Overexpression of splicing factor poly(rC)-binding protein 1 elicits cycle arrest, apoptosis induction, and p73 splicing in human cervical carcinoma cells

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Abstract

Background Splicing factor poly(rC)-binding protein 1 (PCBP1) is a novel tumor suppressor that is downregulated in many cancers thereby regulates tumor formation and metastasis. However, to date, little information has been available on the molecular mechanisms by which PCBP1 evokes apoptosis.

Results Here, we explored the molecular mechanism by which PCBP1 triggers apoptosis in human cervical cancer cells. We testified that overexpression of PCBP1 greatly repressed proliferation of HeLa cells in time-dependent manner. It also induced a significant increase in G2 / M phase arrest and apoptosis. Furthermore, it was shown that overexpression of PCBP1 caused p73 splicing, and thus efficiently downregulated the ratio of Bax / Bcl-2, the release of cytochrome c and the expression of caspase-3.

Conclusion Our results revealed that PCBP1 played a vital role in cycle arrest, apoptosis induction, and p73 splicing in human cervical carcinoma cells and targeting PCBP1 may be a promising approach in cervical cancer therapy.

Background

Cervical cancer ranks as the fourth most frequently diagnosed cancer and the fourth leading cause of cancer death in women [1]. Therefore, research on its pathogenesis is crucial. Poly(rC) binding protein 1 (PCBP1) is a member of the RNA binding protein family and is firstly cloned from human lymphocyte cDNA library in 1994 [2]. With the deepening of research in recent years, PCBP1 has been found to be ubiquitously expressed in many tissues and plays a multifunction role in various life activities, such as intracellular transcription and post-transcriptional regulation, including alternative splicing of pre-mRNA, mRNA stability and translation [3]. In recent years, researches focus on the relationship between PCBP1 and tumors. Studies have been found that PCBP1 acts as a tumor suppressor in tumors and the expression is significantly downregulated in various tumors, including gastric cancer [4], acute myeloid leukemia [5], non-small-cell lung cancer [6], cervical cancer [7] et al. In general, PCBP1 plays a multifunctional role in tumor progress. For instance, PCBP1 has an influence on apoptosis in variety cancers [8, 9]. It is also involved in alternative splicing which dysregulation usually leads to disease and is increasingly associated with tumorigenesis [10, 11]. In addition, PCBP1 negatively regulated the tumor hypoxic microenvironment and inhibited autophagy to further affect the tumor formation and development [8, 12, 13]. Moreover, PCBP1 prevented the process of EMT to reduce cancer metastasis [6]. The above cases all turn out that PCBP1 is involved in the development of tumors as a tumor suppressor, but little information has been available on the molecular mechanisms by which PCBP1 causes cervical cancer apoptosis. In the present study, we would provide some preliminary data to illustrate the distinct functions of PCBP1 in p73 alternative splicing and the mechanisms of PCBP1 on cervical cancer cells apoptosis. The results suggested that PCBP1 may be an attractive novel target for cervical cancer therapy.

Results

The expression of PCBP1 and its spacial distribution

To explore the biologic function of PCBP1, we initially transfected HeLa cells with pEGFP-N1 or pEGFP-N1-PCBP1, and then we verified whether the PCBP1 was successfully transfected and overexpressed in cells. We performed real-time PCR and immunofluorescence experiments. The results demonstrated that PCBP1 mRNA expression was significantly increased in cells transfected with pEGFP-N1-PCBP1 compared to mock group (Fig. 1a) and there was no significant difference in mRNA expression in mock and vector group (Fig. 1a). In addition, further immunofluorescence experiments results showed the PCBP1 protein expression was increased compared with mock and vector group, and the PCBP1 is distributed in both the cytoplasm and the nucleus (Fig. 1b).

Overexpression of PCBP1 and its effects on human cervical carcinoma cells viability

In order to understand the effects of overexpressed PCBP1 in HeLa cells, we examined the proliferation of HeLa cells in different time points after transfection. MTS analysis proved that elevated PCBP1 significantly reduced the cell viability of HeLa cells, and the inhibition of proliferation of PCBP1 transfection. The results were significantly time-dependent (Fig. 2a). Furthermore, the experimental results from the colony formation assay suggested that overexpression of PCBP1 in HeLa cells could significantly repress cell colony formation compared with mock and vector group (Fig. 2b, c).

PCBP1 induces cell cycle arrest and apoptosis

To detect the effect of overexpressed PCBP1, after transfection, we stained the nuclei and then observed the nuclear morphology. We found the nuclear morphology changed and apoptotic bodies appear, this suggested us that apoptosis took place (Fig. 3a).

