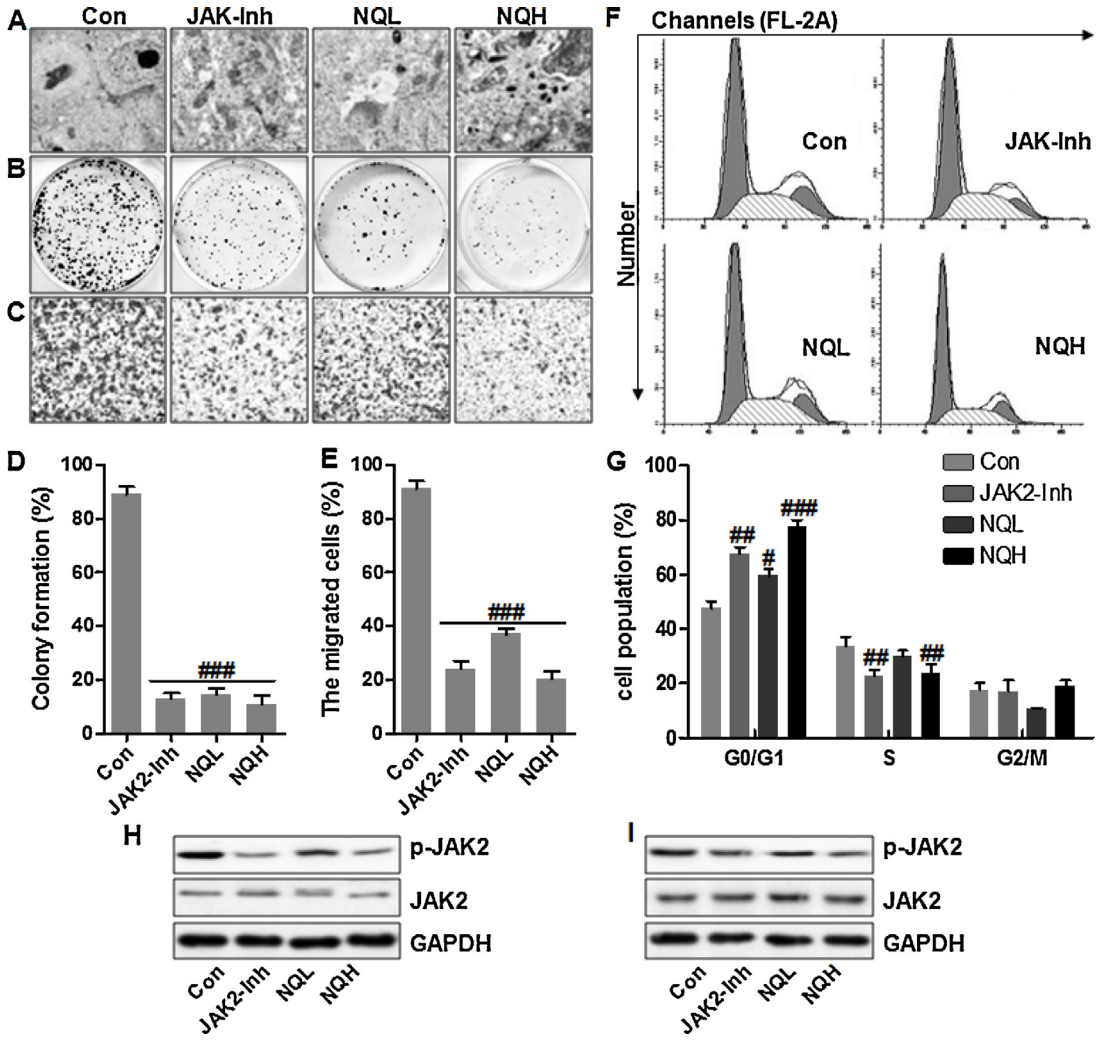

Fig. 4. Quercetin nanoparticle inhibited cervical cancer cell proliferation, migration and invasion. (A) Images of autophagosome formation in cervical cancer cell lines by transmission electron microscopy (TEM); (B) The effect of quercetin nanoparticle on colony formation of Caski cancer cells; (C) Transwell assay was performed to analyze the migrating Caski cancer cells; (D) The evaluation of colony formation; (E) The percentage of migrated Caski cancer cells was calculated; (F) Cell cycle analysis of quercetin nanoparticle-trteated Caski cancer cells; (G) The histogram of quercetin nanoparticle and JAK2-inhibition increased G0/G1-phase population compared with the control group in Caski cells. (H) and (I) were p-JAK2 protein expressed levels via western blot analysis in Caski and Hela cells respectively. Data were presented as mean ± S.E.M. with three independent experiments. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus the control group.

cancer cell proliferation. As shown in Fig. B and D, the colony formation was higher in the control group compared to that in the JAK2-Inh and NQL and NQH group, suggesting that treatment with quercetin nanoparticle could suppress cervical cancer cell proliferation in both two concentrations. In addition, the migrated cells were evaluated. From Fig. 4C and E, JAK2 inhibition could reduce the number of migrated cervical cancer cells, which was consistent with previous results. And notably, after administration with quercetin nanoparticle, the migrated Caski cells were also down-regulated in comparison to the control group in a dose-dependent manner. Next, in order to clarify the underlying mechanisms by which quercetin nanoparticle in inhibiting cervical cancer, the cell cycle distribution of cancer cells was determined, indicating that quercetin nanoparticle resulted in up-regulation of cells in G0/G1 phase during cell cycle, and at the same time lower cells in S phase was observed in cells with lower JAK2 expression and in cells treated with quercetin nanoparticle in both two concentrations (Fig. 4F and G), which elucidated that knockout of JAK2 led to cell cycle arrest during G0/G1 phrase. And in this part, finally we determined the effects of quercetin nanoparticle on JAK2 expression. As shown in Fig. 4H and I, JAK2 activation was down-regulated significantly due to quercetin nanoparticle administration in both two cervical cancer cell lines. The results above indicated that quercetin nanoparticle could inhibit cervical cancer cell proliferation and migration, which might be related with its role in JAK2 suppression.

