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Selective mediation of ovarian cancer SKOV3 cells death by pristine carbon quantum dots/ Cu_2O composite through targeting matrix metalloproteinases, angiogenic cytokines and cytoskeleton

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Abstract

It was shown that some nanomaterials may have anticancer properties, but lack of selectivity is one of challenges, let alone selective suppression of cancer growth by regulating the cellular microenvironment. Herein, we demonstrated for the first time that carbon quantum dots/ Cu_2O composite (CQDs/ Cu_2O) selectively inhibited ovarian cancer SKOV3 cells by targeting cellular microenvironment, such as matrix metalloproteinases, angiogenic cytokines and cytoskeleton. The result was showed CQDs/ Cu_2O possessed anticancer properties against SKOV3 cells with $\text{IC}_{50} = 0.85 \mu\text{g mL}^{-1}$, which was approximately threefold lower than other tested cancer cells and approximately 12-fold lower than normal cells. Compared with popular anticancer drugs, the IC_{50} of CQDs/ Cu_2O was approximately 114-fold and 75-fold lower than the IC_{50} of commercial artesunate (ART) and oxaliplatin (OXA). Furthermore, CQDs/ Cu_2O possessed the ability to decrease the expression of MMP-2/9 and induced alterations in the cytoskeleton of SKOV3 cells by disruption of F-actin. It also exhibited stronger antiangiogenic effects than commercial antiangiogenic inhibitor (SU5416) through down-regulating the expression of VEGFR2. In addition, CQDs/ Cu_2O has a vital function on transcriptional regulation of multiple genes in SKOV3 cells, where 495 genes were up-regulated and 756 genes were down-regulated. It is worth noting that CQDs/ Cu_2O also regulated angiogenesis-related genes in SKOV3 cells, such as Maspin and TSP1 gene, to suppress angiogenesis. Therefore, CQDs/ Cu_2O selectively mediated of ovarian cancer SKOV3 cells death mainly through decreasing the expression of MMP-2, MMP-9, F-actin, and VEGFR2, meanwhile CQDs/ Cu_2O caused apoptosis of SKOV3 via S phase cell cycle arrest. These findings reveal a new application for the use of CQDs/ Cu_2O composite as potential therapeutic interventions in ovarian cancer SKOV3 cells.

Keywords: Ovarian cancer SKOV3 cells, Cellular microenvironment, Matrix metalloproteinases, Cytoskeleton, Angiogenesis, MMP-2/9, VEGFR2, F-actin

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Introduction

In gynecological malignancies, ovarian cancer is the one of the leading causes of death [1, 2]. Although combination of platinum and a taxane-containing agent remains the major treatment method after surgical resection, most patients ultimately succumb to the disorder because of the limited effects of the treatment on cancer growth, relapse, and drug resistance [3, 4]. Consequently, there is an urgent need to exploit more effective treatment means to treat ovarian cancer and delay or prevent recurrences [5].

Compared with traditional treatments, nanomaterials offer new opportunities for the development of diagnostic and therapeutic tools for cancer and other diseases [6–8]. Ag, Au, ZnO, TiO₂, As₂O₃, graphene oxide-silver nanoparticle, and iron core-gold shell nanoparticles have been reported to cause the apoptosis of cancer cells [9–13]. It was shown that some nanomaterials may have anticancer properties, mainly due to toxicity of the nanomaterials, but there is still a lack of nanomaterials that selectively distinguish cancer cells from normal cells.

More and more evidences indicated that cancer progression is closely related to the tumour microenvironment, including the extracellular matrix (ECM) deregulation, blood vessels expanding and immune response suppression [14]. Matrix metalloproteinases (MMPs) are a family of enzymes, have the ability to proteolytically degrade various components of ECM, participate in remodeling of basement membranes and contributed to angiogenesis [14]. The decrease of MMPs expression or enzymatic activities is considered to be the main factor for the inhibition potential of migration and angiogenesis. Angiogenesis, supplies oxygen and nutrients to actively proliferating cancer cells, provides advantages for cancer cells growth, invasion and metastasis [15, 16]. Numerous signaling molecules and pathways associated with angiogenic responses in the tumour microenvironment, play major roles in cancer cell growth and metastasis [17, 18]. Therefore, developing MMPs and angiogenesis inhibitor has become an effective tumor treatment strategy. Most current reports focus on targeting the vascular endothelial growth factor (VEGF). For example, gadolinium metallofullerenol nanoparticles, AgNPs, [19] and hollow mesoporous carbon nanocapsules (HMCNs) [20] have been reported to regulate tumour angiogenesis by VEGF pathway. We found that Fe-MIL-101 have an anti-angiogenesis potential utility by reducing MMP-2/9 expression [21].

In addition, the regulation of cytoskeleton plays a key role in the process of metastasis in cancer cells [22]. Actin, as an important part of cytoskeleton, has the

function of maintaining the communication between cytoplasmic proteins and transmembrane, keeping mechanical strength, and regulating cells locomotion [22]. Recently, several studies have reported the effects of some nanomaterials on actin cytoskeleton in cancer cells. For example, carbon nanomaterial [23], curcumin analog MHMD [24], grapheneoxide nanosheets [25], AgNPs [26], and ZnONPs [27] affected F-actin cytoskeleton through targeting or disruption of F-actin. It has been reported the metal–organic frameworks (IRMOF-3) possesses the ability to disrupt F-actin and tubulin, blocking the rat pheochromocytoma cell division [28]. Recently, we found Cu-MOF has an intrinsic activity of protease-mimicking [29] and disrupts of F-actin in ovarian cancer cells, leading the mitotic catastrophe [30].

Carbon quantum dots (CQDs) are currently eliciting much attention in cancer therapy due to outstanding properties including biocompatibility, low cytotoxicity, water solubility and unique photoluminescence [31]. CQDs and CQDs-based composites can be used for anti-cancer by phototherapy and radiotherapy. For instance, CQDs inhibited human breast cancer cells MCF-7 and MDA-MB-231 by photodynamic therapy triggering the formation of singlet oxygen species [32]. It was reported CQDs could be used as photosensitizers to destroy buried tumors in photodynamic therapy. C–Ag-PEG CQDs inhibited Du145 cells by free radicals in radiotherapy, which reduced the damage of normal cells and increased therapeutic selectivity [33]. It has been reported CQDs can selectively target cancer cells through regulated the expression of major angiogenic cytokines, such as VEGF, FGF, and VEGFR2 [34]. The results were shown that the angiogenesis inhibition rate of 100 µg CQDs was less than 40%. It is an effective strategy for tumor therapy to develop carbon quantum dot composite materials, making use of its synergistic effect to improve the anti-angiogenesis activity.

Cu₂O NPs has been extensively researched to elucidate their significance in cancer therapy. Cu₂O NPs significantly reduce the growth and metastasis of melanoma, improve the viability of tumor-bearing mice, and induce the mitochondrion-mediated apoptosis of cancer cells [35]. Cu₂O NPs was more sensitive to rapidly proliferating HeLa cancer cells than normal human kidney 293 T cells, indicating Cu₂O NPs exert distinct effects on different cells [36]. Since one of the biggest challenges of chemotherapy is that chemotherapy drugs do not effectively distinguish between tumor and normal cells, differential cytotoxicity is very important [36].

Gene expression experiments, as a method for detection of disease markers, has been used to assessment the toxicity mechanism of nanomaterials [37]. For example,

Ag nanoparticles involved regulating gene expression, including oxidative phosphorylation gene, protein synthesis gene, vascular endothelial growth factor-A (VEGFA) gene and fibroblast growth factor2 (FGF2) gene in different cell lines [38, 39]. Single-walled carbon nanotubes also regulated gene expression of relative root growth, influence [40]. Melittin-loaded ZIF8 nanoparticles was found to induce A549 cells apoptosis through regulating the expression of 3383 genes [41]. Recently, we reported that Cu-MOF (HKUST-1) induced mitotic catastrophe through destruction of actin cytoskeleton and changes of gene expression in SKOV3 cells [30].

On the other hand, the proteases naturally expressed by living organisms participate in all stages of tumor progression. In our previous study, we found that the CQDs/Cu₂O composite possessed an intrinsic protease-mimicking activity for hydrolyzing proteins under physiological conditions, ultimately exhibiting a surprisingly higher catalytic activity than Cu₂O and CQDs [42]. Based on the above findings, we investigated the inhibitory mechanism of CQDs/Cu₂O composite on human ovarian cancer SKOV3 cells. It will be attractive to elucidate the potential roles of the CQDs/Cu₂O composite in the regulation of cancer-related proteins. Furthermore, only a few nanoparticles have been reported to interact with the tumour microenvironment. As far as we know, this is the first attempt to reveal CQDs/Cu₂O composite mediates tumor microenvironment and cytoskeleton.