In order to further verify the mechanism of PCBP1 in inhibiting the growth of HeLa cells, we used flow cytometry to detect the cell cycle and apoptosis. The results showed that overexpressed PCBP1 would cause cell cycle arrest (Fig. 3b), and the ratio of G2 / M cells in HeLa cells were significantly increased (Fig. 3d). Then we detected cell apoptosis to prove that whether the decrease in viability of HeLa cells after transfected with pEGFP-N1-PCBP1 was caused by apoptosis. The results showed cells transfected with pEGFP-N1-PCBP1 for 48 h performed more apoptosis than the mock and vector group (Fig. 3c, e). The results of the apoptosis experiment obtained by flow cytometry analysis were statistically analyzed, and the results were also verified.

PCBP1 upregulates Tap73 and downregulates ΔNp73, indicating activation of apoptosis

In order to demonstrate whether p73 is involved in PCBP1 induced cell cycle arrest and apoptosis in human cervical carcinoma cells, western blot and immunofluorescence were used to detect the level of Tap73 and ΔNp73 proteins. We found the level of ΔNp73 was significantly reduced in HeLa cells at 24 h after transfected with pEGFP-N1-PCBP1 compared with the mock group. On the contrary, the level of Tap73 was increased compared with the mock group (Fig. 4a). Quantitative results showed, PCBP1 induced an increase in the Tap73 / ΔNp73 ratio (Fig. 4b). Next, we used immunofluorescent to further verify its spatiotemporal distribution in cells (Fig. 4c). ΔNp73 expression clearly reduced but Tap73 expression obviously upregulated. Moreover, Tap73 mainly detected in the cytoplasm, but ΔNp73 mainly detected in the nucleus. These findings suggested that p73 splicing is involved in PCBP1 induced cell cycle arrest and apoptosis in HeLa cells.

PCBP1 regulates apoptosis via mitochondrial pathway

In order to further verify whether PCBP1 induced apoptosis is associated with mitochondrial apoptosis pathway, we used western blot to examine several key proteins in the mitochondrial apoptosis signalling pathway. As shown in Fig. 5, overexpression of PCBP1 upregulated the ratio of Bax / Bcl-2 to promote cell apoptosis (Fig. 5b). In addition, there was a substantial increase in the expression of cytochrome c at 24 h after transfected with pEGFP-N1-PCBP1 (Fig. 5d). Furthermore, decreased procaspase-3 and increased cleaved caspase-3 both indicated the occurrence of apoptosis (Fig. 5c). Thereby confirming that overexpression of PCBP1 upregulated Bax / Bcl-2 ratio, promoted cytochrome c release and activated caspase-3 to induce apoptosis.

Discussion

Cervical cancer is one of the most diseases threatening women's health, but our understanding of its pathogenesis is still not deep enough. It is imminent to further study its pathogenesis [14]. PCBP1 is an evolutionarily conserved RNA-binding protein that regulates transcription, translation, and alternative splicing of genes [3, 15, 16]. Increasing evidence revealed that PCBP1 is significantly downregulated in gastric cancer [4], acute myeloid leukemia [5], non-small-cell lung cancer [6], and cervical cancer [7] et al. and is involved in tumor metastasis. Subsequently, scientists pointed out that PCBP1 might act as a tumor suppressor in various tumors [17, 18]. For instance, it has been proved that PCBP1 is involved in cell apoptosis. Zhang et al. reported that overexpression of PCBP1 decreased the Bcl-2 expression and caused cancer cells apoptosis [8]. Shi et al. confirmed that PCBP1 increased p27 expression via stabilizing its mRNA to further facilitate cell apoptosis [19]. Ishii et al. reported PCBP1 activated apoptosis through interacted with more severely oxidized RNA [20]. Interestingly, PCBP1 has gained more attention due to its multiple functions in tumor progression, but the real mechanism is relatively unexplored. Therefore, it is worthy to further study the relationship between PCBP1 and cervical cancer. Here, we transfected PCBP1 into HeLa cells and testified that overexpression of PCBP1 greatly repressed proliferation of HeLa cells in time-dependent manner (Fig. 2). It also induced G2 / M phase arrest and significant rise of apoptotic cells (Fig. 3). Overall, these results indicated that elevated PCBP1 is an efficient way to inhibit tumor cell progression, and this will provide a reference for further understanding of the pathogenesis of cervical cancer.