3.4. The effects of quercetin nanoparticle on apoptosis, autophagy and proliferation in cervical cancer cells

In this regard, on the one, we determined how induction of apoptosis by quercetin nanoparticle to ameliorate cervical cancer progression and whether it was associated with JAK2 activation. Flow cytometry was used here to show that quercetin nanoparticle could significantly increase the percentage of apoptotic cancer cells compared with the control group both in the two concentrations (Fig. 5A and B). And the results of 20 μM quercetin nanoparticle displayed similar inhibited effects with JAK2-inhibition. Further, the higher TUNEL positive cells were observed in JAK2-inh group. And also, treatment with quercetin nanoparticle stimulated the TUNEL positive cells, suggesting apoptosis occurrence in tumor cells with quercetin nanoparticle administration, which was likely to be linked with lower JAK2 expressions (Fig. 5C and D). Moreover, our results suggested that quercetin

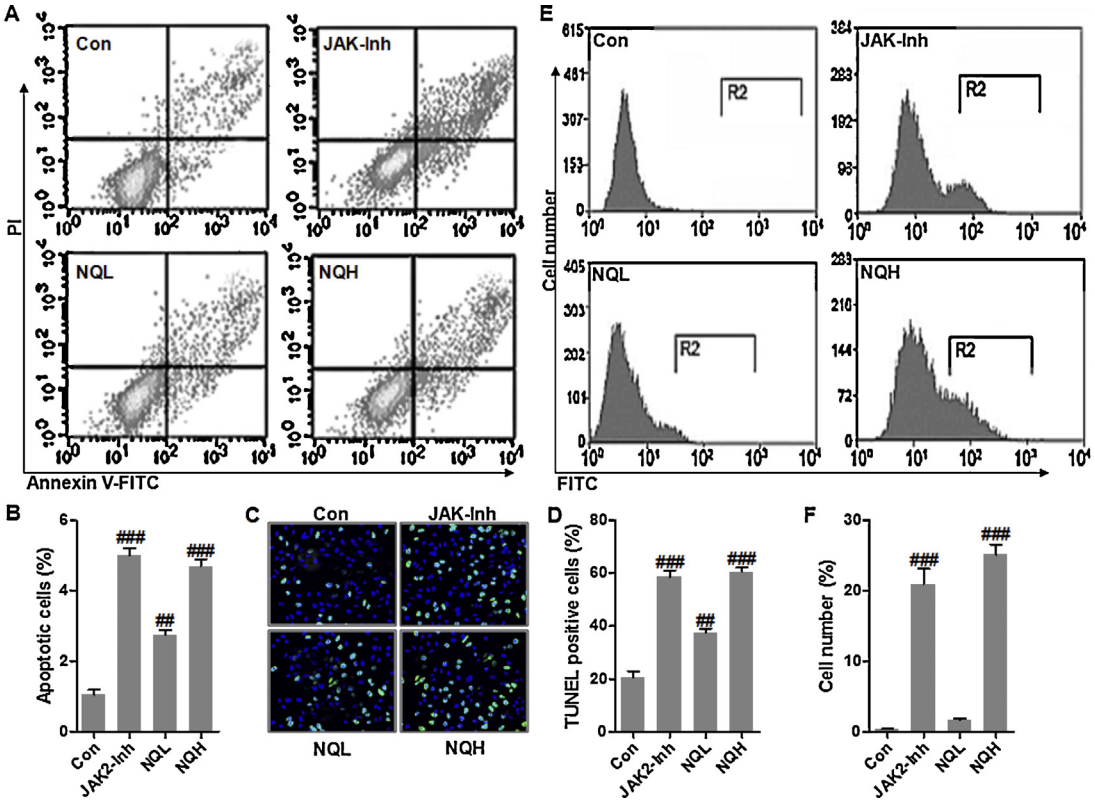

Fig. 5. Quercetin nanoparticle inhibited cervical cancer progression via apoptosis induction. (A) Annexin V-FITC/PI was used to determine the Caski cancer cells apoptosis via flow cytometry; (B) The histogram of aoptotic cells based on flow cytometry assay; (C) TUNEL positive cells were measured by immunofluorescence in Caski cancer cells; (D) The histogram of TUNEL positive cells based on immunofluorescence assay; (E) Caspase-3 activation was analyzed via caspGLOW fluorescein active Caspase-3 staining kit by FACS assays; (F) The evaluation of Caski cells expressed Caspase-3 in different groups. Data were presented as mean ± S.E.M. with three independent experiments. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus the control group.

nanoparticle enhanced Caspase-3 activation significantly compared with the control group by FACS analysis (Fig. 5E and F). Together, the data above suggested that quercetin nanoparticle could inhibit cervical cancer progression via apoptosis induction.