Experiment section

Reagents

CuSO₄, poly-vinylpyrrolidone, and C₆H₁₂O₆ were obtained from Shanghai Tian Scientific Co. Ltd. Cell culture medium (Dulbecco's modified eagle's medium, DMEM) were from Hyclone Laboratories. Fetal bovine serum (FBS) were got from Gibico. Acridine orange, ethidium bromide, FITC-Annexin-V/PI apoptosis assay kit, WST-1 assay kit and Hoechst 33342 were obtained from Beyotime Company of China. Antibody VEGFR2 (Flk-1 [C-1158], sc-504, rabbit polyclonal antibody raised against amino acids 1158–1345 of mouse Flk-1, Santa Cruz Biotechnology, USA). MMP-2 (H-76, sc-10736, rabbit monoclonal antibody against human MMP-2, Santa Cruz Biotechnology, USA). MMP-9 (H-129, sc-10737, rabbit monoclonal antibody against human MMP-9, Santa Cruz Biotechnology, USA). GAPDH (AG019, Primary antibodies used were mouse antibodies specific for the GAPDH, Beyotime Institute of Biotechnology, Jiangsu, China). F-actin (Mouse monoclonal [NH3] to F-actin, Abcam). β -actin (AA128, Primary antibodies used were mouse antibodies specific for the β -actin, Beyotime Institute of Biotechnology, Jiangsu, China). Secondary antibodies goat anti-mouse (A0216,

Beyotime Institute of Biotechnology, Jiangsu, China). Goat anti-rabbit (A0208, 1:1000, Beyotime Institute of Biotechnology, Jiangsu, China).

Synthesis and characterization of CQDs/Cu₂O

Synthesis and characterization of CQDs/Cu₂O have been discussed in our previous publications. [42, 43].

Cell culture

All cell lines including human ovarian cancer cells (SKOV3), human cervical cancer cells (HeLa), human lung adenocarcinoma cells (A549), human colorectal cancer cells (HT-29, HCT116), normal mouse embryonic fibroblasts cells (BABL-3T3), normal human epithelial kidney cells HEK293T, normal mouse macrophage cells J774A1) were purchased from shanghai life science of chinese academy of sciences. The BABL-3T3 cells were grown in high glucose DMEM containing 10% FBS (Fetal bovine serum), while other cells were grown in low glucose DMEM containing 10% FBS. HUVECs were isolated from term umbilical cord veins using collagenase and cultured in DMEM supplemented with 20% FBS. All cell lines were grown at 37 °C in a humidified 5% CO₂ atmosphere. HUVEC cells were used within 6 passages All cells were grown at 37 °C with 5% CO₂ in a humidified incubator.

MTT assay

The cytotoxicity of all cells was detected by MTT assay. 1×10^4 cells were grown in 96-well plates for 24 h. Cells were incubated with 1.56, 3.12, 6.25, 12.5 and 25 $\mu\text{g mL}^{-1}$ of CQDs/Cu₂O for 24–72 h. Cells incubated PBS instead of CQDs/Cu₂O were used as control. Then cells were treated with MTT (20 μL , 5 mg mL^{-1}) for 4 h. 150 μL of DMSO was added after removing the medium. Microplate spectrophotometer (Spectra Max 190) was used to measure the absorbance at 490 nm. The assay was repeated 3 times and all experiments were carried out in duplicate. The inhibition rate (%) = $(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{control}} \times 100\%$. The half-maximal inhibitory concentration (IC₅₀) was measured when the inhibition rate to half that of the control.

WST-1 assay

1×10^4 SKOV3 cells were grown in 96-well plates for 24 h. Cells were incubated with 1.56, 3.12, 6.25, 12.5 and 25 $\mu\text{g mL}^{-1}$ of CQDs/Cu₂O for 24 h. 20 μL of WST-1 was added and incubated 1 h. The absorbance were measured by Microplate spectrophotometer (Spectra Max 190) at 450 nm. The assay was repeated 3 times and all experiments were carried out in duplicate. The inhibition rate (%) = $(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{control}} \times 100\%$. The

half-maximal inhibitory concentration (IC_{50}) was measured when the inhibition rate to half that of the control.

AO/EB staining

After treatment with $12.5 \mu\text{g mL}^{-1}$ CQDs/ Cu_2O , CQDs, or Cu_2O for 24 h, SKOV3 cells were trypsinized and harvested. Cells in suspension were stained with $5 \mu\text{g mL}^{-1}$ AO/EB for 10 min. Then cells were placed on a glass slide and analyzed using an Olympus IX73 fluorescent microscope at 545 nm.

Hoechst 33342 staining

After treatment with $12.5 \mu\text{g mL}^{-1}$ CQDs/ Cu_2O , CQDs, or Cu_2O for 24 h, SKOV3 cells were trypsinized and harvested. Cells were fixed for 10 min by 4% paraformaldehyde after washing 3 times using ice-cold PBS. SKOV3 cells were stained with Hoechst 33342 for 15 min after washing 3 times with PBS. Cells were analyzed using a Olympus IX73 fluorescent microscope at 350 nm.

Flow cytometric analysis of cell cycle

After treatment with CQDs/ Cu_2O , CQDs, or Cu_2O for 24 h, SKOV3 cells were trypsinized and harvested. Cells were fixed with 70% ethanol for 12 h at 4°C , centrifuged (3000 rpm, 15 min) and washed using PBS, stained by PI for 20 min. Cells were subsequently analyzed by flow cytometer. The total number of cells was 10,000.

Apoptosis detection by Annexin V staining

SKOV3 cells were treated with CQDs/ Cu_2O (3.12, 6.25, $12.5 \mu\text{g mL}^{-1}$). Cells were trypsinized and harvested, stained with FITC-Annexin-V and PI for 15 min in dark. Then tested by a flow cytometer. The number of cells was 10,000.

Phalloidin staining

SKOV3 were treated with CQDs/ Cu_2O (3.12, 6.25, $12.5 \mu\text{g mL}^{-1}$). Then cells were fixed using 4% paraformaldehyde, washed with PBS, and permeabilized by 0.1% Triton X-100 for 15 min. After washing by PBS, cells were then incubated with FITC-conjugated phalloidin. Cells were stained using PI for 10 min, then analyzed with Olympus IX73.

Wound healing assay

SKOV3 were seeded onto six-well plates. When cells grown to 90% confluence to form monolayers, cells were scratched by a pipette tip. Subsequently, CQDs/ Cu_2O ($6.25, 12.5, 25 \mu\text{g mL}^{-1}$) were added and incubated with SKOV3 cells for 0, 6, 12 and 24 h. The number of cells migrated into the scratch area was quantified under fluorescent microscope. The migration inhibition rate of a sample was calculated by the following

equation: inhibition percentage = $(N_{\text{Control}} - N_{\text{Sample}}) / N_{\text{Control}} \times 100\%$, where N_{Control} is the average total number of migrated cells in three groups of control, N_{Sample} is the average total number of migrated cells in three groups of CQDs/ Cu_2O .

Tube structure formation assay

HUVEC cells (2×10^4 cells per well), VEGF (10 ng mL^{-1}) and different concentration of CQDs/ Cu_2O were added into 96-well plates which coated by Matrigel for 12 h. Tubular network were observed and quantified by the measuring tool of the software in randomly chosen five fields under a fluorescence microscope (Olympus IX73, Japan). The inhibition (%) = $(\text{length}_{\text{Control}} - \text{length}_{\text{Sample}}) / \text{length}_{\text{Control}} \times 100\%$.

Western blot

SKOV3 cells treated by CQDs/ Cu_2O ($6.25, 12.5, 25 \mu\text{g mL}^{-1}$) for 24 h. After harvested and washed by cold PBS, cells were incubated with cell lysate for 1 h at 4°C on ice. The proteins were extracted by centrifuging for 15 min (14,000 rpm, 4°C). 10% SDS-PAGE was used to separate the extracted proteins. After proteins transferred, membranes were treated with blocking agents. The membranes were incubated with primary antibodies (VEGFR2 1:200, GAPDH 1:3000, MMP-2 1:200, MMP-9 1:200, F-actin 1:200, β -actin 1:1000, GAPDH 1:1000) at 4°C overnight. After washed 3 times, the membranes were incubated with secondary antibodies goat anti-mouse or goat anti-rabbit at room temperature for 1 h. Washing 3 times with PBST, the membranes were treated using chemiluminescence agents.

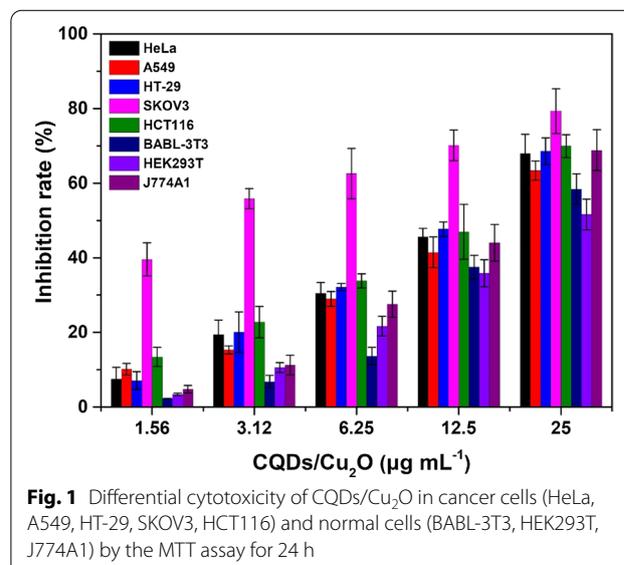


Fig. 1 Differential cytotoxicity of CQDs/ Cu_2O in cancer cells (HeLa, A549, HT-29, SKOV3, HCT116) and normal cells (BABL-3T3, HEK293T, J774A1) by the MTT assay for 24 h

RNA sequencing

SKOV3 cells were treated with CQDs/Cu₂O (3.12, 12.5 μg mL⁻¹) for 24 h. Total RNA of cells treated by CQDs/Cu₂O was extracted by TRIzol reagent. Sequencing libraries were tested by the Illumina HiSeq xten/NovaSeq 4000 sequencer. A fold change of 2 were used as the threshold. $P < 0.05$ was indicated statistically significant.

Statistical analysis

All values represent mean ± SD, n = 3. The significant levels were assessed by Student's t-test. $P < 0.05$ was indicated the statistically significant.