RNA splicing is the key to the pathology of numerous diseases, and experiments have shown that dysregulation of splicing isoforms were increasingly associated with tumor proliferation, metastasis and apoptosis [21, 22]. Additionally, PCBP1 is related to alternative splicing. In pancreatic cancer, upregulated PCBP1 reduced tumor metastasis by interacting with integrin β1 to regulate its alternative splicing [11]. Moreover, overexpression of PCBP1 inhibited the tumor invasion and metastasis in HepG2 cells via regulating exon inclusion of CD44 [10]. In our study, we transfected PCBP1 into HeLa cells and indicated that overexpressed PCBP1 obviously enhanced the expression of Tap73 and decreased ΔNp73 expression (Fig. 4). Tap73 and ΔNp73 are two variants of p73, which is a structural homolog of p53 and acts as a tumor suppressor. This gene often encodes two opposing variants: the transcriptionally active TAp73 and the dominant-negative ΔNp73 [23]. ΔNp73 overexpressed in a variety of cancers and it is correlated with poor prognosis [24, 25]. Additionally, ΔNp73 can interact with wild-type p53 or Tap73 to efficiently counteract wild-type p53 and TAp73 mediated apoptosis, and growth suppression [26]. Therefore, ΔNp73 has become a novel tumor-specific molecular target for cancer because of its anti-apoptotic functions. TAp73 contains the NH2-terminal domain and plays a similar role to p53 as a tumor suppressor [27, 28]. TAp73 is relevant to DNA damage and upregulates proapoptotic Bcl-2 family members and causes apoptosis via the mitochondrial pathway [29]. Recently, some studies elucidated that the ratio between Tap73 and ΔNp73 might contribute to tumorigenesis and resistance to chemotherapy and determined the fate of the cell [30]. Our results showed overexpressed PCBP1 upregulated the ratio of Tap73 / ΔNp73 and caused HeLa cell apoptosis. These data are evidenced by our finding that that PCBP1 is involved in p73 gene splicing and it will induce cell apoptosis via upregulation of Tap73 / ΔNp73 ratio in human cervical cancer (Fig. 4). This may have a certain inspiration for cancer treatment. Taken together, our data indicated that PCBP1 is an important gene and a tumor suppressor in cervical cancer.

To further study the mechanism of PCBP1 induced apoptosis, we examined the expression of some related proteins after transfection of PCBP1. Indeed, our data revealed that overexpressed PCBP1 significantly decreased anti-apoptosis Bcl-2 expression and increased the Bax / Bcl-2 ratio (Fig. 5). It has been confirmed that Bax / Bcl-2 ratio regulated cytochrome c release from mitochondria [31]. In addition, we further detected the expression level of cytochrome c, which can activate the caspase-3 and downstream cell death pathway. We discovered that procaspase-3 expression levels were significantly reduced, while cytochrome c levels were elevated. In light of our previous work, Tap73 / ΔNp73 ratio also plays an important role in regulating apoptosis via mitochondrial pathway [32, 33]. PCBP1 may initiate a mitochondria-mediated apoptotic pathway by inducing p73 alternative splicing. As expected from the above results, we believe that PCBP1 plays a pivotal role in arresting cell cycle, inducing apoptosis, regulating p73 splicing in human cervical carcinoma cells, and it induced splice regulation of p73 may be another downstream signaling pathway independent of p53. In conclusion, our results suggested that PCBP1 could be used as a potential candidate for cervical cancer therapy and it has broad prospects as a molecular therapeutic target for cervical cancer. However, this also requires the use of tumor-bearing animal models and clinical trials to further study the effects of PCBP1.

Materials And Methods

Cell culture and transfection

The human cervical carcinoma HeLa cells were obtained from the First Hospital of Lanzhou University and cultured in Dulbecco's modified Eagle's medium (DMEM, Minghai Biochem, Lanzhou, China) supplemented with 10% fetal bovine serum (Minghai Biochem, Lanzhou, China) at a culture temperature of 37℃, 5% CO_2 in incubator (Thermo, USA). DNA transfection was carried out using Exfect2000 transfection reagent (Vazyme, Nanjing, China) as a mediator according to the manufacturer's instruction. The pEGFP-N1-PCBP1 and non-targeting negative control pEGFP-N1 was purchased from Invitrogen (Invitrogen Life Technologies, CA, USA). To transfect HeLa cells with plasmid vector, cells were plated into either 60 mm dish or a 100 mm dish and allowed to adhere for 24 h. Exfect2000 transfection reagent was utilized for the transfection. After pEGFP-N1 or pEGFP-N1-PCBP1 transfection, cells were cultured for 5 h and then the medium was replaced with fresh medium supplemented with 10% fetal bovine serum. Cells were harvested 24–48 h after transfection.

Cell survival detection

Cell viability was investigated using the methyl tetrazolium salt (MTS) assay. Cells were plated into 96-well plates (Promega, Beijing, China) and incubated 24 h. Then used transfection reagent to transfect pEGFP-N1 or pEGFP-N1-PCBP1 and cultured 24 h and 48 h to detect viability. 20 μl / well of MTS solution was added to each 100 μl of DMEM medium, and incubated for 60 min at a 37℃ constant temperature incubator. Then the absorbance was detected with multifunction microplate reader (Tecan Infinite M200, Swiss) at 490 nm. The survival rate of cells in each well was shown as a percentage of control.