Next, the possible molecular mechanism by which quercetin nanoparticle suppressed cervical cancer growth was investigated. Here, immunoinfluorecence assay was conducted to determine Caspase-3 activity. As shown in Fig. 6A and B, Caspase-3 expressed highly in JAK2-Inh, NQL and NQH groups in comparison to the normal control group, suggesting that apoptosis could be induced by quercetin nanoparticle via JAK2 suppression. Next, we investigated the molecular mechanism by which quercetin nanoparticle via inhibiting JAK2 expression could be effective in cervical cancer suppression. As shown in Fig. 6C, STAT5 activation was determined by western blot. JAK2 inhibition and quercetin nanoparticle inactivated STAT5 obviously. Previous studies have suggested that STAT5 could alter anti-apoptotic gene Bcl-2 expression, affecting apoptosis in cells. And accordingly, Bax and Bad, as pro-apoptotic genes, are altered based on Bcl-2 expressed levels. Subsequently, Cyto-c and Apaf-1 are changed, leading to Caspase-3 activity. In Fig. 6C, we found down-regulation of STAT5 by JAK2-inhibition and quercetin nanoparticle administration, inactivating Bcl-2 expression. Inhibited Bcl-2 activity enhanced Bax, Bad, Cyto-c and Apaf-1 activation. Finally, cleaved Caspase-3 was stimulated, resulting in apoptosis in cervical cancer cells. In addition, JAK2 alteration affects PI3K/AKT signaling pathway, which will influence autophagy and proliferation in the end by Cyclin-D1. As shown in Fig. 6D, we found that PI3K was reduced for JAK2 inhibition and quercetin nanoparticle treatment. Thus, AKT was inactivated, leading to phosphorylated GSK high expression and down-regulation of Cyclin-D1, which slowed down proliferation of cervical cancer cells.

As it was shown in Fig. 6E, AKT-regulated mTOR signaling pathway was determined by western blot, and we found that mTOR activation was down-regulated for high expression of TSC. With the down-regulation mTOR activation, enhancement of 4EBP was observed in JAK2 inhibitor-, NQL- and NQH-treated cervical cancer cells, which then reduced eIF4E activation, attributing to proliferation inhibition. In addition, p70S6K and S6RP, as important factors leading to proliferation in cells, were also reduced significantly in JAK2 suppression and quercetin nanoparticle-treated Caski cancer cells compared to the control group (Fig. 6E). The results above significantly suggested that quercetin nanoparticle could inhibit cervical cancer progression via suppression of JAK2-regulated apoptotic signaling pathway, which could be a potential therapeutic strategy inhibiting the cervical cancer development.

3.5. Quercetin nanoparticle suppressed tumor xenograft growth and development in vivo experiments

In this part, the human cervical cancer Caski tumor xenograft models were performed to demonstrate the effects of quercetin nanoparticle on inhibiting tumor growth. Compared to the control group, quercetin nanoparticle in two concentrations significantly inhibited tumor growth and size for five weeks, and also in the JAK2-Inh group, similar result was observed (Fig. 7A and B). Further, the tumor weight was also estimated. Decreased tumor weight was observed for the JAK2 inhibition and quercetin nanoparticle treatment in the end of the experiments in vivo

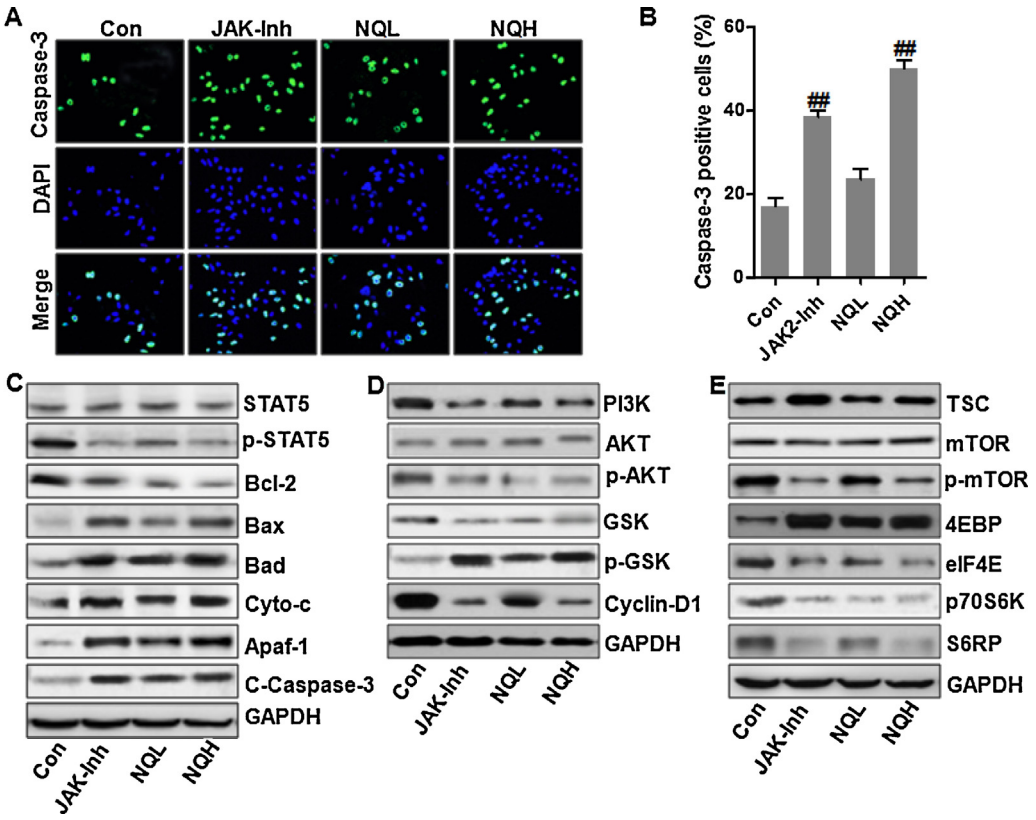

Fig. 6. The effects of quercetin nanoparticle on apoptosis, autophagy and proliferation in cervical cancer cells. (A) Caspase-3 positive cells were measured via immunofluorescence in cervical cancer cells treated by JAK2-inhibition and different concentrations of quercetin nanoparticle; (B) The histogram of Caspase-3 levels in Caski cells; (C) Western blot assays of quercetin nanoparticle induced apoptosis through STAT5/Bcl-2-induced Caspase-3 activation; (D) Western blot assays of quercetin nanoparticle induced autophagy via PI3 K/AKT-related signaling pathway; (E) The protein levels of mTOR-related signaling pathway affecting cell proliferation. Data were presented as mean ± S.E.M. with three independent experiments. $^{\#\#}P < 0.01$ versus the control group.

