Cytotoxicity of curcumin against CD44± prostate cancer cells: Roles of miR-383 and miR-708

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Abstract

Cancer stem cells (CSCs) remaining in the tumor tissues after applying treatments may cause recurrence or metastasis of prostate cancer (PC). Curcumin has the promising potential to target CSCs. Here, we aim to evaluate the cytotoxic effects of curcumin on the expression of miR-383-5p and miR-708-5p and their target genes in $CD44^+$ CSCs and $CD44^-$ non-CSCs isolated from the PC3 prostate cancer cell line.

We used MTT assay to determine the optimal cytotoxic dose of curcumin on $CD44^\pm$ PC cells. Then, we assessed nuclear morphological changes using DAPi staining. We used Annexin V-FITC/PI to quantify apoptotic cell death. qRT-PCR was also used to detect miRNA and gene expression levels after curcumin treatment.

Curcumin significantly enhanced the apoptosis in both $CD44^-$ and $CD44^+$ PC cells in a dose-dependent manner (*P*-value < 0.05). The cytotoxicity of curcumin against $CD44^-$ cells (IC_{50} = 40.30 ± 2.32 μM) was found to be more effective than $CD44^+$ cells (IC_{50} = 83.31 ± 2.91 μM). Also, curcumin promoted miR-383-5p and miR-708-5p overexpression while downregulating their target genes LDHA, PRDX3, and RAP1B, LSD1, respectively.

Our findings indicate that curcumin, by promoting the expression of tumor suppressors, miR-383-5p and miR-708-5p, and inhibiting their target genes, induced its cytotoxicity against $CD44^\pm$ PC cells. We trust that curcumin could be established as a promising adjuvant therapy to current PC treatment options with more research in the clinical field.

Introduction

According to GLOBOCAN 2018, prostate cancer (PC) incidence is almost 1.3 million per year. In 2018, PC accounted for 359,000 deaths worldwide [1]. Furthermore, importantly, it causes physical and emotional damage to patients. A more and deeper understanding of PC's pathogenesis and molecular basis is critical to improving treatments [2]. Despite its high prevalence, there is insufficient data about PC etiology. In addition to ethnic and genetic predisposition, obesity has also been associated with a high frequency of PC. The probability of PC increases more than 2.5 fold if first-degree families are affected. It is estimated that genetic factors play a critical role in 40% of early-onset PCs and 5-10% of all PCs [3]. The prognosis of patients diagnosed with localized lesions of PC is good, but the survival rate of metastatic cases drops down to 31%. Radical surgery, radiation therapy, cryotherapy, and androgen-deprivation therapy are commonly used to treat PC according to the stage of the tumors. Although applying appropriate treatments abolish the main part of cancer stem cells (CSCs) remaining in the tumor tissues may cause recurrence or metastasis of the tumor [4]. CSCs, a small subset of tumor cells, have the ability of self-renewal and asymmetric division, making them less vulnerable to chemotherapy and radiotherapy. So, the reason behind the failure of chemotherapy is the inability of current chemotherapeutics to eliminate CSCs [5]. Several markers such as CD44, CD133, and ABCG2 have been introduced to identify prostate CSCs. CD44 is one of the main prostate CSC tumor markers, and its high

expression has been associated with tumor proliferation, stemness gene expression, and metastatic colonization [4].

microRNAs (miRNAs) are small non-coding RNAs that alter gene expression by post-transcriptional silencing of the genes. miRNAs such as miR-708-5p and miR-383-5p have been remarkably downregulated in PC. Recent studies have approved that CD44 is a direct target of miR-708 and miR-383. miR-708 is a negative regulator of $CD44^+$ prostate CSCs, which can be used to indicate prognosis and diagnosis of PC. Induction of ectopic expression of miR-383 has inhibited the tumorigenesis process and metastasis in $CD44^+$ prostate CSCs. Downregulation of miR-708 and miR-383 has been correlated with poor prognosis, tumor growth, and recurrence in clinical specimens. Together, overexpressing mir-708 and miR-383 could serve as an excellent attempt to treat metastatic PC [6, 7].

Natural products are valuable resources that have been frequently used to discover and produce anti-cancer drugs [8]. Curcumin or diferuloylmethane is a bioactive metabolite derived from the *Curcuma longa L.* plant, primarily used in Chinese traditional medicine. Multiple studies have shown that curcumin and its analogs have promising potential to target CSCs [9, 10]. However, studies suggest that curcumin causes anti-cancer effects by regulating miRNAs and their downstream molecular pathways [11, 12], the role of curcumin in the regulation of miR-708 and miR-383 expression has not been elucidated yet.

According to these facts, our objective is to assess the molecular mechanism of cytotoxicity of curcumin and the efficacy of curcumin on the regulation of miR-708-5p, miR-383-5p, and their target genes in prostate CSCs and non-CSCs.

Study Methodology

Drugs and reagents

Curcumin (C1386) and MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, M2128) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Curcumin was dissolved in dimethylsulfoxide (DMSO) and stored at -20º C.

Cell culture

The human prostate cancer cell line, PC3, was obtained from the National Cell Bank of

Iran (NCBI, Pasteur Institute, Tehran, Iran). Cells were grown in RPMI 1640 medium (Gibco, UK), containing 10 % fetal bovine serum (FBS, Gibco), penicillin, and streptomycin. Finally, The cells were incubated in a humidified atmosphere of 5% CO_2 at 37˚C.

Isolation of $CD44^{\pm}$ cells by magnetic-activated cell sorting (MACS)

We used MACS for both positive selection ($CD44^+$ CSCs) and negative selection ($CD44^-$ non-CSCs). Concisely, PC3 prostate cancer cells were trypsinized and collected. Then, 10^6 cells were stained with anti-

human CD44 primary antibody (Miltenyi Biotec, 130-095-180). After incubating the cell suspension with Anti-PE Micro Bead (Miltenyi Biotec,130-048-801), the cells were passed through a MACS column within a magnetic field. $CD44^+$ cells carrying the magnetic beads were retained inside the column adsorbed onto the surface. We sorted the cells twice to achieve more than 95% pure cells.