Quantitative RT-PCR analysis and agarose gel electrophoresis

Total RNA was extracted from cells with TRIzol reagent (Takara Biotech Co., Ltd.) and the complementary DNA (cDNA) was synthesized by using Transcriptor First Strand cDNA Synthesis System kit, real-time PCR analysis of PCBP1 and the reference gene β-Actin was treated by using a SYBR Green reaction kit (TIANGEN, China) in real-time PCR instrument (Thermo, USA), according to instruction. All experiments were carried out in triplicate and analyzed using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method. Products were run in 1% agarose gel and the band intensity was scanned. The results were normalized by β-Actin levels.

Colony formation assay

The cells were harvested for 24 h after transfection, and seeded into 60 mm dishes. After incubated at 37 °C for 10–14 days, the culture was terminated when macroscopic clones appeared in the dishes. The clone was fixed with paraformaldehyde and stained with crystal violet. Count clones containing more than 50 cells, calculated clone formation rate and collected images. Each experiment was performed in triplicate.

Cell cycle assay

After pEGFP-N1 or pEGFP-N1-PCBP1 transfection and cultured for 24 h, then collected the cells, fixed with pre-cooled 70% ethanol in PBS, and overnight at 4℃. Then, the supernatant was centrifuged at 800 rpm for 4 min. 100 μl of propidium iodide (PI, Sigma, USA) was added after washing three times with PBS, and the cells were incubated for 30 min in the dark. The samples were collected with a minimum of 20,000 cells and analyzed with a flow cytometer FlowSight (Amnis, Seattle, WA, USA). The results were analyzed with FlowJo 7.6 software and each experiment was repeated at least three times.

Cell apoptosis assay

We used 4', 6-diamidino-2-phenylindole (DAPI) (Vector, Laboratories, USA) staining to observe nuclear morphology after pEGFP-N1 or pEGFP-N1-PCBP1 transfection. HeLa cells were expressed PCBP1 after transfection and cultured for 48 h. First, cells were collected and washed twice with PBS and fixed with the 4% paraformaldehyde for 20 min. And then permeabilized with 0.5% Triton X-100 / PBS for 15 min on ice and finally treated with DAPI. Finally fluorescence microscope was used to observe after the tablet was sealed with anti-fluorescence quench sealing solution.

In order to determine the apoptosis rate more accurately, Annexin V-FITC / PI double staining flow cytometry was used to detect apoptosis. Cells were collected and washed twice with PBS. Next, 75 μl of binding buffer was added to the cell suspension, and then 5 μl of annexin V and 5 μl of PI (Annexin V-FITC Apoptosis Detection Kit I, BD, USA) were also added to the cell suspension. The samples were detected with a flow cytometer FlowSight (Amnis, Seattle, WA, USA) after incubated in the dark for 15 min at RT, and immediately analyzed on IDEAS Application v6.0.

Western blot analysis

After pEGFP-N1 or pEGFP-N1-PCBP1 transfection and cultured for 24 h and 48 h, cells were collected. Whole proteins were lysed from the cells using RIPA lysis buffer (Solarbio, China) added PMSF. Western blot analysis was performed according to standard procedures. Proteins were fractionated by 10% SDS-PAGE and transferred to a methanol activated PVDF membrane (GE Healthcare, Beijing, China). Antibodies against Tap73, ΔNp73 (Imgenex, San Diego, USA), β-Actin (Bioss, Beijing, China) and Bax, Bcl-2, Cytochrome c, procaspase-3, cleaved caspase-3 (Santa Cruz, CA, USA) were used according to the instruction. HRP-linked anti-mouse or anti-rabbit IgG antibodies (Bioss, Beijing, China) were used as secondary antibodies. The results were normalized by β-Actin levels.

Immunofluorescence

Cells were transfected with pEGFP-N1 or pEGFP-N1-PCBP1 and harvested at 48 h. First, cells were washed in 0.01 M PBS (pH 7.4), fixed with paraformaldehyde, permeabilized with 0.5% Triton X-100 / PBS for 15 min on ice, and blocked with 5% BSA for 60 min at RT. Incubated with antibodies against PCBP1 (Santa Cruz, CA, USA) at 4℃ overnight, then added fluorescent secondary antibody and incubated at RT for 1 h in the dark. Finally, 0. 01 M PBS was used to wash three times for 5 min each wash and DAPI was added. After added glycerol, the samples were detected with a confocal laser microscope (LSM, Carl Zeiss AG, Germany).

Statistical analysis

Data are presented as means ± SD. Statistical analysis were showed on the means of the data obtained from at least three independent experiments. Student's t-tests program in Microsoft Excel was used to compare the differences between the mock group, vector group and the PCBP1 group. $P < 0.05$ was considered significant.