(Fig. 7C). In addition, we specially determined the KI-67, Cyclin-D1, p-STAT3 and Caspase-3 expression via immunohistochemistry. Interestingly, we found that higher numbers of KI-67, Cyclin-D1, and p-STAT3-positive cells in tumor samples (Fig. 7D–G), which were in consistent with the former results in vitro experiments. In addition, Caspase-3 was assessed, and it was stimulated with high number by JAK2 inhibition and quercetin nanoparticle stimulation (Fig. 7D and H) with significant difference in comparison to the control group, resulting in apoptosis to block cervical cancer growth. Collectively, the results above further confirmed our supposing that quercetin nanoparticle could, at least partly, suppress cervical cancer progression through apoptosis- and proliferation-related signaling pathway through JAK2 inhibition.

3.6. Quercetin nanoparticle inhibited cervical cancer growth via inducing activation of apoptosis- and inhibiting proliferation-related signaling pathway

To examine whether quercetin nanoparticle could block tumor growth through apoptosis and autophagy, western blot analysis was performed and we found that, in accordance with experiments in vivo, we found lower expressed levels of p-STAT5, Bcl-2, and higher cleaved-Caspase-3 expressions for JAK2 suppression and quercetin nanoparticle administration (Fig. 8A). Also, PI3K/AKT-regulated GSK and mTOR signaling pathway were also found to be up-regulated in the control group notably (Fig. 8B). However, inhibiting JAK2 activity or administrated with quercetin nanoparticle could reverse this abnormal expression of GSK and mTOR. Additionally, many other genes, attributing to apoptosis and inhibition of proliferation, including Bax, Bad, Cyto-c, Apaf-1, Cyclin-D1, 4EBP, eIF4E, p70S6K and S6RP were measured via RT-PCR in different groups of JAK2-Inh, NQL and NQH (Fig. 8C). The results showed that JAK2 inhibition and quercetin nanoparticle treatment could stimulate apoptosis-related genes expression, such as Bax, Bad, Cyto-c and Apaf-1. Contrastly, Cyclin-D1, 4EBP, eIF4E, p70S6K and S6RP, which are genes promoting cell proliferation, were found to be inhibited by JAK2 suppression and quercetin nanoparticle administration. Further, autophagy was determined by transmission electron microscopy. It was notable that the cell morphology was damaged highly in JAK2-Inh, NQL and NQH groups, inhibiting proliferation of cervical cancer cells (Fig. 8D). In the part, LC3 was measured finally, which was a marker of autophagy, and it was expressed significantly after JAK2 inhibition and quercetin nanoparticle treatment in cervical cancer tissues (Fig. 8E). Thus, here our results suggested the potential role of JAK2 in promoting cervical tumor growth. And quercetin nanoparticle might be an effective therapeutic strategy in clinical treatment of cervical cancer via suppressing JAK2 activation.

4. Discussion

Cervical cancer is the second most common malignant tumor in female in the world. With the improvement in diagnostic technology and medical treatment, the outcome of patients with cervical cancer has been significantly improved [1,20]. However, more strategies are needed to be explored to ameliorate cervical cancer across the world. Dietary flavonoid quercetin is known to promote optimal health, partly via its anti-oxidation effect against reactive oxygen species, and anti-inflammatory response in tissue injury [21]. In addition, it could improve cell or tissue activity via