MTT assay

Concisely, the $CD44^{\pm}$ prostate cancer cells were seeded in 96 well plates. The $CD44^-$ cells were incubated in a medium containing 0-70 μM curcumin. The $CD44^+$ prostate cancer cells were treated with 0-150 μM curcumin. After 48 h of incubation, the medium was replaced with 180 mL FBS-free medium and 20 ml MTT (Sigma, M2128). Then the cells were incubated for 4 h at 37 ºC. Then, 200 mL DMSO (ScharlauChemie, and Barcelona, Spain) was used to dissolve formazan crystals. Finally, the absorbance was measured at 570 nm using an ELISA reader.

Apoptosis assessment

The cytotoxicity and nuclear morphological changes induced by curcumin were examined by DAPi staining. Briefly, the $CD44^{\pm}$ cells (5×10^4 cells/well) were seeded into 6-well plates. The $CD44^-$ and $CD44^+$ cells were treated with 15- 60 μM and 50-150 μM curcumin, respectively. Then, the cells were fixed and stained with DAPi.

Quantification of gene expression by real-time PCR

Total RNA was extracted from $CD44^{\pm}$ prostate cancer cells using TRIzol reagent (Invitrogen). The total RNA was reverse-transcribed into cDNA using a Viva 2-steps RT-PCR kit (Vivantis, USA). Briefly, we used SYBR Green PCR Master Mix (EURx, Ltd, da s, Poland) to perform qRT-PCR, and gene expression was analyzed using a Light Cycler 96 real-time PCR System (Roche Applied Science) [13, 14]. Fold changes in gene expression were calculated using the ΔΔCt method relative to GAPDH gene expression. The primers are listed in Table 1.

Quantitation of microRNAs by real-time PCR

The expression levels of miR-383-5p and miR-708-5p were measured using BON-miR miRNA cDNA Synthesis Kit (Cat: BON209001). The following primers were used in qPCR reactions: miR-383-5p (TAGATCAGAAGGTGATTG), miR-708-5p (GGAAGGAGCTTACAATCTA), and U6 (AAGGATGACACGCAAA). miRNA expression fold changes were evaluated using the comparative ΔΔCt method [15].

Flow cytometry

Human $CD44^{\pm}$ prostate cancer cells were cultivated at a density of 5×10^5 cells/well in a 6-well plate. The $CD44^{\pm}$ cells were treated with subtoxic concertation of curcumin for 48h. The cells were counterstained with Annexin V-FITC (fluorescein isothiocyanate)/PI (propidium iodide) (IQ products, Netherlands) staining assay. Briefly, the cells were centrifuged and rinsed with calcium-binding buffer. Annexin V/FITC

was added to the cell suspension. Then, the cells were washed with calcium-binding buffer, and propidium iodide (PI) was added. Finally, analysis was performed by a flow cytometer (Partec CyFlow®).

Statistical analysis

Data analysis was performed using SPSS V.21 software. One-way ANOVA with Tukey's post hoc test was used to determine the statistical comparisons. A *P*-value less than 0.05 was considered significant. The standard curves were drawn using sigma plot V .11 software. (Systat Software, San Jose, CA).

Results

Curcumin induces cytotoxicity against $CD44^-$ cells more than $CD44^+$ prostate cancer cells.

First, we sorted $CD44^{\pm}$ cells from the PC3 prostate cancer cell line using the MACS method. $CD44^+$ PC3 cells are shown in Fig. 1. Then, $CD44^{\pm}$ prostate cancer cells were treated with various concentrations of curcumin. The cell survival rate was determined using an MTT cell proliferation assay. The IC_{50} values of curcumin for $CD44^-$ and $CD44^+$ prostate cancer cells were 40.30 ± 2.32 μM and 83.31 ± 2.91 μM, respectively.

Our results confirmed that curcumin inhibited both $CD44^-$ and $CD44^+$ prostate cancer cells in a dose-dependent manner. The cytotoxicity of curcumin against $CD44^-$ cells was more effective than $CD44^+$ cells. Indeed, $CD44^+$ cells were more resistant to curcumin treatment. Cell survival curves after treatment with curcumin are demonstrated in Fig. 2.

The role of curcumin treatment on morphology and apoptosis

We assessed nuclear morphological changes using DAPi staining. In a dose-dependent manner, curcumin significantly enhanced apoptosis in both $CD44^-$ and $CD44^+$ PC3 cells. Autophagic cell morphology was also seen after treatment with high doses of curcumin in $CD44^{\pm}$ PC3 cells. (Fig. 3 and Fig. 4).

Indeed, we used Annexin V-FITC/PI to quantify apoptotic cell death and to evaluate whether curcumin could inhibit cell growth by induction of apoptosis. Flow cytometric results revealed that curcumin treatment significantly increased apoptotic cell rate compared to the control group in both $CD44^-$ and $CD44^+$ PC3 cells (*P*-value < 0.001). As shown in Fig. 5a and 5b, 15 and 30 μM concentrations of curcumin increased apoptotic rate in $CD44^-$ PC3 cells by 30.15 ± 4.03 % and 39.72 ± 1.61 %, respectively. In $CD44^+$ PC3 cells, 50 and 80 μM concentrations of curcumin increased apoptotic rate by 34.57 ± 1.30 % and 43.86 ± 1.52 %, respectively (Fig. 5c and 5d).

Curcumin promoted the expression of miR-383-5p and miR-708-5p while downregulating their target genes.