Availability Of Data And Materials

All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors report no conflict of interest in this work.

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Contributions

YC, CD and HZ conceived the manuscript. YC, CD, CX and HC consulted the literature and wrote the initial draft of the manuscript. TC, GM, XZ, JY, FW, HL and HY participated in writing the manuscript. All authors reviewed the manuscript.

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Declarations
Declarations.

Figures

Fig. 1

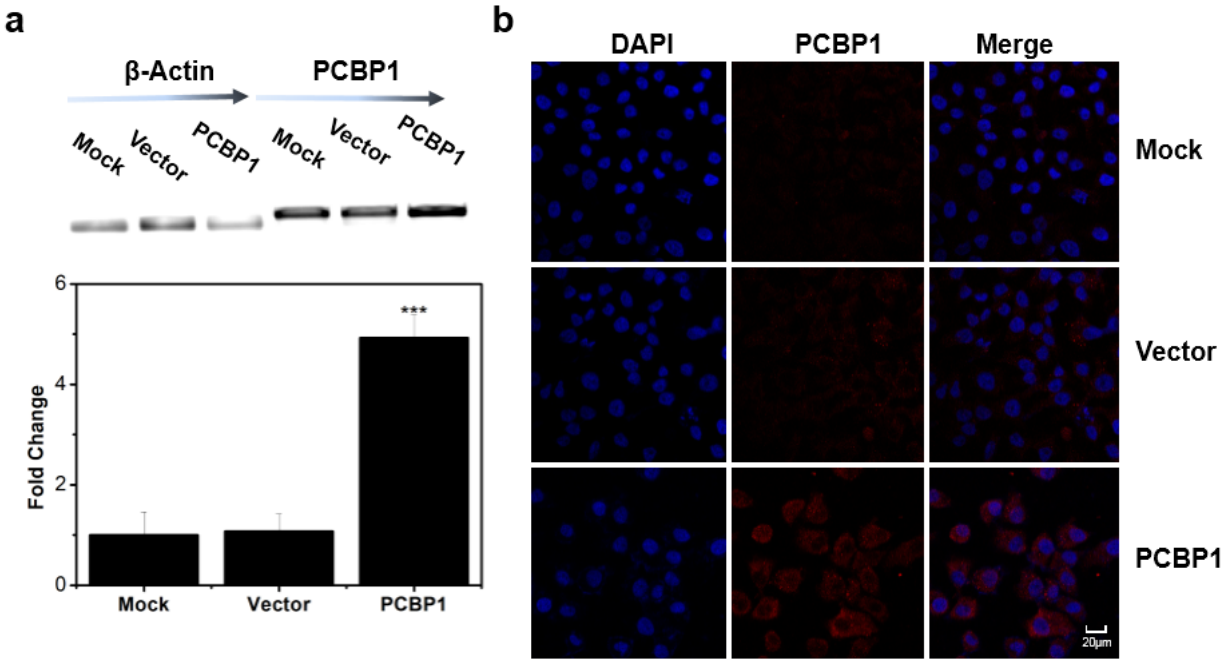

Figure 1

The expression of PCBP1 mRNA and protein and its spacial distribution. a Expression of PCBP1 mRNA was determined in HeLa cells, the data was normalized to β-Actin expression. b PCBP1 protein is distributed in both the cytoplasm and the nucleus. All experiments were repeated at least three times. The data are expressed as the mean ± SD. *** P < 0.001 (vs. mock group).