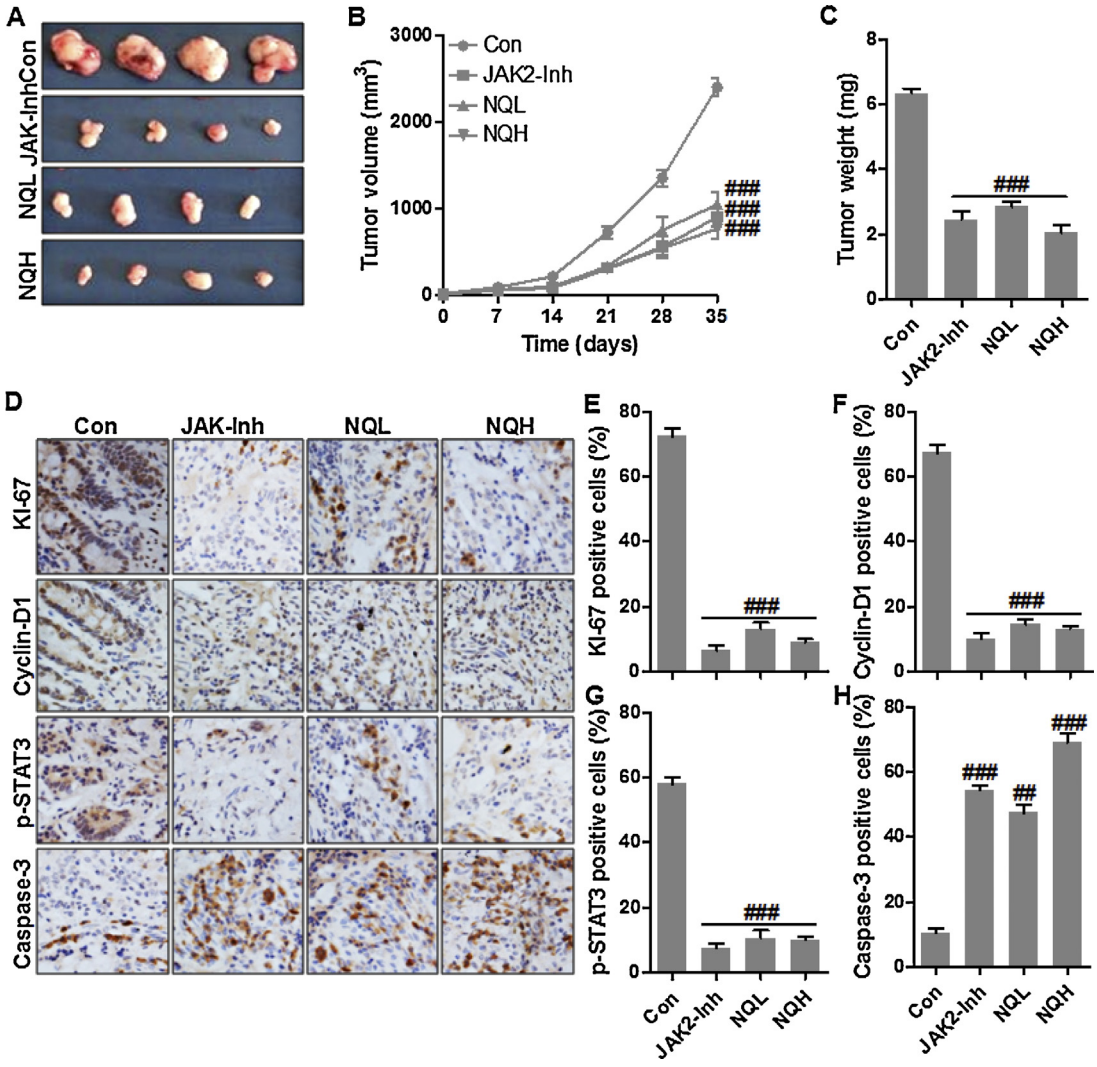

Fig. 7. Quercetin nanoparticle suppressed tumor xenograft growth and development in vivo experiments. (A) Nude mice were injected with Caski cells treated with or without JAK2-inhibition and quercetin nanoparticle. Tumor size was photographed at day 35; (B) Tumor volume was measured twice per week; (C) Tumor weight was measured in the end of the experiments. The mice were sacrificed finally to demonstrate the effects of quercetin nanoparticle on tumor inhibition. (D) The number of KI-67, Cyclin-D1, p-STAT3 and Caspase-3 positive cells was assessed via immunochemistry; The evaluation of (E) KI-67, (F) Cyclin-D1, (G) p-STAT3 and (H) Caspase-3 positive cells based on the immunochemical assays. Data were presented as mean ± S.E.M. with three independent experiments. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus the control group.

apoptotic regulation. And quercetin has received increasing attention as a pro-apoptotic flavonoid with specific, and almost exclusive, effects on tumor cells [22]. Due to the potential effects of quercetin on suppressing cancer progression, it was involved here to illustrate the possible mechanism by which cervical cancer was inhibited. Further, studies before have suggested that nanoparticulate drug delivery systems have been known to better the bioavailability of drugs on intranasal administration compared with only drug solutions [23]. In addition, quercetin nanoparticle has been investigated, which was desirable in systematic use, including targeting tumors as well as inflammatory sites [24]. However, to our knowledge, it was the first time quercetin nanoparticle was used in inhibiting cervical cancer progression.

In our study, first we found that JAK2 inhibition could be effective in suppressing cervical cancer progression, which could be a potential target for cervical cancer treatment. Also, previous studies have suggested that JAK2 regulates many signaling pathways that modulate cell apoptosis, autophagy and proliferation [25,26]. And studies before suggested that JAK2 expressed highly in many tumors, and inhibiting JAK2 signaling pathway could suppress lung cancer cells and bladder tumor growth, indicating its role in cancer progression [27]. And our data also indicated that quercetin nanoparticle could significantly suppress cervical cancer development from inhibiting JAK2 activation. As shown in our results, quercetin nanoparticle ameliorated cervical cancer growth via inhibition of cancer cells invasion, migration and proliferation, which was similar to the results of JAK2 inhibition.

JAK2 has been well known to activate STATs, inducing inflammation, proliferation and invasion as well as migration in cells [28]. Constitutive activation of STAT3, resulting in an unregulated increase in cell proliferation and reduction in cell apoptosis, is strongly correlated with the development of numerous types of cancer [29,30]. Therefore, inhibiting cell proliferation and promoting apoptosis by the suppression of STAT3 activation has been a major focus in the development of anticancer drugs. In our study, we found that JAK2 knockout inactivate STAT3 and STAT5 activity. Subsequently, anti-apoptotic gene Bcl-2 was down-regulated significantly. In the end, pro-apoptotic gene Bax and Bad were promoted, contributing to Cyto-c and Apaf-1 activity, which led to Caspase-3 up-regulation. Caspase-3 is a hallmarker for induction of apoptosis [31]. Western blot and immunofluorescence assays suggested that Caspase-3