We used miRBase and Target scan bioinformatic algorithms to search for potential target genes of miR-383-5p and miR-708-5p. According to this in silico analysis and previous studies, we confirmed LDHA and PRDX3 as potential targets of miR-383-5p, and RAP1B and LSD1 as potential targets of miR-708-5p.

To investigate the possible involvement of miR-708-5p and miR-383-5p in curcumin's anti-PC function, we detected these miRNAs' expression by real time-PCR. As shown in Fig 6a and 6d, curcumin significantly increased the expressions of miR-383-5p in both $CD44^+$ and $CD44^-$ prostate cancer cells.

Then, we conducted real-time PCR to investigate whether curcumin could inhibit target genes of miR-383-5p in the $CD44^{\pm}$ PC3 cells. As shown in Fig. 6b and 6c, both 15 and 30 μM concentrations of curcumin significantly inhibited LDHA and PRDX3 (downstream targets of miR-383-5p) in $CD44^-$ cells. Treatment of the $CD44^+$ cells with 80 μM curcumin significantly inhibited LDHA and PRDX3, but 50 μM curcumin did not significantly alter the expression of LDHA and PRDX3 (Fig 6e and 6f).

Also, we observed that curcumin was significantly increased the expression of miR-708-5p in the $CD44^{\pm}$ PC3 cells (Fig 7a and 7d). In $CD44^-$ cells, curcumin (15 and 30 μM) significantly inhibited the expression of downstream target genes of miR-708-5p, RAP1B, and LSD1 (Fig 7b and 7c). In $CD44^+$ cells, 80 μM curcumin significantly inhibited the expression of RAP1B and LSD1. However, 50 μM curcumin only inhibited the expression of LSD1, but the alteration in the expression of RAP1B was statistically insignificant (Fig 7e and 7f).

Altogether, in $CD44^+$ PC cells, the inducing effects of curcumin on the overexpression of miR-708-5p (Fold change = 3.14 ± 0.30) was more significant than miR-383-5p (Fold change = 2.07 ± 0.26). However, in $CD44^-$ PC cells, the expression of miR-383-5p (Fold change = 2.88 ± 0.79) was more significantly induced by curcumin than miR-708-5p (Fold change = 2.23 ± 0.56).

Discussion

Many medicinal plants are used as attempts to prevent and treat PC. Natural bioactive molecules derived from these plants possess chemopreventive and chemotherapeutic features. Recent studies on plant extracts of tomato, soy, ginger, and garlic have shown that bioactive molecules derived from these extracts could diminish chemoresistance and tumor growth [16–19]. Curcumin is a phytochemical product whose anti-PC effects on the inhibition of proliferation, invasion, cellular adhesion, and angiogenesis of many tumors have been confirmed [20].

Here, we found that curcumin exerted its cytotoxic effects against both prostate $CD44^+$ CSCs and $CD44^-$ non-CSCs through induction of apoptosis in a dose-dependent manner. Also, our results revealed that $CD44^+$ cells were more resistant to curcumin than $CD44^-$ cells. As mentioned earlier, recent studies introduced miRNAs as the new target of curcumin in the treatment of cancer [11]. Especially, curcumin by regulating microRNA expression patterns in prostate CSCs has been shown to mitigate invasive and proliferative characteristics of PC. Similar to our findings, Zhang et al. revealed that curcumin suppressed

proliferative and invasive characteristics of human prostate CSCs through induction of expression of specific miRNAs in the DLK1-DIO3 miRNA cluster [21]. Also, Liu et al. showed that curcumin treatment arrested cell cycle and inhibited proliferation and metastasis in the human prostate CSCs. They proposed that curcumin upregulates miR-145 and decreases proliferative characteristics of human prostate CSCs by inhibiting Oct4 expression [22].

In this study, we used curcumin as an attempt to induce the expression of tumor suppressor miRNAs, miR-708-5p and miR-383-5p in the human prostate $CD44^+$ CSCs and $CD44^-$ non-CSCs. miR-708 was first introduced as a therapeutic target of PC by Saini et al. They have revealed that induction of miR-708 expression in $CD44^+$ PC cells suppressed tumorigenicity. In contrast, the knockdown of miR-708 increased tumor growth in $CD44^-$ PC cells [6]. Our results showed that curcumin significantly increased the expression level of miR-708-5p in both $CD44^+$ and $CD44^-$ cells in a dose-dependent manner.

The therapeutic roles of miR-708 also have been confirmed in many cancers, including prostate, breast, cervical and pancreatic carcinoma [23–25]. Similar to our study, many attempts have been made to induce the expression of miR-708 in different cancers. The low expression of miR-708 is involved in the neuroendocrine differentiation of PC, which causes a poor prognosis in patients. Sham et al. found that the overexpression of EZH2 inhibits miR-708. So, EZH2/miR-708 pathway could serve as an important target in treating the neuroendocrine PC. They also found that induction of miR-708 reduces the frequency of $CD44^+$ neuroendocrine PC cells [26]. Metformin, an anti-diabetic biguanide agent, has shown promising effects in inducing miR-708 expression in prostate and breast cancer. By targeting miR-708/NNAT and miR-708/CD47 pathways, metformin induces apoptosis and inhibits chemoresistance and self-renewal in prostate and breast cancers [27, 28]. In another study, glucocorticoids commonly prescribed as an adjuvant regimen for ovarian cancer patients have been shown to reduce invasive characteristics of ovarian cancer by inducing expression of miR-708 and inhibiting RAP1B [29].

Also, our results showed that curcumin suppressed the expression of RAP1B and LSD1, two target genes of miR-708, which are also crucial in the pathogenesis of PC. Previous studies have shown that LSD1 suppresses PC cell viability [30]. Furthermore, RAP1B expression is correlated with immune infiltration of tumors in various cancers [31].