Fig. 2

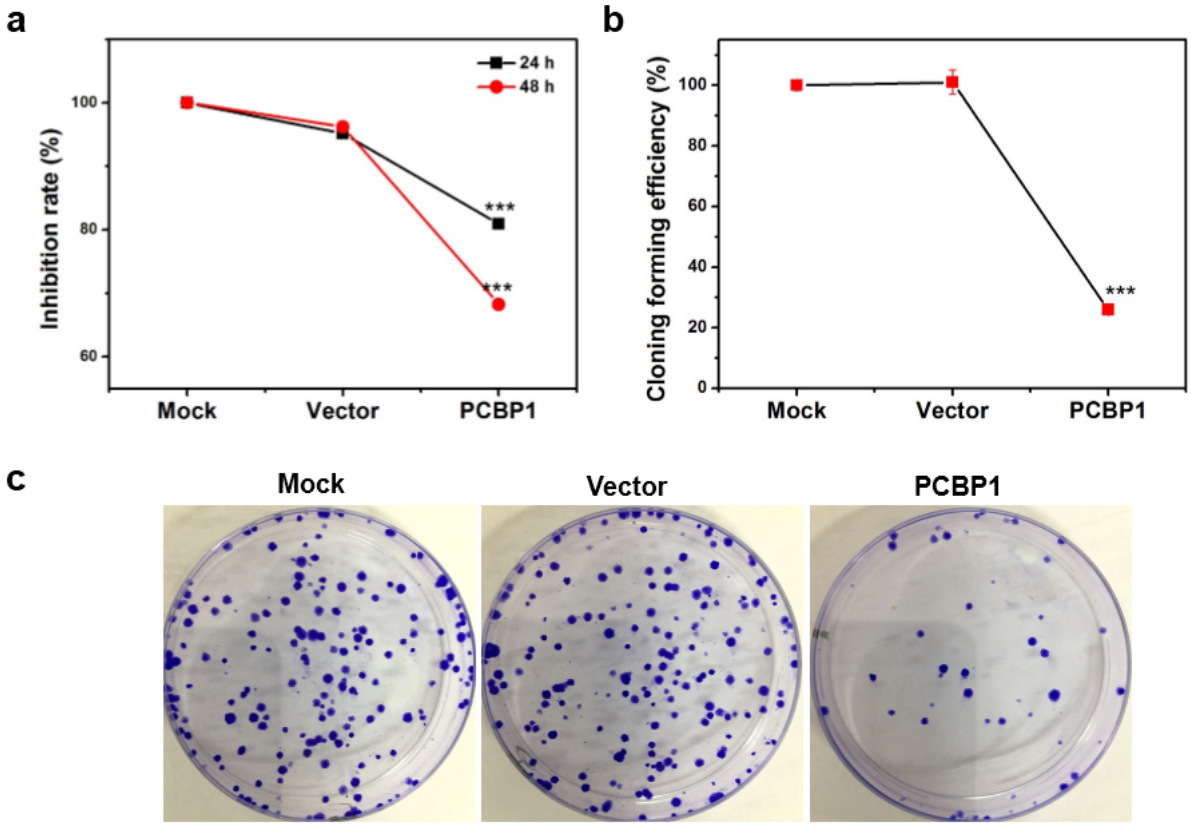

Figure 2

Overexpression of PCBP1 significantly inhibited human cervical carcinoma HeLa cells viability and colony formation. a The cell viability transfected with pEGFP-N1-PCBP1 was detected by MTS assay. b Quantification of the colony counts. c Representative images of the colony formation potential of HeLa cells treated with transfection. All experiments were repeated at least three times. The data are performed as the mean ± SD. *** P < 0.001 (vs. mock group).