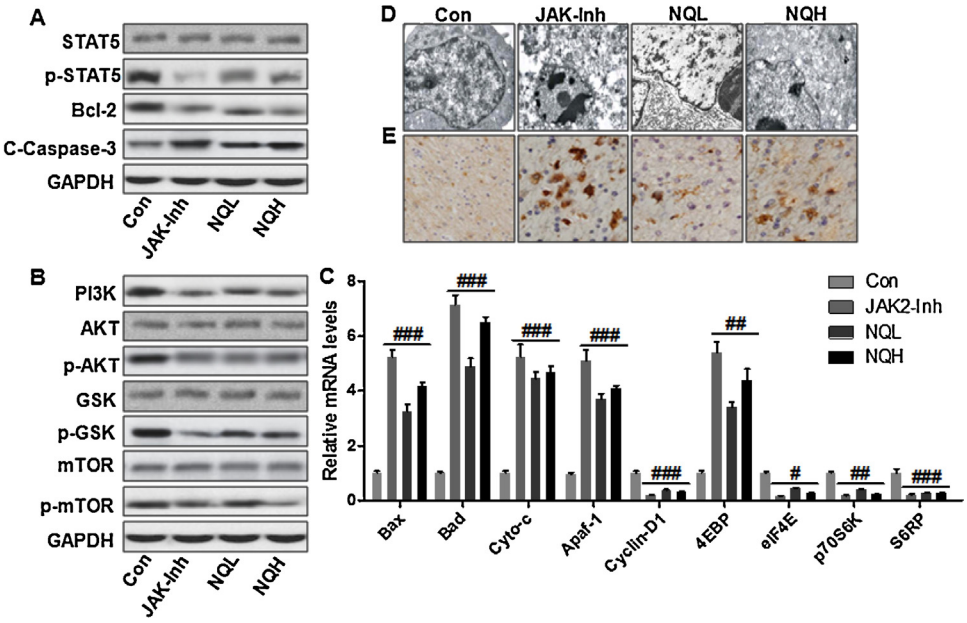

Fig. 8. Quercetin nanoparticle inhibited cervical cancer growth via inducing activation of apoptosis- and inhibiting proliferation-related signaling pathway. (A) Western blot assays of apoptosis signaling pathway; (B) Western blot analysis on cell proliferation; (C) RT-qPCR analysis of apoptosis- and autophagy-associated signals in tumor tissues injected with Caski cancer cells with or without JAK2-inhibition and quercetin nanoparticle treatment; (D) Images of autophagosome formation in tumor tissues by transmission electron microscopy (TEM) in different groups; (E) Autophagy marker of LM3 expressed highly in tumor tissues with JAK2 inhibition and quercetin nanoparticle administration compared to the control group. Data were presented as mean ± S.E.M. with three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus the control group.

was stimulated in our study due to JAK2 inhibition and notably, quercetin nanoparticle displayed similar role in stimulating Caspase-3, which might be related to its effects on JAK2 suppression. And the data here was consistent with previous studies, further confirming that quercetin nanoparticle might performed its role in controlling tumor growth by apoptotic induction via STATs-regulated Bcl-2 signaling pathway regulated by JAK2. Further, quercetin nanoparticle promoted cell cycle arrest in G0/G1 phase, suppressed tumor development and growth, and induced apoptosis, supporting the importance of quercetin nanoparticle in impeding cervical cancer.

Moreover, we found that quercetin nanoparticle could reduce PI3K activation, which was a key cause resulting in AKT inactivity. Thus, with the down-regulation of PI3K levels, AKT was also reduced. The AKT pathway regulates diverse cellular processes, including cell proliferation, differentiation, apoptosis and tumorigenesis [32,33]. Activated AKT inhibits GSK activity, triggering Cyclin-D1 expression, which plays a crucial role in cell proliferation in a variety of cancer cells, such as breast cancer and ovarian cancer cells [34–36]. In accordance with our supposing, the data here indicated suppressed Cyclin-D1 activation, and then the liver cancer cell proliferation was inhibited, which illustrated that quercetin nanoparticle was able to block liver cancer progression via limiting cell proliferation. The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is ubiquitously expressed in mammalian cells. mTOR has been well known to be associated with tumor development in many cancers [37,38]. And AKT/mTOR signaling pathway was involved in a variety of tumor types [39]. Similarly, our study suggested that mTOR was down-regulated for quercetin nanoparticle. Notably, decreased mTOR activation enhanced 4EBP and then eIF4E was found to be reduced via western blot and RT-PCR assays, which were a main reason for cell autophagy induction. And also, mTOR could directly promote proliferation of liver cancer cells via p70S6K and S6RP stimulation. JAK2 knockout inactivated mTOR activity, and accordingly reduced p70S6K and S6RP expression, leading to proliferation inhibition of liver cancer cells. Similarly, autophagy in liver cancer cells was also determined in our study. In addition, LC3 was reported to be an essential marker for autophagy [40,41]. Here, in our study, we found that LC3 was enhanced in JAK2-inhibition and quercetin nanoparticle-treated cells via immunochemistry, demonstrating that quercetin nanoparticle could induce autophagy in cervical cancer. The results above suggested that quercetin nanoparticle might be an essential strategy to improve cervical cancer progression through induction of apoptosis, autophagy and inhibiting cervical cancer cell proliferation via inhibition of JAK2 activation.

Collectively, JAK2 over-expressed in cervical cancer cell lines. And inactivated JAK2 expression by quercetin nanoparticle was found to prevent cervical cancer progression by apoptotic induction, autophagy and inhibiting proliferation of cervical cancer cells. The results here indicated that quercetin nanoparticle could suppress cervical cancer progression by STATs-regulated Bcl-2/Caspase-3 signaling pathway and PI3K/AKT-related GSK and mTOR pathways, thus leading to apoptosis, autophagy and proliferation inhibition of cancer cells, which was associated with JAK2 activation. Our results supplied new knowledge of cervical cancer treatment and provide potential therapeutic opportunities for cervical cancer inhibition.

Acknowledgements

This study was funded by Henan Research Program of Foundation and Advanced Technology, (No. 162300410090), the Initial Funding of Young Teachers in Zhengzhou University (No. 1411329028) and the General Program of National Natural Science Foundation of China (Nos. 81372371, and 81202278).

References

[1] D. Lorusso, F. Petrelli, A. Coinu, F. Raspagliesi, S. Barni, A systematic review comparing cisplatin and carboplatin plus paclitaxel-based chemotherapy for recurrent or metastatic cervical cancer, Gynecol. Oncol. 133 (2014) 117–123.