Results and discussion

MTT assays

The effect of CQDs/Cu₂O, Cu₂O on cell cytotoxicity was tested in cancer cells (HeLa, A549, HT-29, SKOV3, HCT116), and normal cells (BABL-3T3, HEK293T, J774A1) by the MTT assay. Cells were treated with CQDs/Cu₂O or Cu₂O (1.56, 3.12, 6.25, 12.5, 25 μg mL⁻¹) for 24–72 h. The effect of CQDs was detected in HeLa, SKOV3 and BABL-3T3 cells by MTT assay. The results were shown CQDs were non-toxic and biocompatibility,

which is agreed with previous findings [34]. As shown in Fig. 1, CQDs/Cu₂O displayed cytotoxicity in a concentration-dependent manner in all tested cells, and SKOV3 cells displayed the highest inhibitory rate for proliferation among all cells. To further verify the results of MTT assay, a second mitochondrial activity-based assay, WST-1, was employed to measure the cytotoxicity of CQDs/Cu₂O in SKOV3 cells. WST data showed that CQDs/Cu₂O inhibited the growth of SKOV3 cells in a dose-dependent manner, the value of IC₅₀ obtained by WST assay (IC₅₀ = 1.46 μg mL⁻¹) was consistent with the result from MTT assay (IC₅₀ = 1.50 μg mL⁻¹). It was suggested the MTT assay can be used to evaluate the cytotoxicity of CQDs/Cu₂O.

Interestingly, when the concentration of CQDs/Cu₂O was lower than 12.5 μg mL⁻¹, a discriminative difference was identified for the cytotoxicity between SKOV3 cancer cells and other cancer cells, even normal BABL-3T3, HEK293T and J774A1 cells. This is important because developing an anticancer drug that can effectively distinguish between tumor and normal cells is currently the greatest challenge [21].

In general, the CQDs/Cu₂O composite displayed cytotoxic activity against cancer and normal cells in the IC₅₀ (half maximal inhibitory concentration) range of 0.85 to 22.4 μg mL⁻¹ (Table 1). The IC₅₀ value of CQDs/Cu₂O

Table 1 Comparison of the IC₅₀ (μg mL⁻¹) of CQDs/Cu₂O composite and Cu₂O in different cell lines

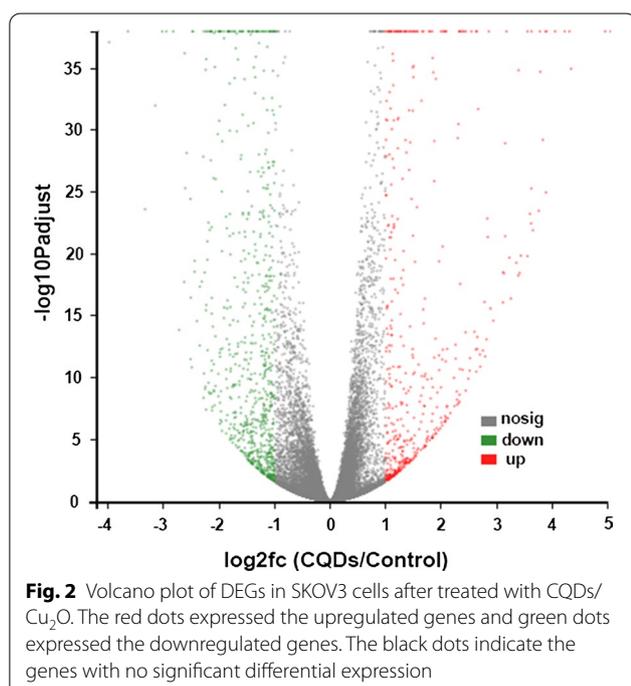
| Cells | IC ₅₀ (CQDs/Cu ₂ O) | | | IC ₅₀ (Cu ₂ O) | | |
|----------|---|------|------|--------------------------------------|------|------|
| | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| HeLa | 10.6 | 9.5 | 4.9 | 27.8 | 23.9 | 28.1 |
| A549 | 15.8 | 12.3 | 10.5 | 31.3 | 24.7 | 23.6 |
| HT-29 | 7.1 | 4.0 | 2.5 | 16.2 | 14.3 | 15.5 |
| SKOV3 | 1.5 | 0.9 | 0.85 | 15.7 | 13.9 | 12.2 |
| HCT116 | 9.4 | 6.9 | 5.8 | 15.0 | 11.7 | 14.8 |
| BABL-3T3 | 18.9 | 17.8 | 16.9 | 29.5 | 27.4 | 18.3 |
| HEK293T | 22.4 | 16.3 | 11.7 | 24.8 | 19.6 | 15.9 |
| J774A1 | 20.1 | 16.4 | 14.2 | 23.8 | 18.9 | 16.6 |

Table 2 Comparison of the IC₅₀ (μg mL⁻¹) of CQDs/Cu₂O, OXA and ART in different cell lines

| Cells | IC ₅₀ (CQDs/Cu ₂ O) | | | IC ₅₀ (OXA) | | | IC ₅₀ (ART) | | |
|----------|---|------|------|------------------------|------------|-----------|------------------------|------------|-----------|
| | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| HeLa | 10.6 | 9.5 | 4.9 | 84.8 [21] | 28.5 [21] | 20.0 [21] | 126.5 [21] | 62.7 [21] | 43.6 [21] |
| A549 | 15.8 | 12.3 | 10.5 | 219.8 [21] | 128.3 [21] | 27.0 [21] | 160.7 [21] | 66.0 [21] | 31.1 [21] |
| HT-29 | 7.1 | 4.0 | 2.5 | 47.81 | 32.65 | 26.82 | 63.41 | 50.24 | 43.60 |
| SKOV3 | 1.5 | 0.9 | 0.85 | 241.5 [21] | 120.8 [21] | 64.6 [21] | 280.8 [21] | 121.1 [21] | 96.9 [21] |
| HCT116 | 9.4 | 6.9 | 5.8 | 62.9 | 41.2 | 28.6 | 40.3 | 22.6 | 21.4 |
| BABL-3T3 | 18.9 | 17.8 | 16.9 | 77.8 [21] | 34.2 [21] | 13.8 [21] | 118.2 [21] | 52.4 [21] | 36.6 [21] |

Table 3 The raw data from RNA-Seq analysis of the CQDs/Cu₂O and control group

| Sample | Raw reads | Clean reads | Total mapped | Q30 (%) | GC content (%) |
|----------|------------|-------------|--------------------|---------|----------------|
| Control1 | 56,611,380 | 54,934,068 | 52,445,514(95.47%) | 93.71 | 50.23 |
| Control2 | 68,457,650 | 66,554,960 | 63,613,738(95.58%) | 93.97 | 50.25 |
| Control3 | 60,574,334 | 59,402,794 | 58,070,632(97.76%) | 96.09 | 50.49 |
| CQDs1 | 63,142,992 | 61,528,818 | 58,951,428(95.81%) | 94.14 | 50.40 |
| CQDs2 | 65,707,806 | 63,946,010 | 61,244,702(95.78%) | 93.97 | 50.23 |
| CQDs3 | 65,006,850 | 63,690,950 | 62,268,500(97.77%) | 96.08 | 50.88 |



against cells was lower than Cu₂O and CQDs were non-toxic. It was indicated that the cytotoxicity of the CQDs/Cu₂O composite was higher than that of the CQDs and Cu₂O. In addition, the leakage of copper into the medium was undetectable at 37 °C after 72 h by ICP-AES (coupled plasma-atomic emission spectrometry) measurement, suggesting CQDs/Cu₂O displayed high stability and the CQDs/Cu₂O plays a leading roles in cytotoxic activity. Moreover, the IC₅₀ value of CQDs/Cu₂O against SKOV3 cells was lower than above mentioned cancer cells and normal cells, which was approximately threefold lower than other tested cancer cells and approximately 12-fold lower than normal cells. It was further suggested CQDs/Cu₂O was sensitive to SKOV3 cells.