Since CD44 is a hyaluronic acid receptor, it has a crucial role in the adhesion and migration of cancerous cells. At first, Bucay et al. showed that miR-383-5p, which is highly downregulated in PC, could be essential for PC metastasis and stemness by directly targeting CD44. The restoration of miR-383 expression in PC cells has proven anti-metastatic and anti-tumorigenic effects [7].

Our results revealed that curcumin significantly increased the expression levels of miR-383-5p in both $CD44^+$ and $CD44^-$ cells. In a similar study, Lv et al. showed that allicin, a phytochemical extracted from garlic, induces miR-383 expression in gastric cancer. Allicin targets the miR-383/ERBB4 axis to inhibit invasion of gastric cancer [32]. A recent study conducted by Huang et al. also approved that induction of miR-383 expression could inhibit growth and metastasis in PC [33]. Conversely, downregulation of miR-

383 by TMPO-AS1 in lung adenocarcinoma and pancreatic carcinoma and MIR4435-2HG in head and neck squamous cell carcinoma leads to tumor progression [34–36].

Although tumor suppressive roles of miR-383 have been confirmed in various cancers such as malignant melanoma and glioma, Wan et al. showed that high miR-383 expression in cholangiocarcinoma is associated with advanced tumor stages, metastasis, and poor prognosis [37–39].

Our results also confirmed the downregulation of PRDX3 and LDHA, target genes of miR-383, by curcumin in $CD44^{\pm}$ PC cells. PRDX3 and LDHA are typically overexpressed in PC. PRDX3, acting as an antioxidant, protects PC cells from reactive oxygen species and promotes the survival of cancerous cells [40]. LDHA plays a vital role in the Warburg effect (aerobic glycolysis), which provides a sufficient amount of metabolites for the rapid growth of cancerous cells. Vieira et al. showed that overexpression of LDHA in the clinical specimen of PC patients is associated with therapeutic resistance [41]. In another study, the MYC-LDHA axis was shown to be important in PC pathogenesis, and FAM46B by targeting this axis induces apoptosis and inhibits glycolysis in PC cells [42]. Altogether, suppression of expression of PRDX3 and LDHA is another aspect of promising roles of curcumin in PC treatment.

Conclusion

Our present study revealed that curcumin suppressed cellular proliferation and induced apoptosis in $CD44^{+}$ and $CD44^{-}$ PC cells. We also showed that curcumin, by promoting the expression of miR-383 and miR-708, and inhibiting their target genes, induced its cytotoxicity against $CD44^{\pm}$ PC cells. Considering that traditional cancer chemotherapeutic agents and radiotherapy cannot eradicate CSCs, cytotoxicity of curcumin against $CD44^{+}$ human prostate CSCs is a remarkable feature. We trust that curcumin could be established as a promising adjuvant to current PC treatment options with more research in the clinical field.

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Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

All authors contributed to the study conception and design. NN conceived the idea and supervised the study. Material preparation, data collection and analysis were performed by N.N, M-A.V, F.J, K.N-K, and R.P. The first draft of the manuscript was written by R.P and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Tables

Table 1 Primers used in qRT-PCR reactions

	Forward	Reverse	Product size
LDHA	ATGGCAACTCTAAAGGATCAGC	CCAACCCCAACAACTGTAATCT	86
PRDX3	CAAGCAAAATTATTCAGCACCA	CCCCTTAAAGTCATCAAGGCT	129
RAP1B	TTTATTCCATCACAGCACAGTCC	TTTCTGTTAATTTGCCGCACTAGG	262
LSD1	GGCTCGGGGCTCTTATTCCTA	CCCCAAAAACTGGTCTGCAAT	226
GAPDH	ACATCATCCCTGCCTCTACTG	CCTGCTTCACCACCTTCTTG	180