Fig. 3

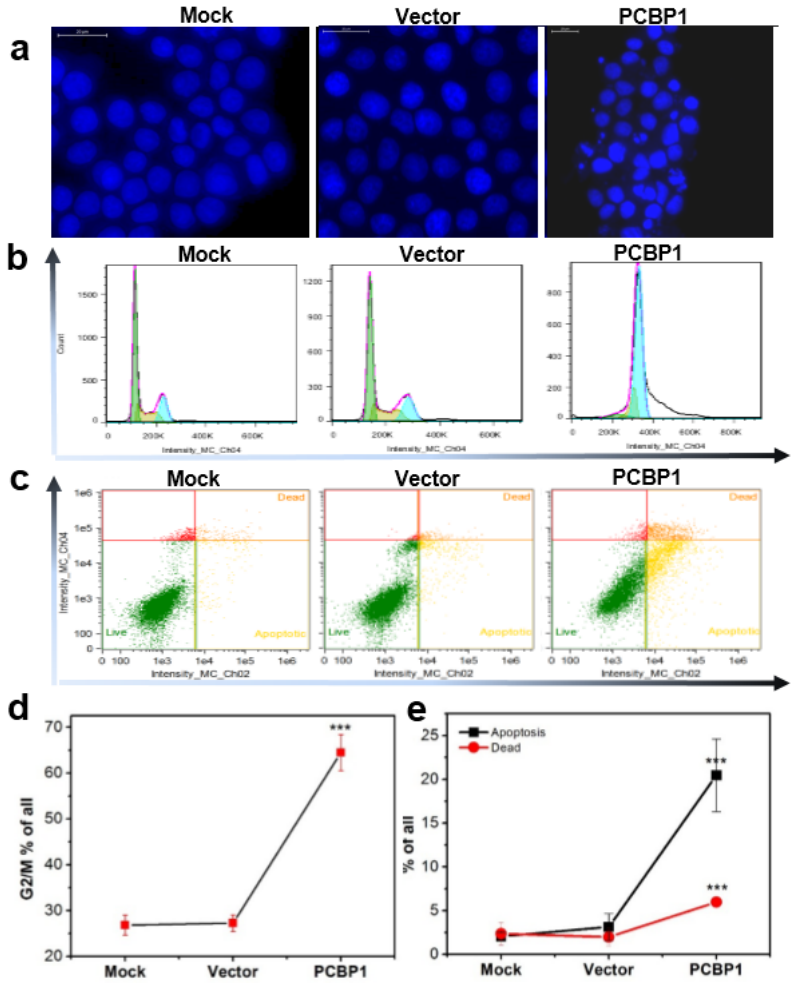

Figure 3

Overexpression of PCBP1 induced cell cycle arrest and apoptosis in human cervical carcinoma HeLa cells. a Nuclear morphology was observed after transfection in human cervical carcinoma HeLa cells. b Flow cytometry analysis of cell cycle arrest in HeLa cells transfected with PCBP1. c Apoptotic effect of overexpression of PCBP1 in HeLa cells. d Percentages of G2 / M phase of cell population was shown. e Total percentages of apoptotic and dead rate of cell population was calculated. All experiments were repeated at least three times. The data are expressed as the mean ± SD, *** P < 0.001 (vs. mock group). ** P < 0.01 (vs. mock group).

Fig. 4

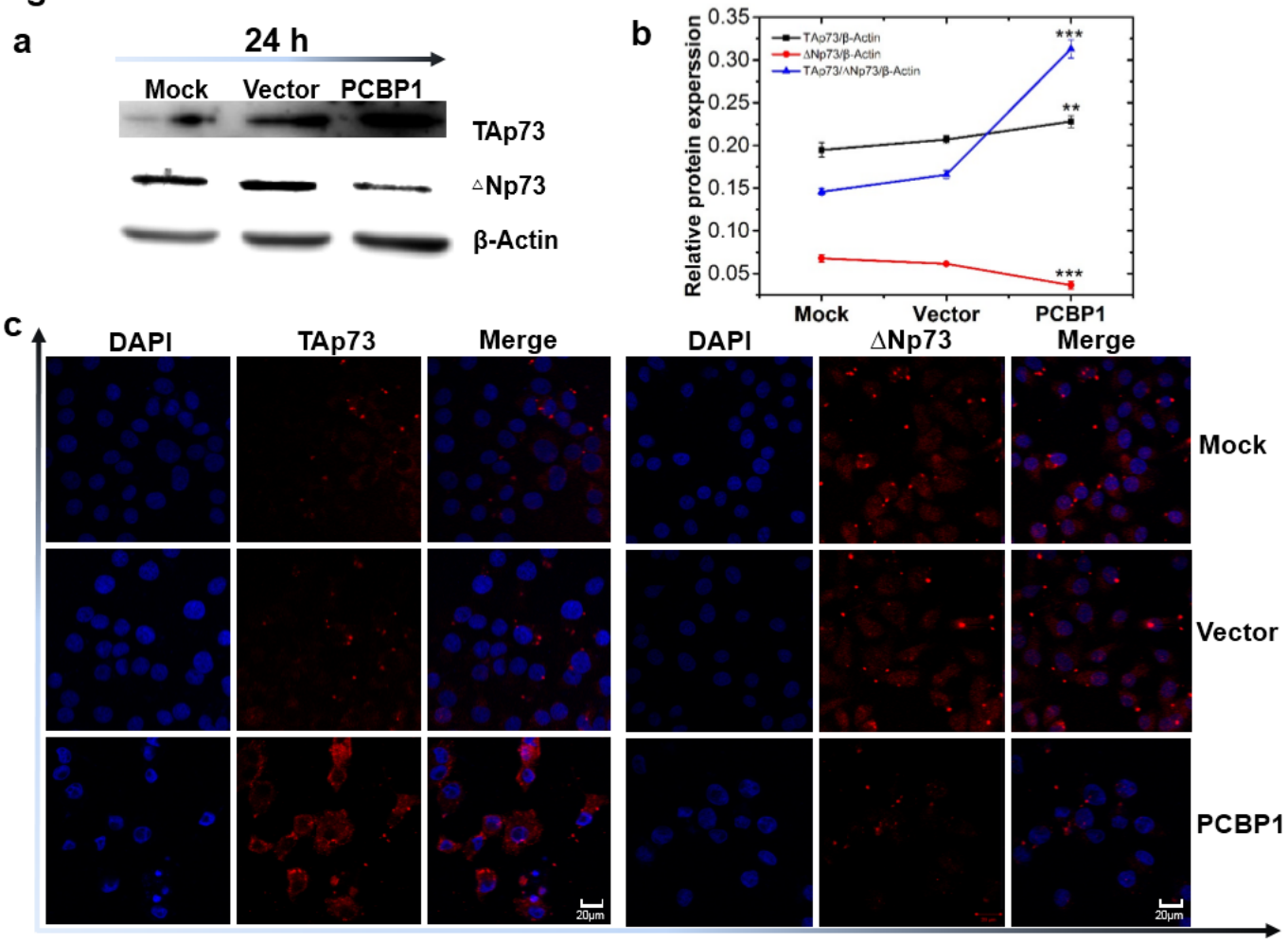

Figure 4

Overexpression of PCBP1 increased the protein ratio of Tap73/ΔNp73 in human cervical carcinoma HeLa cells. a Western blot results. b Quantitative analysis of Tap73 and ΔNp73 proteins in HeLa cells by western blot analysis. c The expression and localization of Tap73 and ΔNp73 proteins in HeLa cells detected by fluorescent microscopy. All experiments were repeated at least three times. The data are expressed as the mean ± SD. *** P < 0.001 (vs. control group). ** P < 0.01 (vs. mock group).