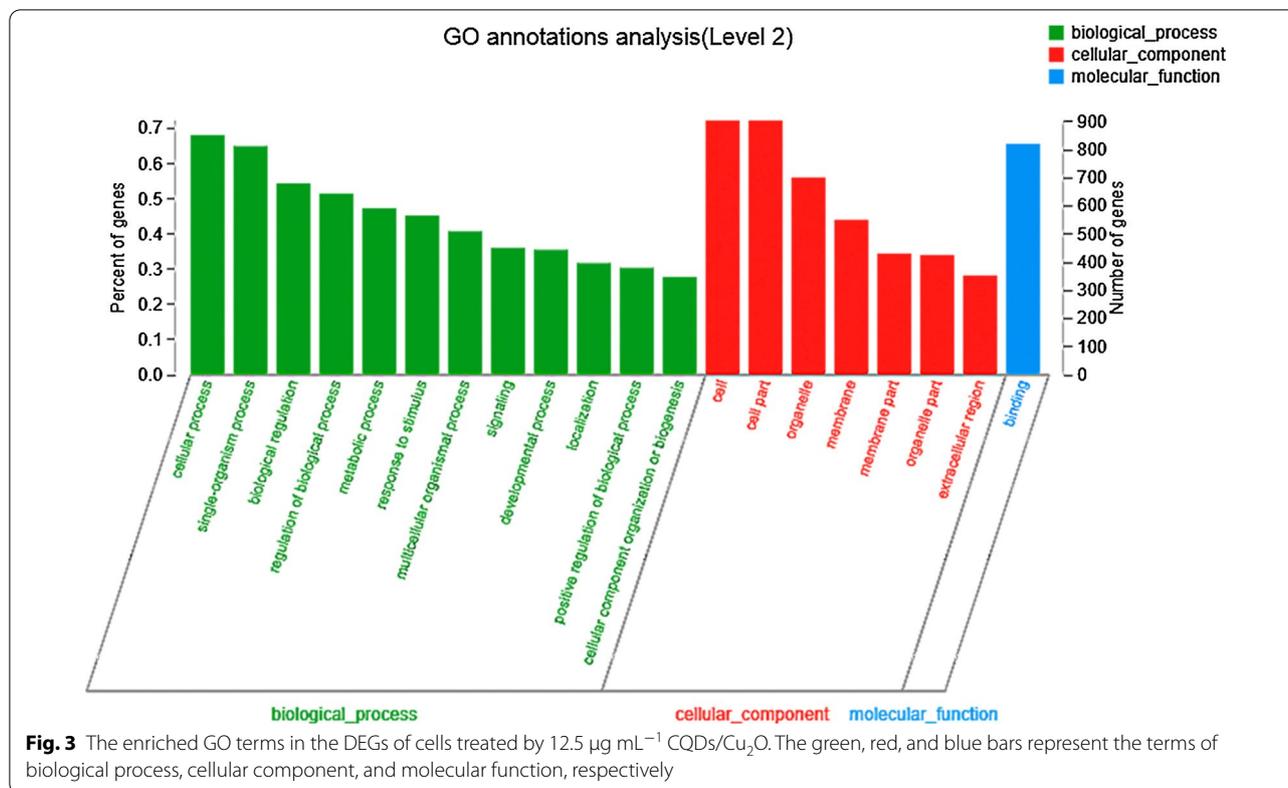
On the other hand, compared with commercial anti-cancer agents such as ART and OXA at different times, the cytotoxicity of CQDs/Cu₂O was better than that of ART and OXA against cancer cells (Table 2). The

IC₅₀ of CQDs/Cu₂O in SKOV3 cells (IC₅₀ = 0.85 μg mL⁻¹) was approximately 75-fold lower than that of OXA (IC₅₀ = 64.6 μg mL⁻¹) [21] and 114-fold lower than that of ART (IC₅₀ = 96.9 μg mL⁻¹) [21] at 72 h. Therefore, CQDs/Cu₂O may have a potential utility for effectively distinguishing between SKOV3 cells and other tested cells.

RNA-Seq data and identification of differentially expressed genes (DEGs)

Six sequencing libraries including CQDs/Cu₂O treatment libraries (CQDs1, CQDs2, CQDs3) and control group libraries (Control1, Control2, Control3) were successfully constructed and subsequently sequenced (Table 3). The RNA-Seq of six samples produced 37.95 million raw reads and 37.05 million high quality clean reads. It was shown that 95.47–97.77% of the reads were successfully mapped to the Homo genome (Table 3). Compared to the control, fragments per kilobase of transcript per million fragments (FPKM) analysis indicated that a total of 1251 transcripts at a fold change > 2 and false discovery rate (FDR) < 0.05 were differentially expressed, among which, 495 genes were upregulated while 756 were downregulated (Fig. 2).

In order to determine the differentially expressed genes at lower doses and elucidate the mechanism of selective inhibition of SKOV3 cells by CQDs/Cu₂O, the RNA sequencing analysis were performed after SKOV3 cells treated with 3.12 μg mL⁻¹ CQDs/Cu₂O for 24 h. Compared to the control, fragments per kilobase of transcript per million fragments (FPKM) analysis indicated that a total of 1011 transcripts at a fold change > 2 and false discovery rate (FDR) < 0.05 were differentially expressed, among which, 381 genes were upregulated while 630 were downregulated. There was no significant difference between the high concentration group and the low concentration group. On the one hand, the enriched GO terms in the DEGs of cells treated by 3.12 μg mL⁻¹ CQDs/Cu₂O have also been provided in supporting information (Additional file 1: Figure S1). As shown in Additional file 1: Figure S1, the distribution of enriched



GO terms of cells treated by $3.12 \mu\text{g mL}^{-1}$ CQDs/Cu₂O was similar with that treated by $12.5 \mu\text{g mL}^{-1}$ CQDs/Cu₂O. Moreover, DEGs analysis results showed that the angiogenesis-related genes (Maspin and TSP1) were significantly upregulated after $3.12 \mu\text{g mL}^{-1}$ CQDs/Cu₂O treatment, which was also consistent with those of higher doses treatment.

GO and KEGG analysis of DEGs

Gene Ontology (GO) was used to analyze the obtained DEGs, where three main ontologies such as biological process, cellular component, and molecular function performed. As shown in Fig. 3, the top 20 most enriched GO terms are summarized. In the category of biological process, the most abundant groups were “cellular process”, “single-organism process”, and “biological regulation”. Within the cellular component category, the “cell”, “cell part”, and “organelle” were identified as the most enriched GO terms. The molecular functional groups of DEGs were also related to “binding”.

GO enrichment analysis was also used to identify significantly enriched BP, CC, or MF terms in DEGs during CQDs/Cu₂O treatment. As shown in Table 4, there were 10, 8, and 6 ontology terms enriched in BP, CC, and MF, respectively. The enriched BP terms mainly included the regulation of the apoptotic process, programmed cell

death, and cell motility, indicating that CQDs/Cu₂O plays a key role in the induction of SKOV3 cell apoptosis and the regulation of cell movement. The enriched CC terms mainly included proteinaceous extracellular matrix and integral components of the plasma membrane, suggesting that CQDs/Cu₂O affected the extracellular matrix and plasma membrane. The enriched MF terms included calcium ion binding, nucleic acid binding, receptor binding, etc.

To determine the effect of CQDs/Cu₂O on the functions of DEGs, the KEGG data base was used to analyze annotated pathways of DEGs. The result indicated that DEGs were significantly enriched in 20 pathways after treated with CQDs/Cu₂O in Fig. 4 (P corrected < 0.05). According to the number of DEGs, the three pathways such as steroid biosynthesis, focal adhesion, and ECM-receptor interaction were the most enriched pathways. Based on the lowest P value, steroid biosynthesis was among the most notable enriched pathways.

CQDs/Cu₂O induced apoptosis of SKOV3 cells

Transcriptome analysis revealed that apoptotic process and programmed cell death were significantly affected. AO/EB (acridine orange/ethidium bromide), Hoechst 33342, and FITC-Annexin-V/PI were used to demonstrate apoptosis. AO is absorbed by viable and nonviable

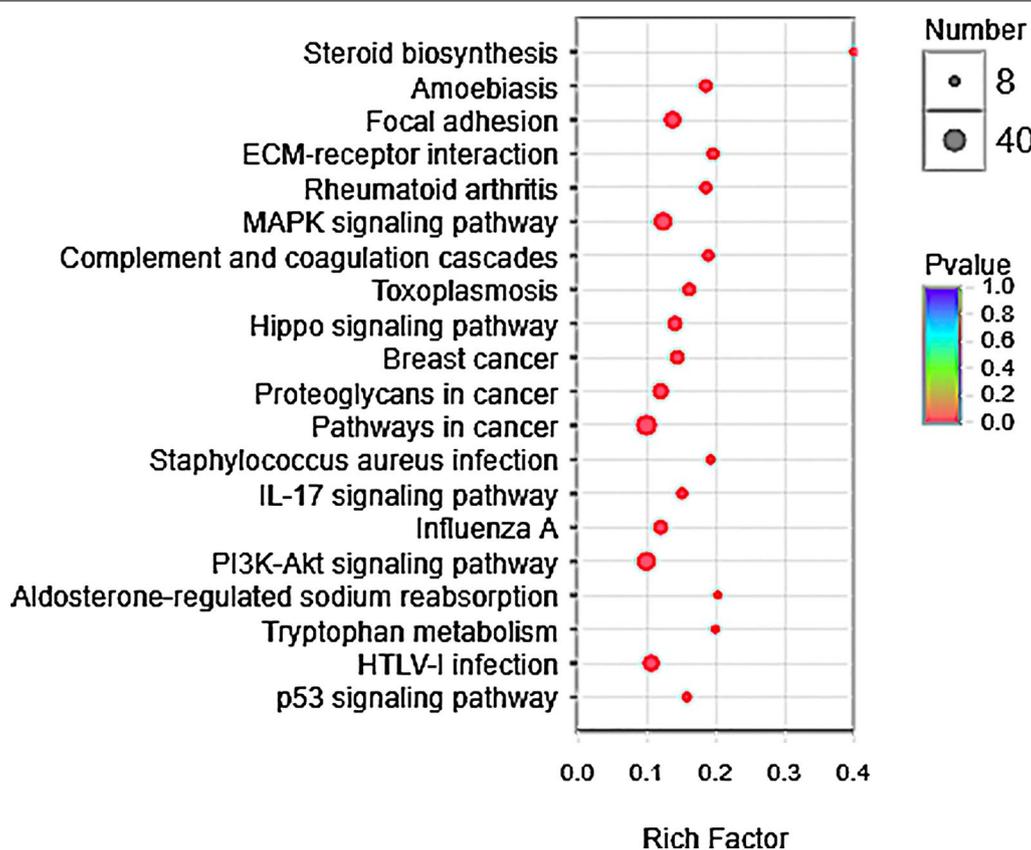


Fig. 4 A bubble diagram of the significant enrichment pathways through KEGG analysis of DEGs ($P < 0.05$). The size and color of bubble indicated the number of gene enriched in certain pathway and the P -value, respectively. The rich factor = (the number of DEGs mapped to a certain pathway) / (the total number of genes mapped to this pathway)

cells to emit green fluorescence, while EB is absorbed by nonviable cells to emit red fluorescence. Therefore, viable cells displayed uniform bright green fluorescence with organized structure. The apoptotic cells showed red to orange fluorescence with fragmented chromatin, and the necrotic cells showed red fluorescence with swollen [44]. As shown in Fig. 5, cells appeared healthy with a green nucleus in the control group. Some of the cells incubated with the CQDs/Cu₂O samples had an orange nuclei and fragmented chromatin, indicating that they were either in necrosis or at the late stage of apoptosis. In addition, only few necrotic cells with uniformly red nuclei and an organized structure appeared after treatment with the same concentration of Cu₂O. However, there was no significant change in the CQD group. These results indicate that CQDs/Cu₂O induces necrosis or apoptosis of SKOV3 cells, and the effect of the CQDs/Cu₂O composite is better than that of CQDs and Cu₂O.