Figures

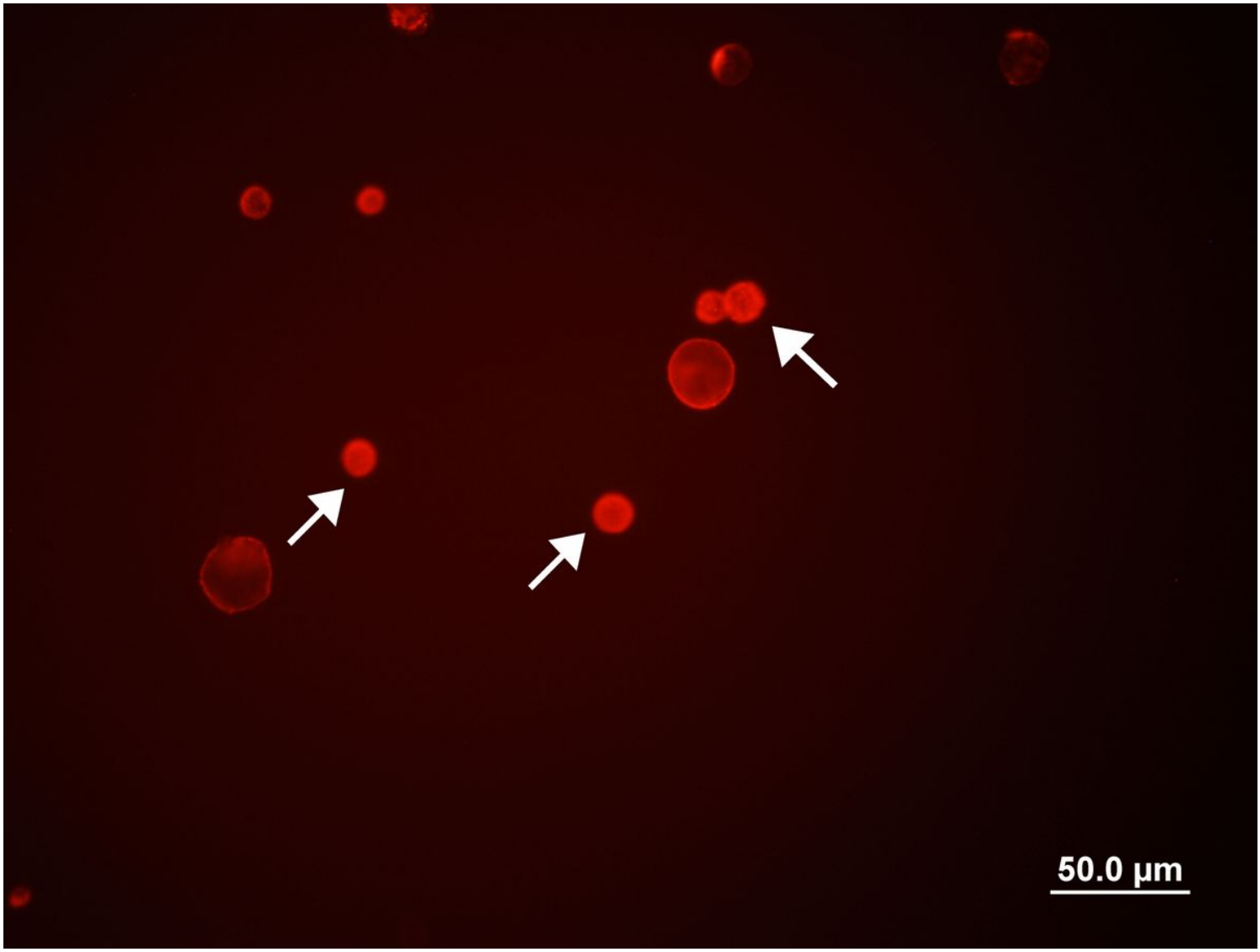

Figure 1

Isolation of $CD44^{\pm}$ cells from PC3 cell line using MACS. $CD44^{+}$ cells were isolated and identified by fluorescence microscopy. The arrows show $CD44^{+}$ prostate CSCs.

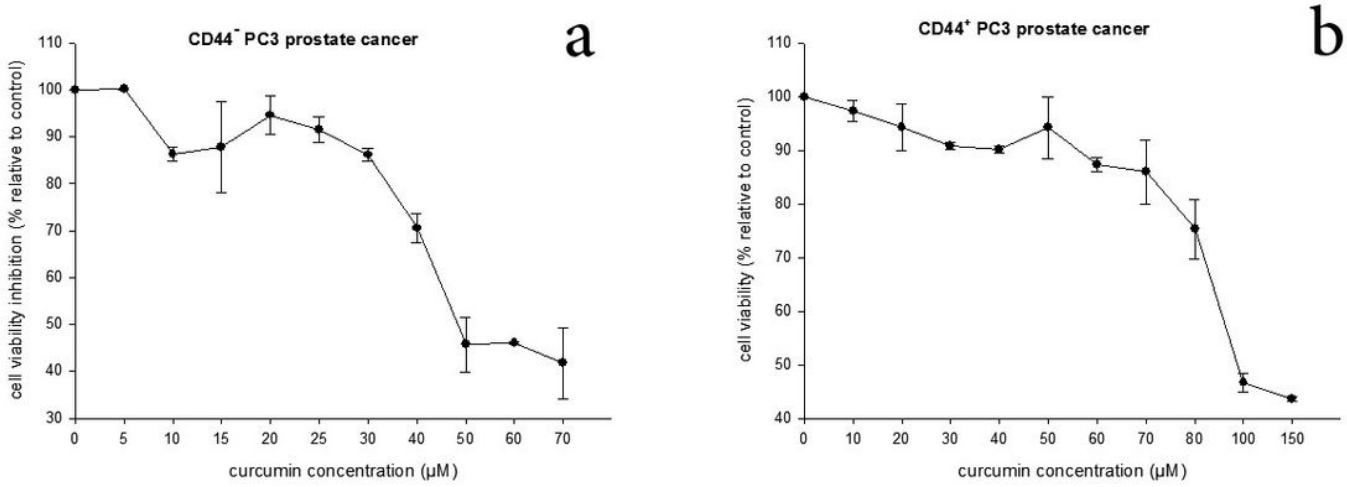

Figure 2

Standard curve of the viability of CD44$^{\pm}$ PC3 cells treated with different concentrations of curcumin.