[2] A. Adefuye, A.A. Katz, K.J. Sales, The regulation of inflammatory pathways and infectious disease of the cervix by seminar fluid, Patholog. Res. Int. 2014 (2014) 748740.
[3] L. Xu, J.H. Liu, J. Zhang, N. Zhang, Z.H. Wang, Blockade of autophagy aggravates endoplasmic reticulum stress and improves Paclitaxel cytotoxicity in humancervical cancer cells, Cancer Res. Treat. 47 (2015) 313–321.
[4] N.K. Saxena, D. Sharma, X. Ding, S. Lin, F. Marra, D. Merlin, F.A. Anania, Concomitant activation of the JAK/STAT, PI3 K/AKT, and ERK signaling is involved in leptin-mediated promotion of invasion and migration of hepatocellular carcinoma cells, Cancer Res. 67 (2007) 2497–2507.
[5] H. Xiong, Z.G. Zhang, X.Q. Tian, D.F. Sun, Q.C. Liang, Y.J. Zhang, R. Lu, Y.X. Chen, J. Y. Fang, Inhibition of JAK1, 2/STAT3 signaling induces apoptosis, cell cycle arrest, and reduces tumor cell invasion in colorectal cancer cells, Neoplasia 10 (2008) 287–297.
[6] R. Bolli, A.B. Stein, Y. Guo, O.L. Wang, G. Rokosh, B. Dawn, J.D. Molkentin, S.K. Sanganalmath, Y. Zhu, Y.T. Xuan, A murine model of inducible, cardiac-specific deletion of STAT3: its use to determine the role of STAT3 in the upregulation of cardioprotective proteins by ischemic preconditioning, J. Mol. Cell. Cardiol. 50 (2011) 589–597.
[7] A. Elsasser, K. Suzuki, S. Lorenz-Meyer, C. Bode, J. Schaper, The role of apoptosis in myocardial ischemia: a critical appraisal, Basic Res. Cardiol. 96 (2001) 219–226.
[8] M. Cargnello, J. Tcherkezian, P.P. Roux, The expanding role of mTOR in cancer cell growth and proliferation, Mutagenesis 30 (2015) 169–176.
[9] M. Kobori, Y. Takahashi, M. Sakurai, Y. Akimoto, T. Tsushida, H. Oike, K. Ippoushi, Quercetin suppresses immune cell accumulation and improves mitochondrial gene expression in adipose tissue of diet-induced obese mice, Mol. Nutr. Food Res. 60 (2016) 300–312.
[10] L.K. Stewart, J.L. Soileau, D. Ribnicky, Z.Q. Wang, I. Raskin, A. Poulev, M. Majewski, W.T. Cefalu, T.W. Gettys, Quercetin transiently increases energy expenditure but persistently decreases circulating markers of inflammation in C57BL/6J mice fed a high-fat diet, Metabolism 57 (2008) S39–S46.
[11] K. Muller, D.A. Fedosov, G. Gompper, Margination of micro- and nano-particles in blood flow and its effect on drug delivery, Sci. Rep. 4 (2014).
[12] S.S. Bhattacharyya, S. Paul, A. De, D. Das, A. Samadder, N. Boujedaini, A.R. Khuda-Bukhsh, Poly (lactideco-glycolide) acid nanoencapsulation of a synthetic coumarin: cytotoxicity and bio-distribution in mice, in cancer cell line and interaction with calf thymus DNA as target, Toxicol. Appl. Pharmacol. 253 (2011) 270–282.
[13] M.L. Maitland, R.L. Schilsky, Clinical trials in the era of personalized oncology, CA Cancer J. Clin. 61 (2011) 365–381.
[14] A. Minaei, M. Sabzichi, F. Ramezani, H. Hamishehkar, N. Samadi, Co-delivery with nano-quercetin enhances doxorubicin-mediated cytotoxicity against MCF-7 cells, Mol. Biol. Rep. 43 (2016) 99–105.
[15] C. Wang, L. Su, C. Wu, J. Wu, C. Zhu, G. Yuan, RGD peptide targeted lipid coated nanoparticles for combinatorial delivery of sorafenib and quercetin against hepatocellular carcinoma, Drug. Dev. Ind. Pharm. 4 (2016) 1–29.
[16] K. Bishayee, A.R. Khuda-Bukhsh, S.O. Huh, PLGA-loaded gold-nanoparticles precipitated with quercetin downregulate HDAC-Akt activities controlling proliferation and activate p 53-ROS crosstalk to induce apoptosis in hepatocarcinoma cells, Mol. Cells 38 (2015) 518–527.
[17] G. Sharma, J. Park, A.R. Sharma, J.S. Jung, H. Kim, C. Chakraborty, D.K. Song, S.S. Lee, J.S. Nam, Methoxy poly(ethylene glycol)-poly(lactide) nanoparticles encapsulating quercetin act as an effective anticancer agent by inducing apoptosis in breast cancer, Pharm. Res. 32 (2015) 723–735.
[18] K.M. Lee, M.K. Hwang, D.E. Lee, K.W. Lee, H.J. Lee, Protective effect of quercetin against arsenite-induced COX-2 expression by targeting PI3K in rat liver epithelial cells, J. Agric. Food Chem. 58 (2010) 5815–5820.
[19] J. Tan, S. Wang, J. Yang, Y. Liu, Coupled particulate and continuum model for nanoparticle targeted delivery, Comput. Struct. 122 (2013) 128–134.
[20] K.C. Nwankwo, U.U. Aniebue, E.N. Aguwa, A.N. Anarado, E. Agunwah, Knowledge attitudes and practices of cervical cancer screening among urban and rural Nigerian women: a call for education and mass screening, Eur. J. Cancer Care (Engl.) 20 (2011) 362–367.