The apoptotic effects of CQDs/Cu₂O on SKOV3 cells were determined by staining with Hoechst 33342. Nuclear condensation & DNA fragmentation are

considered to be typical features of apoptosis. SKOV3 cells were treated with 12.5 $\mu\text{g mL}^{-1}$ CQDs/Cu₂O for 24 h. As shown in Fig. 6, cells displayed a uniformly blue fluorescence in the control group and CQDs group. CQDs/Cu₂O treated cells showed nuclear shrinkage, cytoplasmic blebbing, and chromatin condensation, including bright blue dots in the nuclei, thereby representing nuclear fragmentation, whereas a small number of cells were chromatin condensation in Cu₂O group. These observations further indicated CQDs/Cu₂O can induce SKOV3 cells apoptosis, which is better than both CQDs and Cu₂O.

A flow cytometric analysis via FITC-Annexin-V/PI assay was used to further confirm the CQDs/Cu₂O-induced apoptosis. As shown in Fig. 7a, as the concentration of CQDs/Cu₂O increased from 3.12 ~ 12.5 $\mu\text{g mL}^{-1}$, the proportion of cells in the upper right quadrants increased from 3.5% to 48.4%, however, the proportions of other quadrant cells did not change significantly. This data also confirm that CQDs/Cu₂O can effectively induce SKOV3 cell apoptosis.

Table 4 GO enrichment analysis of the DEGs during CQDs/Cu₂O treatment (P value < 0.01)

| Description | GO ID | Term Type | Number | P-value uncorrected | P-value corrected |
|---|------------|-----------|--------|---------------------|-------------------|
| Regulation of apoptotic process | GO:0042981 | BP | 138 | 1.89891E-10 | 0 |
| Regulation of programmed cell death | GO:0043067 | | 138 | 2.3484E-10 | 0 |
| Regulation of locomotion | GO:0040012 | | 91 | 2.44938E-10 | 0 |
| Regulation of cell death | GO:0010941 | | 150 | 2.78134E-10 | 0 |
| Regulation of localization | GO:0032879 | | 216 | 2.82001E-10 | 0 |
| Response to organic substance | GO:010033 | | 168 | 3.03004E-10 | 0 |
| Positive regulation of response to stimulus | GO:0048584 | | 184 | 3.05792E-10 | 0 |
| Regulation of cell motility | GO:2000145 | | 84 | 3.254E-10 | 0 |
| Positive regulation of signal transduction | GO:0009967 | | 134 | 3.27693E-10 | 0 |
| Anatomical structure development | GO:0048856 | | 293 | 1.89891E-10 | 0 |
| Proteinaceous extracellular matrix | GO:0005578 | CC | 57 | 5.76E-11 | 0 |
| Extracellular matrix | GO:0031012 | | 70 | 1.49E-10 | 0 |
| Integral component of plasma membrane | GO:0005887 | | 129 | 1.9E-10 | 0 |
| Plasma membrane part | GO:0044459 | | 227 | 2.99E-10 | 0 |
| Intrinsic component of plasma membrane | GO:0031226 | | 136 | 3.4E-10 | 0 |
| Extracellular space | GO:0005615 | | 153 | 3.46E-10 | 0 |
| Extracellular region | GO:0005576 | | 219 | 3.7E-10 | 0 |
| Extracellular region part | GO:0044421 | | 321 | 3.82E-10 | 0 |
| Calcium ion binding | GO:0005509 | MF | 79 | 6.87E-10 | 0 |
| Nucleic acid binding | GO:0003676 | | 145 | 1.02E-09 | 0 |
| Heparin binding | GO:0008201 | | 26 | 1.12E-07 | 0 |
| Receptor binding | GO:0005102 | | 127 | 1.67E-07 | 0 |
| Sulfur compound binding | GO:1901681 | | 32 | 2.52E-07 | 0 |
| Glycosaminoglycan binding | GO:0005539 | | 29 | 9.63E-07 | 0.002 |

Determination of the cell cycle

The cell cycle, which includes DNA replication, mitosis, and cytokinesis, is a major event in cell division. It has been proven that deregulation of the cell cycle is related to numerous carcinogenic processes [45]. Nanomaterials, such as Se, Ag, ZnO, Ag-ZnO, TiO₂ NPs, have been reported to arrest cell cycle to suppress cancer cell proliferation [46–49]. Cu-based nanomaterials also play a critical role in regulating the cell cycle. For example, cuprous oxide nanoparticles (CONPs) suppressed the proliferation of HeLa cells and caused cell cycle arrest in G0/G1 [36]. A previous study reported that Cu-MOF (HKUST-1) has the potential to inhibit the proliferation of SKOV3 by cell cycle arrest in G2/M. [30] To determine whether apoptosis is involved in the cell cycle effect of CQDs/Cu₂O, SKOV3 cells were treated by CQDs/Cu₂O and subjected to flow cytometry analysis (Fig. 7b). As shown in Fig. 7b, CQDs/Cu₂O led to accumulation of cells in the S phase (9.24 ± 0.9% in control group versus 23.8 ± 0.5% in 12.5 μg mL⁻¹ CQDs/Cu₂O treated group (*P* < 0.001), while there was no significant change in the G0/G1 and

G2/M phase. The results showed that the SKOV3 cells are arrested at S phase after treatment by CQDs/Cu₂O, indicating that mitosis and proliferation of cancer cells were inhibited.

Effects of CQDs/Cu₂O on the SKOV3 migration

Ovarian cancer is the one of the leading causes of death in gynecological malignancies. Local recurrence and metastasis are considered to be the main causes of treatment failure for ovarian cancer. It was critical to suppress the metastasis of cancer cells for the development of effective drugs and therapies. GO enrichment analysis revealed that CQDs/Cu₂O regulated cell motility. Hence, the wound-healing assay was used to detect the amount of migrated cells and the width of scratch after treatment with CQDs/Cu₂O. As shown in Fig. 8a, the width of scratch increases with increasing concentrations of CQDs/Cu₂O and prolonging reaction time. As well as the amount of migrated cells decreased after treated with CQDs/Cu₂O (Fig. 8b). The migration inhibition rate increased from 61.8 to 79.4%

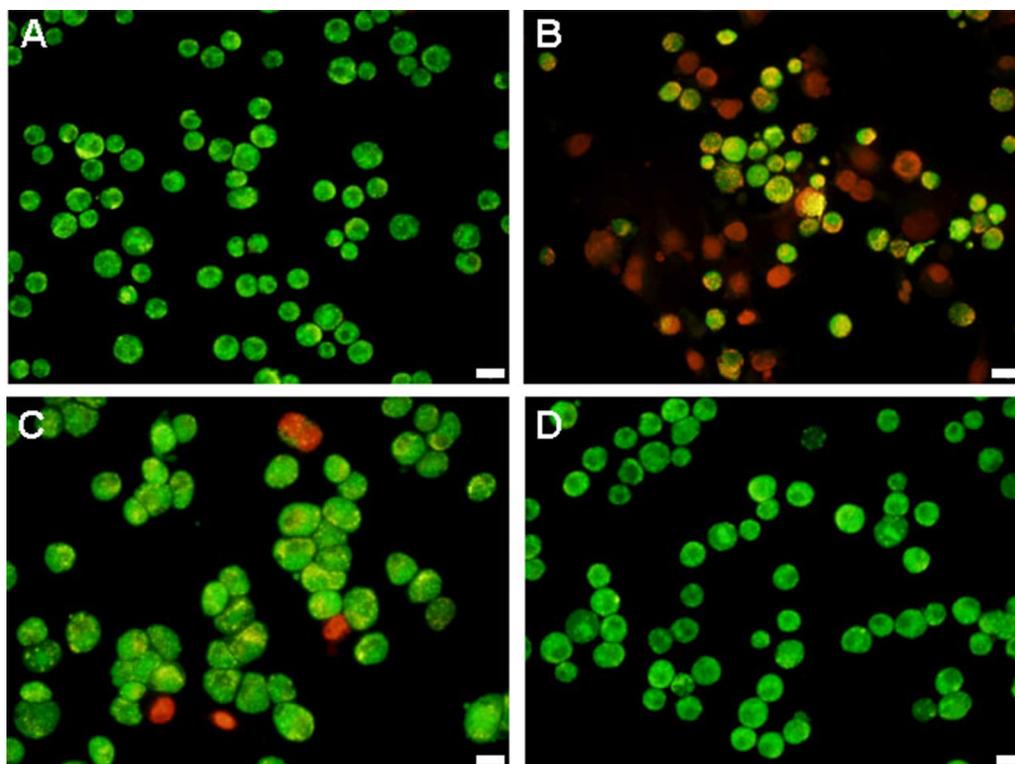


Fig. 5 Fluorescence photomicrographs changes by AO/EB staining of SKOV3 cells after treatment with CQDs/Cu₂O (B), CQDs (C), or Cu₂O (D) for 24 h compared to controls (A), respectively. Scale bar 20 μ m.

after incubation with CQDs/Cu₂O (Fig. 8c). When cells treated with 25 μ g mL⁻¹ CQDs/Cu₂O, the migration inhibition rate doesn't show significant change at 6, 12 and 24 h, mainly due to the strong cytotoxicity of high concentration of CQDs/Cu₂O, and the inhibitory effect on cell migration was shown in a short time and maintained for a period of time. CQDs/Cu₂O was demonstrated to exhibit remarkable concentration- and time-dependency, suggesting that it has the potential to suppress SKOV3 cell migration.