Fig. 5

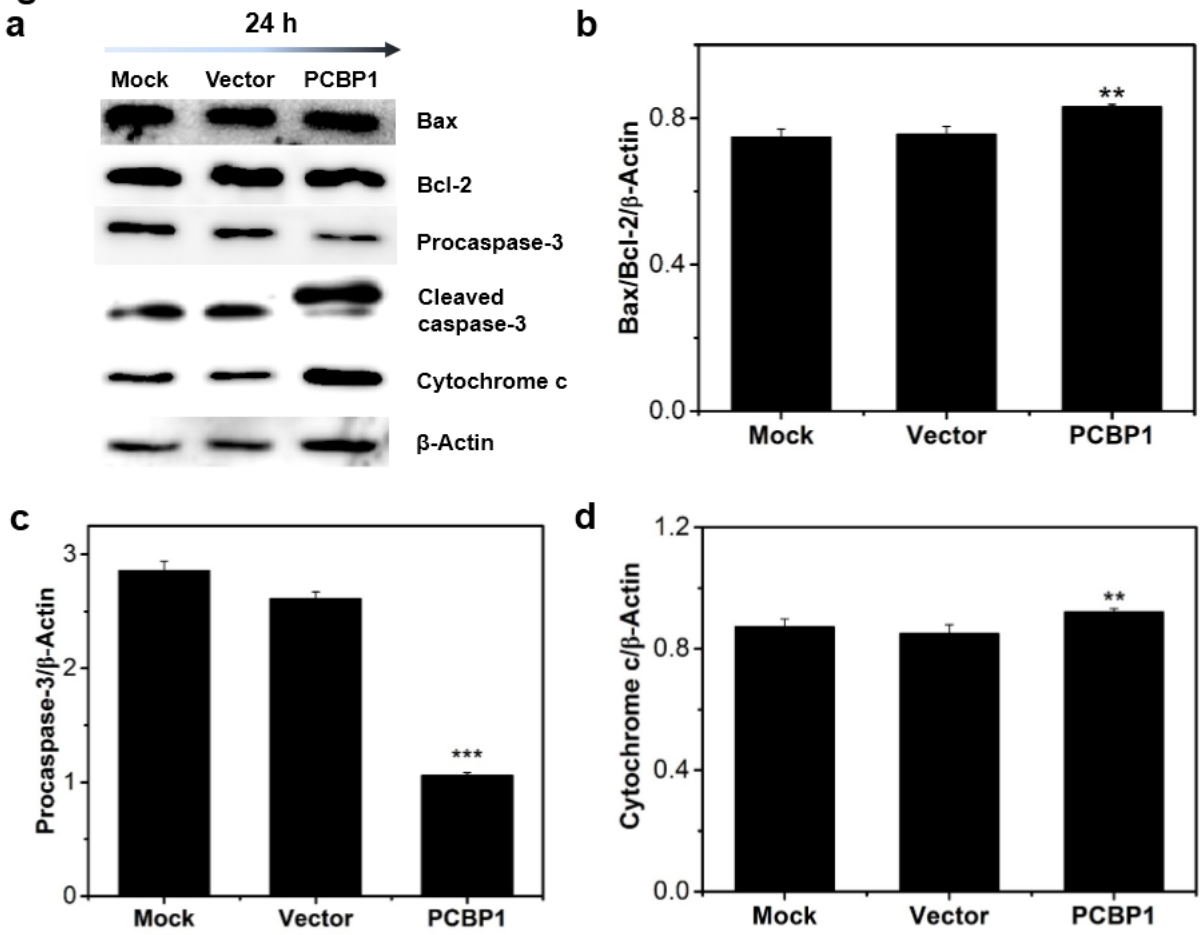

Figure 5

Overexpression of PCBP1 induced apoptosis through mitochondrial pathway in human cervical carcinoma HeLa cells. a Western blot results. b Quantitative analysis of Bax / Bcl-2 ratio in HeLa cells. c Quantitative analysis of procaspase-3 expression in HeLa cells. d Quantitative analysis of cytochrome c expression in HeLa cells. All experiments were repeated at least three times. The data are expressed as the mean ± SD. *** $P < 0.001$ (vs. mock group). ** $P < 0.01$ (vs. mock group).