[21] G. Bureau, F. Longpre, M.G. Martinoli, Resveratrol and quercetin, two natural polyphenols, reduce apoptotic neuronal cell death induced by neuroinflammation, J. Neurosci. Res. 86 (2008) 403–410.
[22] Y.S. Dong, J.L. Wang, D.Y. Feng, H.Z. Qin, H. Wen, Z.M. Yin, G.D. Gao, C. Li, Protective effect of quercetin against oxidative stress and brain edema in an experimental rat model of subarachnoid hemorrhage, Int. J. Med. Sci. 11 (2014) 282–290.
[23] S. Wang, Y. Zhou, J. Tan, J. Xu, J. Yang, Y. Liu, Computational modeling of magnetic nanoparticle targeting to stent surface under high gradient field, Comput. Mech. 53 (2014) 403–412.
[24] A.C. Puhl, M. Fagundes, K.C. dos Santos, I. Polikarpov, M.F. das Gracas, F. da Silva, J.B. Fernandes, P.C. Vieira, M.R. Forim, Preparation and characterization of polymeric nanoparticles loaded with the flavonoid luteolin, by using factorial design, Int. J. Drug Deliv. 3 (2011) 683–698.
[25] C.J. Menet, L.V. Rompaey, R. Geney, Advances in the discovery of selective JAK inhibitors, Prog. Med. Chem. 52 (2013) 153–223.
[26] J. Lim, B. Taoka, R.D. Otte, K. Spencer, C.J. Dinsmore, M.D. Altman, G. Chan, C. Rosenstein, S. Sharma, H.P. Su, et al., Discovery of 1-amino-5*H*-pyrido(4,3-b) indol-4-carboxamide inhibitors of janus kinase 2 (JAK2) for the treatment of myeloproliferative disorders, J. Med. Chem. 54 (2011) 7334–7349.
[27] L. Fernandez, A. Flores-Morales, O. Lahuna, D. Sliva, G. Norstedt, L.A. Haldosen, A. Mode, J.A. Gustafsson, Desensitization of the growth hormone-induced Janus kinase 2 (Jak 2)/signal transducer and activator of transcription 5 (Stat5)-signaling pathway requires protein synthesis and phospholipase C, Endocrinology 139 (1998) 1815–1824.
[28] Y. Cui, A. Hosui, R. Sun, K. Shen, O. Gavrilova, W. Chen, M.C. Cam, B. Gao, G.W. Robinson, L. Hennighausen, Loss of signal transducer and activator of transcription 5 leads to hepatosteatosis and impaired liver regeneration, Hepatology 46 (2007) 504–513.
[29] M. Bartoli, D. Platt, T. Lemtalsi, X. Gu, S.E. Brooks, M.B. Marrero, R.B. Caldwell, VEGF differentially activates STAT3 in microvascular endothelial cells, FASEB J. 17 (2003) 1562–1564.
[30] H. Yu, D. Pardoll, R. Jove, STATs in cancer inflammation and immunity: a leading role for STAT3, Nat. Rev. Cancer 9 (2009) 798–809.
[31] P.J. Real, A. Sierra, A. de Juan, J.C. Segovia, J.M. Lopez-Vega, J.L. Fernandez-Luna, Resistance to chemotherapy via STAT3-dependent overexpression of Bcl-2 in metastatic breast cancer cells, Oncogene 21 (2002) 7611–7618.
[32] P.G. Longo, L. Laurenti, S. Gobessi, S. Sica, G. Leone, D.G. Efremov, The Akt/Mcl-1 pathway plays a prominent role in mediating antiapoptotic signals downstream of the B-cell receptor in chronic lymphocytic leukemia B cells, Blood 111 (2008) 846–855.
[33] J. Guillermet-Guibert, K. Bjorklof, A. Salpekar, C. Gonella, F. Ramadani, A. Bilancio, S. Meek, A.J. Smith, K. Okkenhaug, B. Vanhaesebroeck, The p110beta isoform of phosphoinositide 3-kinase signals downstream of g protein-coupled receptors and is functionally redundant with p110gamma, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 8292–8297.
[34] U. Maurer, F. Preiss, P. Brauns-Schubert, L. Schlicher, C. Charvet, GSK-3—at the crossroads of cell death and survival, J. Cell Sci. 127 (2014) 1369–1378.
[35] J. Tan, L. Geng, E.M. Yazlovitskaya, D.E. Hallahan, Protein kinase B/Akt-dependent phosphorylation of glycogen synthase kinase-3β in irradiated vascular endothelium, Cancer Res. 66 (2006) 2320–2327.
[36] A.L. Gartel, A.L. Tyner, The role of the cyclin-dependent kinase inhibitor p21 in apoptosis, Mol. Cancer Ther. 1 (2002) 639–649.
[37] A.K. Jonassen, M.N. Sack, O.D. Mjos, D.M. Yellon, Myocardial protection by insulin at reperfusion requires early administration and is mediated via AKT and p70s6 kinase cell-survival signaling, Circ. Res. 89 (2001) 1191–1198.
[38] Z. Sheng, L. Ma, J.E. Sun, L.J. Zhu, M.R. Green, BCR-ABL suppresses autophagy through ATF5-mediated regulation of mTOR transcription, Blood 118 (2011) 2840–2848.
[39] A. Burchert, Y. Wang, D. Cai, N. von Bubnoff, P. Paschka, S. Müller-Brüsselbach, O.G. Ottmann, J. Duyster, A. Hochhaus, A. Neubauer, Compensatory PI3-kinase/Akt/ mTor activation regulates imatinib resistance development, Leukemia 19 (2005) 1774–1782.
[40] Y. Zhen, W. Li, Impairment of autophagosome-lysosome fusion in the buff mutant mice with the VPS33A mutation, Autophagy 11 (2015) 1608–1622.
[41] A. Bernard, D.J. Klionsky, Toward an understanding of autophagosome-lysosome fusion: the unsuspected role of Atg14, Autophagy 11 (2015) 583–584.