We found that the expression of MMP-2/9 sharply decreased after treatment with CQDs/Cu₂O for 24 h by western blot analysis (Fig. 8d). It has been reported the decrease of MMPs expression is considered to be the main factor for the inhibition potential of migration and angiogenesis. Carbon nanocapsules (HMCNs) and fullerene-based nanoparticle Gd@C₈₂(OH)₂₂ serve as potent migration inhibitors by downregulating MMP-2/9 [20, 50]. The results further indicated that CQDs/Cu₂O effectively suppressed SKOV3 cell migration by regulating the expression of metalloproteinase.

Effects of CQDs/Cu₂O on HUVEC blood vessel formation

Angiogenesis is required for tumor progression and metastasis to provide oxygen and nutrients. Targeting angiogenesis has become a unique perspective and strategy in anticancer therapy. DEGs analysis results showed that the SERPINB5 (known as Maspin) and THBS1 (known as TSP1) genes were significantly upregulated after CQDs/Cu₂O treatment. To confirm whether CQDs/Cu₂O has an anti-angiogenesis effect, a blood vessel formation in vitro model system was selected and the effect of CQDs/Cu₂O on new blood vessel formation in HUVECs cells was assessed. VEGF was used to induce endothelial cell proliferation, migration, and differentiation into blood vessel structures. As shown in Fig. 9a a robust and complete blood vessel produced after HUVECs cells treated with VEGF within 12 h. CQDs/Cu₂O effectively inhibited the length of blood vessel. A reduction of approximately 40~70% in total blood vessel length per field following treatment with 3.12 to 12.5 μ g mL⁻¹ CQDs/Cu₂O for 6 h. When the time was extended to 12 h, the inhibition percentage increased from 60 to 90%, suggesting a dose-dependent and a time-dependent decrease. The viability of HUVEC cells was also determined by MTT

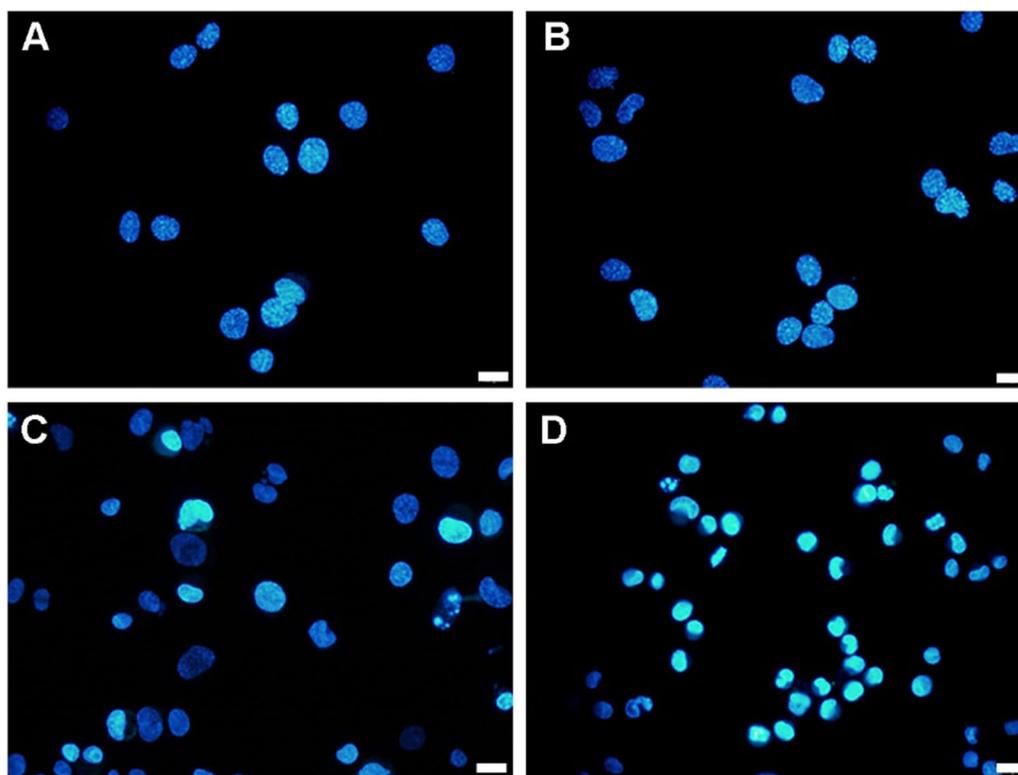


Fig. 6 Fluorescence photomicrographs changes by Hoechst 33342 staining of SKOV3 cells after treatment with CQDs (B), Cu₂O (C) and CQDs/Cu₂O (D) for 24 h compared to controls (A), respectively. Scale bar 20 μm

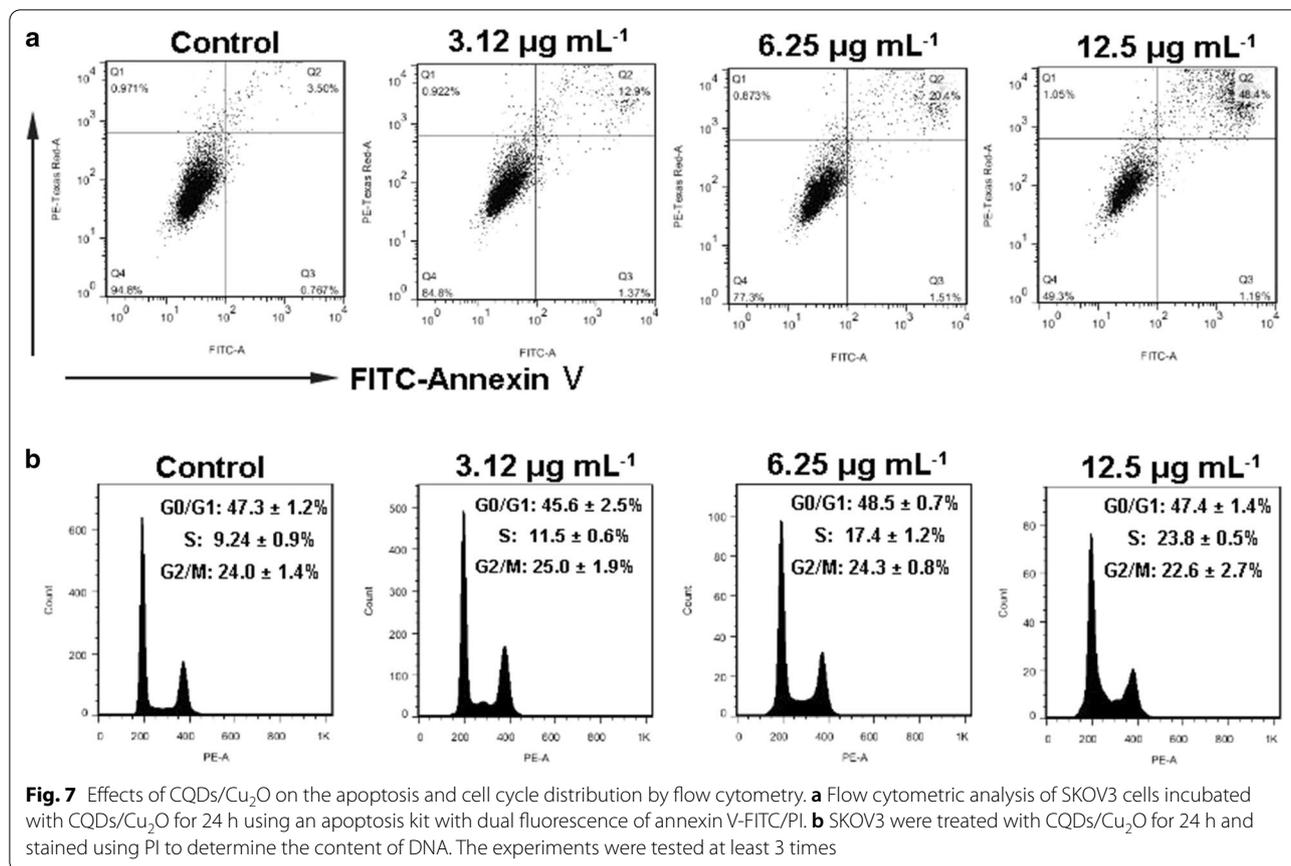
assay. It was shown that the inhibition rate was lower than 20% when HUVEC cells were treated with CQDs/Cu₂O for 12 h (Additional file 1: Figure S2), indicating that this concentration of CQDs/Cu₂O was non-toxic over a short period and antiangiogenic activity was not caused by the cytotoxicity of CQDs/Cu₂O.