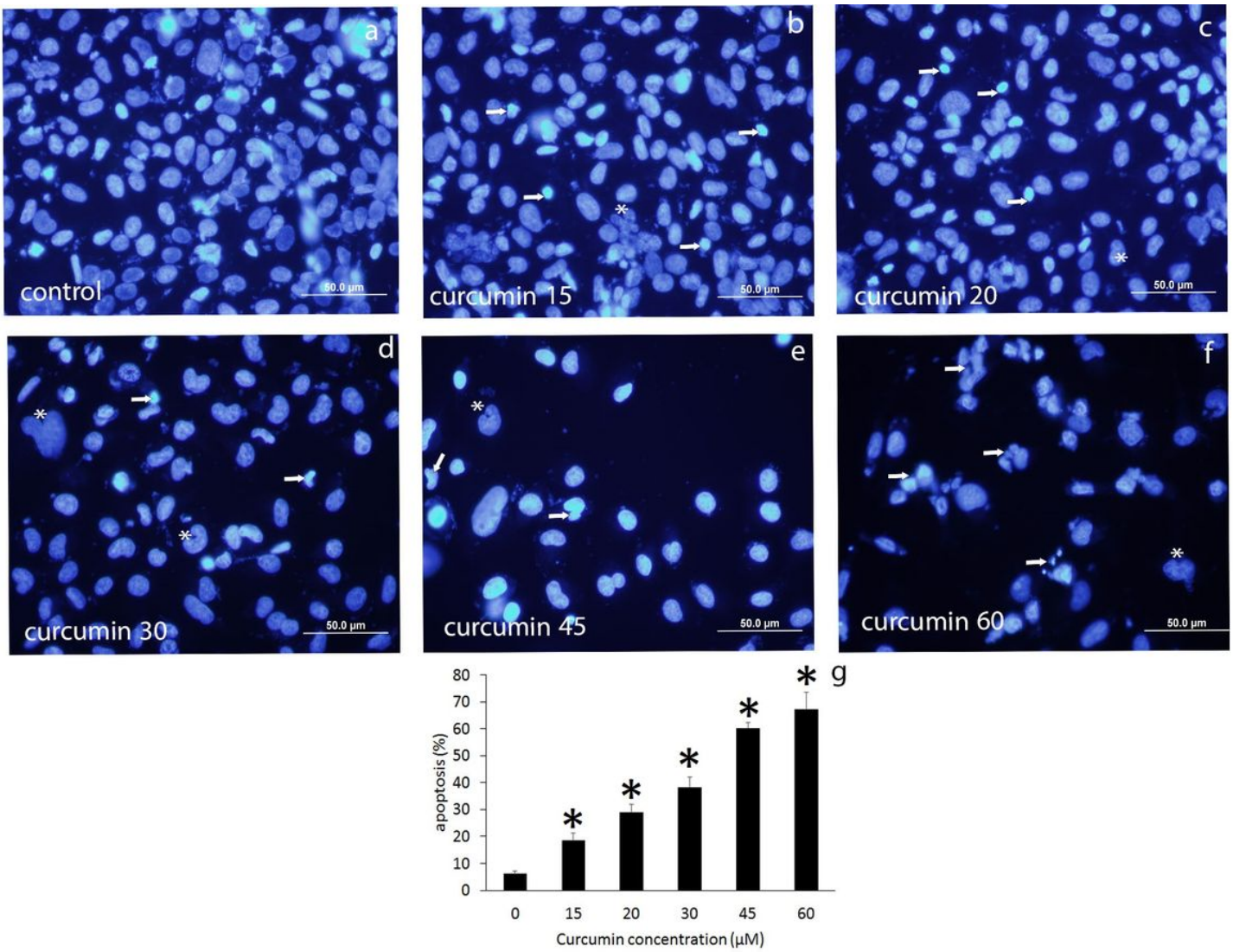

Figure 3

DAPi staining of CD44^{-} PC3 cells treated with 15 – 60 μM curcumin. Apoptotic cells are shown with arrows. Autophagic cells are marked with asterisks (a-g). *P-value < 0.005 vs. control.