Moreover, semaxanib (SU5416, 87 μM), as a selective inhibitor of VEGF, was used as the experimental control. CQDs/Cu₂O also had better antiangiogenic activity than SU5416 and Cu₂O (Fig. 9b). In Fig. 9c, the length of blood vessel induced by CQDs/Cu₂O (12.5 μg mL⁻¹) was about 1.8-fold shorter than the length of blood vessel induced by SU5416. The length was approximately 4.2-fold shorter than that caused by Cu₂O. CQDs/Cu₂O inhibited tube formation by ~90%, which was more than that with Cu₂O (53%) and SU5416 (80%) (Fig. 9d). It has been reported 80 μg mL⁻¹ AgNPs [19] suppressed about 80% blood vessel formation of bovine retinal endothelial cells. 12.5 μg mL⁻¹ Fe-MIL-101 [21] effectively suppressed about 60% blood vessel formation of HUVEC cells. It was suggested that CQDs/Cu₂O possessed better anti-angiogenic activity than AgNPs and Fe-MIL-101.

A variety of growth factors and cytokines are involved in regulating angiogenesis. VEGF, as one of the most important pro-angiogenic factors, combined to receptors VEGFR1 and VEGFR2, stimulating endothelial cell migration and blood vessel formation. [13, 17, 51] As shown in Fig. 8d, the expression of VEGFR2 decreased after treatment with CQDs/Cu₂O. Altogether, our results indicated that angiogenesis mediated by VEGFR2 and MMP-2/9, indicating that CQDs/Cu₂O may be a potential candidate in anti-angiogenic therapy.

CQDs/Cu₂O induced alterations in the cytoskeleton of SKOV3 cells

The cytoskeleton, which has a variety of biopolymer networks such as F-actin, microtubules, and intermediate filaments, plays a fundamental role in all eukaryotic cells [52]. Dynamic regulation of the F-actin cytoskeleton is essential for many physiological cellular processes, including cell adhesion, migration, division, and apoptosis of cancer cells. We investigated the effect of CQDs/Cu₂O on the F-actin cytoskeleton of SKOV3 cells. First, we observed the morphological changes of the F-actin cytoskeleton by FITC-conjugated phalloidin and PI immunofluorescence staining. As shown in Fig. 10,



SKOV3 cells in the control group displayed normal actin structure, single intact nucleus, and short F-actin bundles arranged around the cell membrane. After treated by CQDs/Cu₂O, a large number of fluorescence spots were scattered throughout the cytoplasm, gradually disappeared as the CQDs/Cu₂O concentration increased and the fluorescence intensity decreased, which significantly differed from that in normal F-actins. CQDs/Cu₂O might therefore exhibit enhanced damage and disruption of F-actin. We also evaluated the content of F-actin cytoskeleton after treated by CQDs/Cu₂O. The results of western blot demonstrates that the expression of F-actin in SKOV3 cells treated with CQDs/Cu₂O significantly was significantly lower than that in the control group

(Fig. 8d). It has been reported that some nanoparticles exhibit depolymerization effects or disrupt the cytoskeletal architecture and affect cell division, such as GO nanosheets [24], ZnO nanoparticles [25], gold nanoparticles [27], silver nanoparticles [27], and MOFs (IRMOF-3) [28]. Compared to the above nanoparticles and agents, we could demonstrate the disruption of the cytoskeleton filament owing to CQDs/Cu₂O exposure and prove that the expression of the F-actin cytoskeleton decreased at the protein level. Hence, the suppression of SKOV3 migration by CQDs/Cu₂O may be related to the down-regulation of F-actin. It may be unique perspective and strategy in anticancer therapy.

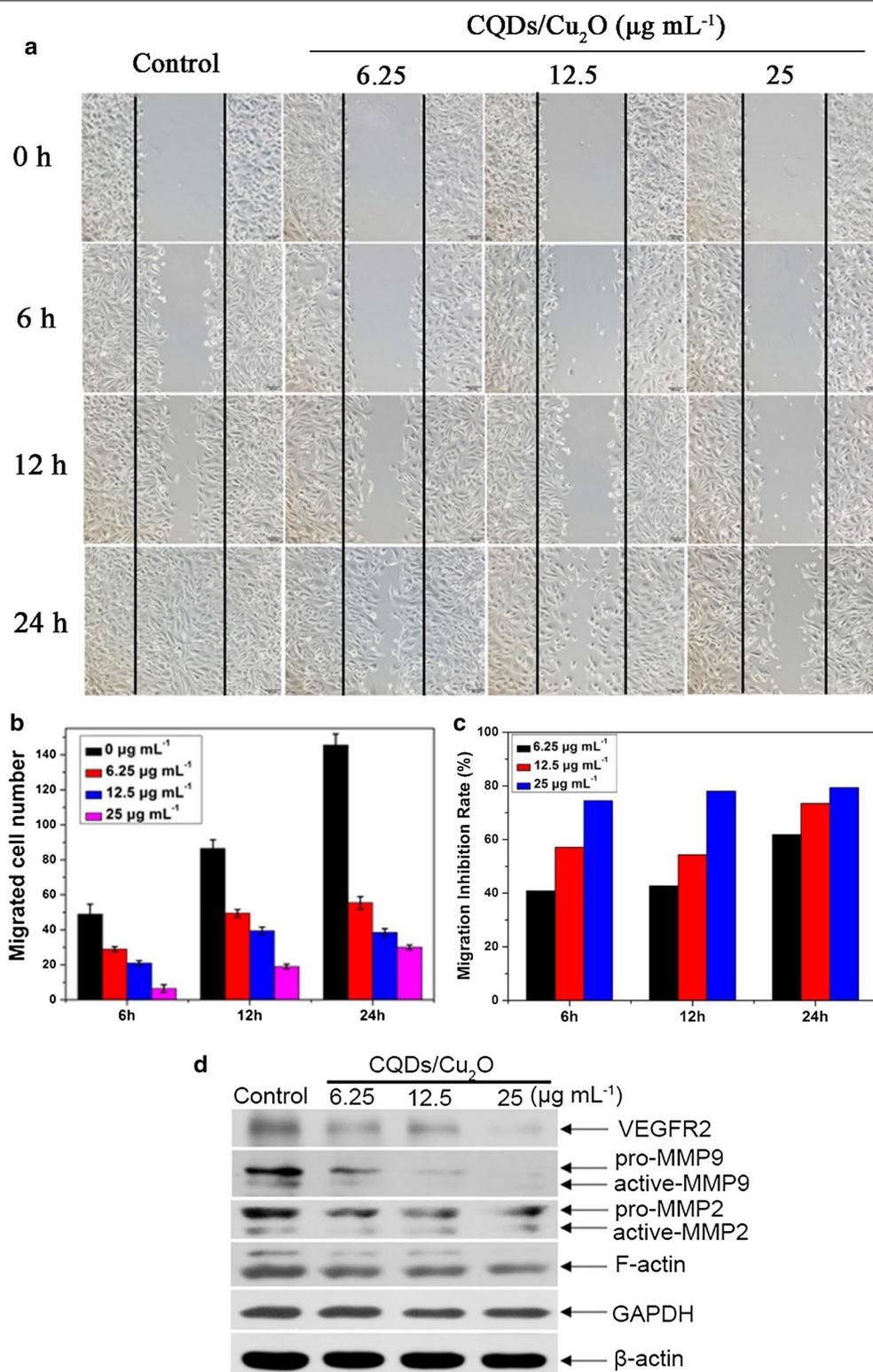


Fig. 8 Effect of CQDs/Cu₂O on SKOV3 cell migration by the wound-healing assay. **a** The “scratch” was produced by scraping the monolayer SKOV3 cells using a pipette tip after grown to form a confluent monolayer. (Scale bar 100 µm). The relative migration activity (**b**) and the inhibition rate of migration (**c**). The experiments were tested at least 3 times

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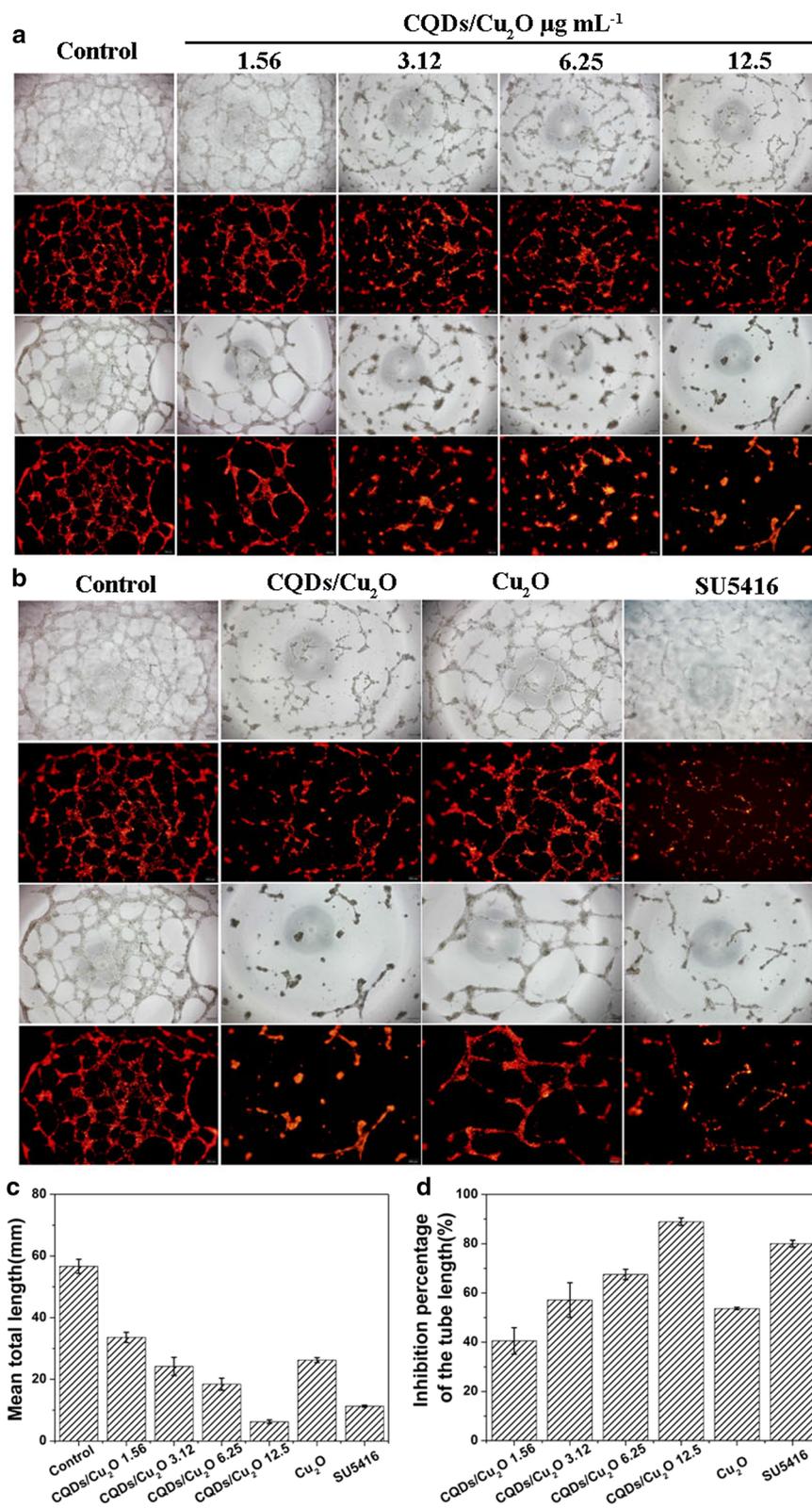


Fig. 9 Dil-labeled HUVECs were inoculated in Matrigel-coated wells and induced blood vessel formation. Scale bar, 200 µm. **a** CQDs/Cu₂O composite inhibited blood vessel formation. Scale bar, 200 µm. **b** The effect of 12.5 µg mL⁻¹ CQDs/Cu₂O (87 µM), Cu₂O (87 µM) and SU5416 (87 µM) on blood vessel formation. **c** Cumulative tube length in four fields/well and the inhibition percentage of the tube length

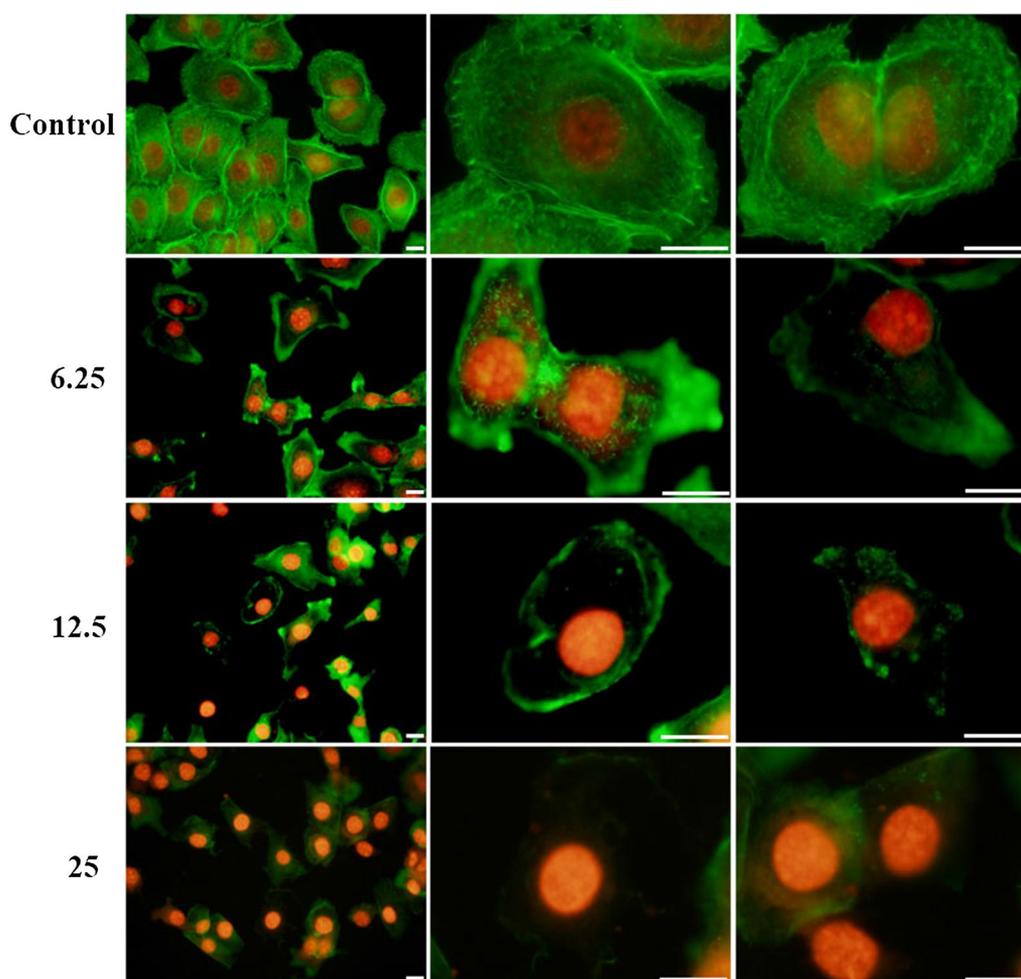


Fig. 10 Effects of CQDs/Cu₂O-stimulated disruption of the cytoskeleton. SKOV3 cells were stained using FITC-conjugated phalloidin and PI after treatment with CQDs/Cu₂O (0, 6.25, 12.5, 25 μg mL⁻¹) for 24 h. Images are representative of at least three experiments, Scale bar, 20 μm

Conclusions

In summary, we demonstrated for the first time that CQDs/Cu₂O composite displayed a greater sensitivity in SKOV3 cells than HeLa, A549, HT-29, HCT116 cancer cells and normal cells, such as BABL-3T3, HEK293T and J774A1, where the IC₅₀ value of CQDs/Cu₂O against SKOV3 cells was approximately threefold lower than other tested cancer cells and approximately 12-fold lower than normal cells. Amazingly, the IC₅₀ of CQDs/Cu₂O was approximately 114-fold and 75-fold lower than the IC₅₀ of commercial artesunate (ART) and oxaliplatin (OXA), indicating CQDs/Cu₂O possess strong antitumor activity than chemotherapeutics OXA and ART. This should be one of the most fascinating features of CQDs/Cu₂O.

Interestingly, CQDs/Cu₂O suppressed migration and angiogenesis processes in vitro mainly through down-regulation the expression of VEGFR2 and MMP-2/9, which may be one of the main reasons for the selective inhibition tumor, since angiogenesis is an effective tumor treatment strategy to provide oxygen and nutrients for tumour progression and metastasis. CQDs/Cu₂O also exhibited stronger antiangiogenic effects than commercial antiangiogenic inhibitor (SU5416). Furthermore, CQDs/Cu₂O induces alterations in the cytoskeleton of SKOV3 cells by disruption of F-actin cytoskeleton which is critical for numerous physical cellular processes, including cell adhesion, migration, division, and cancer cell apoptosis. It is worth noting that CQDs/Cu₂O also regulated angiogenesis-related genes in SKOV3 cells,

such as Maspin and TSP1 gene, to suppress angiogenesis. This is first time to elucidate selectively mediation of CQDs/Cu₂O in SKOV3 cells via transcriptome analysis, providing significant insights into the development and clinical application of CQDs/Cu₂O.

From the above reasons, a probable mechanism for inhibition SKOV3 by CQDs/Cu₂O has been proposed. CQDs/Cu₂O selectively mediated of ovarian cancer SKOV3 cells death mainly through downregulation the expression of MMP-2, MMP-9, F-actin, and VEGFR2, meanwhile CQDs/Cu₂O induced apoptosis of SKOV3 via S phase cell cycle arrest.

Our findings reveal a new role of CQDs/Cu₂O in the selective and restrictive ovarian cancer SKOV3 cells. These findings could open a new avenue for using CQDs/Cu₂O composite as potential therapeutics for cancer chemotherapy.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12951-021-00813-8>.

Additional file 1: Figure S1. The enriched GO terms in the DEGs of cells treated by CQDs/Cu₂O (3.12, 12.50 µg mL⁻¹). The green, red, and blue bars represent the terms of biological process, cellular component, and molecular function, respectively. **Figure S2.** The viability of HUVEC cells after treated with CQDs/Cu₂O by the MTT assay for 12 h.

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Authors' contributions

BL and JW conceived the idea, supervised all aspects of this project, and contributed to the paper and wrote the manuscript. DC designed the experiments, carried out the part of experiments, and wrote major part of the manuscript. MN carried out major part of the experiments. They analyzed the data, were involved in discussions, critical assessment, and manuscript improvements. TL, YY, CX and ZH synthesized and characterized the samples. TL and DN contributed to revised manuscript, carried out part of the experiments and prepared the response sheet. All authors read and approved manuscript.

Declarations

Competing interests

There are no conflicts to declare.

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