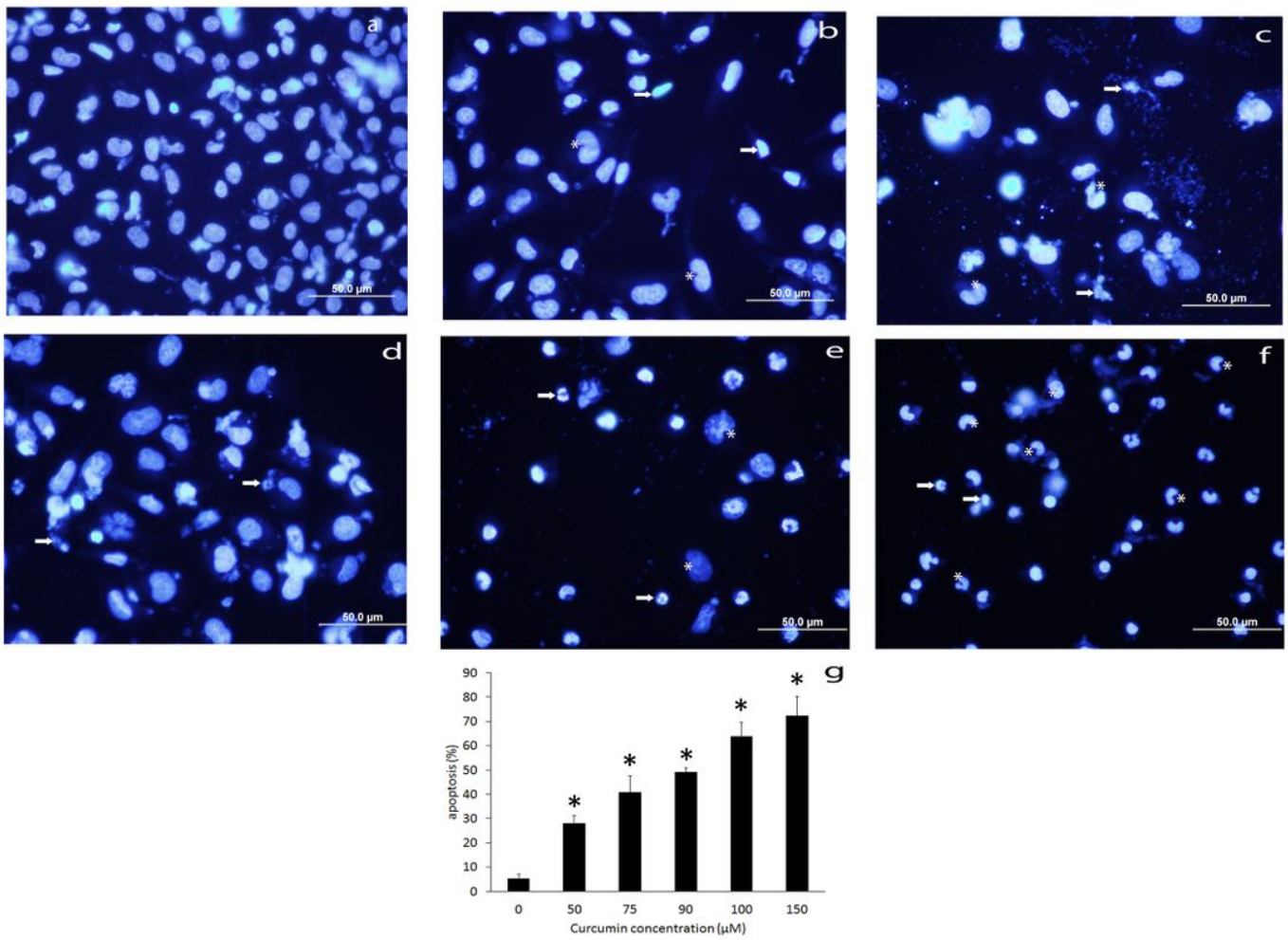

Figure 4

DAPi staining of CD44^{+} PC3 cells treated with 50 – 150 μM curcumin. Apoptotic bodies are shown with arrows and autophagic cells are marked with asterisks (a-g). *P-value < 0.005 vs. control.

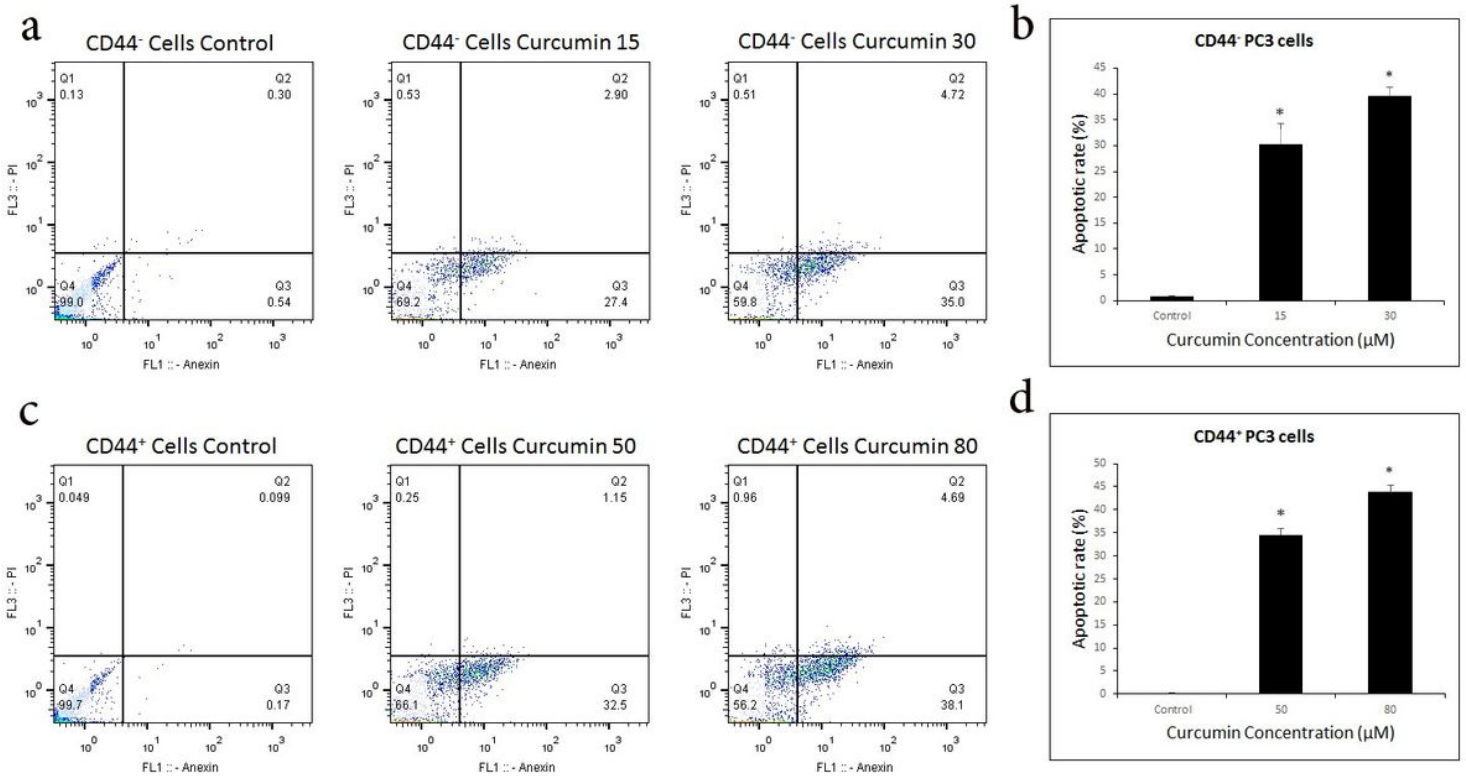

Figure 5

Effects of curcumin on the apoptosis of CD44$^{\pm}$ PC3 cells. (A, C) Flow cytometric analysis and (B, D) apoptotic rates of CD44$^{\pm}$ PC3 cells treated with subtoxic concentrations of curcumin for 48h. Live cells: Q4, Apoptosis: Q2 and Q3, Necrotic cells: Q1. *P-value < 0.001 vs. control. PI, propidium iodide.

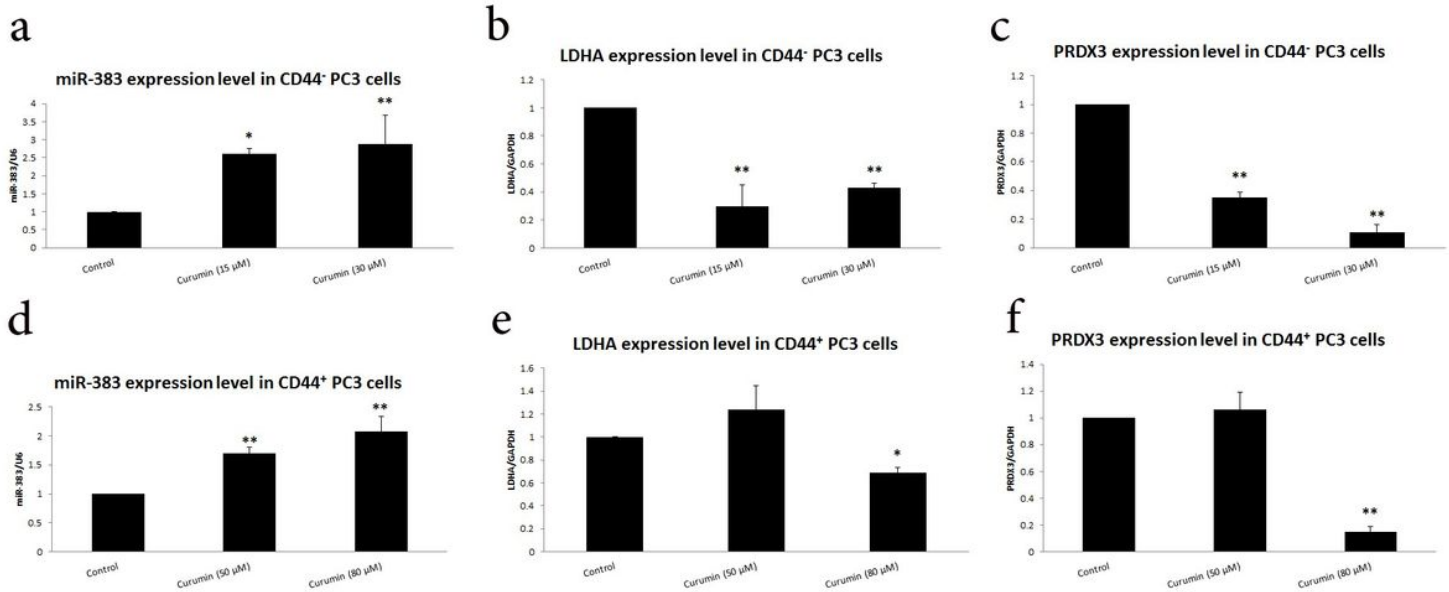

Figure 6

Curcumin induced the expression of miR-383 while suppressing the expression of LDHA and PRDX3 in CD44$^{\pm}$ PC3 cells. The CD44$^{\pm}$ PC3 cells were exposed to the subtoxic concentrations of curcumin according to IC_{50} values. qRT-PCR was used for the detection of miRNA and gene expression levels after curcumin treatment for 24h. All tests are performed three times. **P*-value < 0.05 and ***P*-value < 0.01

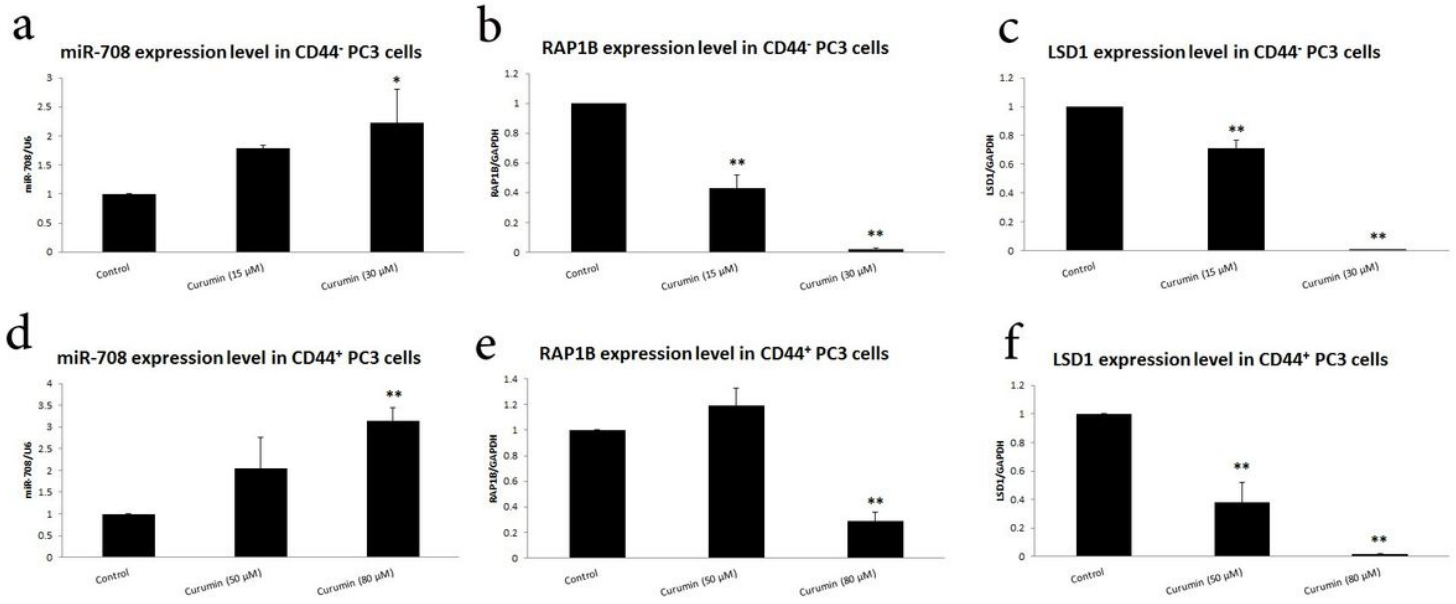

Figure 7

Curcumin induced the expression of miR-708-5p while suppressing the expression of RAP1B and LSD1 in CD44$^{\pm}$ PC3 cells. The cells were exposed to a subtoxic concentration of curcumin. qRT-PCR was used for the detection of miRNA and gene expression levels after curcumin treatment for 24h. All tests are performed three times. **P*-value < 0.05 and ***P*-value < 0.01