Effects of high expression of Csk-binding protein on morphology and biological function of Jurkat cells

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Abstract

BACKGROUND: The linkage and synergistic effect of adaptor proteins can effectively regulate signal transduction of T cells, which can form a limit or amplification cascade to realize the complex immune function of T cells. C-terminal Src kinase (Csk)-binding protein (Cbp) is an adaptor protein, which mainly exert the negative feedback regulation of Src kinase activity. This negative feedback effect depends on Y317 of Cbp, which may be involved in the SH2 domain of Csk.

OBJECTIVE: To explore the effects of high expression of Cbp on ultrastructure and related biological function of Jurkat cells.

METHODS: The virus particles were constructed with expressing enhanced green fluorescent protein (EGFP) only and Cbp-EGFP fusion protein to transfect Jurkat cells. There were untransfected group (Jurkat group), negative control group (transfected with expression of EGFP virus only), and Cbp group (transfected with Cbp-EGFP virus).

RESULTS AND CONCLUSION: Confocal microscope showed that cell transfection efficiency was more than 95% and Cbp was located on the cell membrane. Optical microscope showed after transfection with Cbp-EGFP virus, more Jurkat cells shrunk, with poor size uniformity. Apoptosis detection showed that after transfection with Cbp-EGFP virus, the number of apoptotic and necrotic cells was greatly increased. Cbp mRNA expression was increased, Csk expression was decreased obviously and lymphocyte-specific protein tyrosine kinase expression was increased. So, in Jurkat cells, the high expression of Cbp can decrease the uniformity of cells and increase the necrosis cells, thus inhibiting the signal transduction.

Subject headings: Apoptosis; T-Lymphocytes; Jurkat Cells; Adaptor Proteins, Signal Transducing
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INTRODUCTION

Adapter protein molecules refer to the proteins which have neither enzyme activity nor transcription activity[1] but contain conservative structures such as SH2 and SH3 domain or tyrosine/proline motif. These molecules mediate the reaction between protein and protein, thus forming multiple protein signal complexes[2]. Conservative domain of adapter protein molecules can react with the above binding motifs and the complementary protein molecule chaperones, for example, the combination between SH2 and PTB domain with tyrosine phosphatase residues of target proteins. SH3 and WW domains can be recognized and bind proline motif in complementary protein molecule chaperones[3-4]. Due to the synergy of adapter protein molecules, signal transduction of T cells can be efficiently and accurately controlled, forming cascade amplification or restrictions, which finally realizes the complex immune function of T cells[5].

C-terminal Src kinase (Csk)-binding protein (Cbp), also known as phosphoprotein associated with glycosphingolipid-enriched microdomain[6], is a transmembrane protein first found in 2000[6-7]. It is widely expressed on the surface of a variety of cell membranes, including T lymphocytes. Human Cbp has a total length of 432 amino acids, including extra-membrane domain, transmembrane domain and intramembrane domain. There are 10 tyrosine distributed on the intramembrane domain, which is a potential phosphorylation site[8]. Cbps are considered to be involved in control of negative feedback of Src kinase activity. It is also considered that activated member of Src family phosphorylates Y314 locus of Cbp, thus recruiting Csk to the cell membrane to achieve its inhibitory effects[9]. Clinical studies addressing lung cancer and

esophageal cancer have shown that Cbp expression is decreased while exogenous Cbp gene can inhibit the proliferation of tumor cells[10-11]. Overexpression of Cbp in breast cancer cell lines can inhibit epidermal growth factor-induced Src tyrosine kinase[12]. Also, it is found in research of blood cells that Cbp can recruit Csk thus inhibiting activity of Src family kinase[13-14]. Malsuoka *et al* [15] observed dynamic changes in the interaction of Csk with Cbp by utilizing fusion proteins. Since Cbp can participate into the negative feedback regulation of Src kinase family, we constructed a Cbp-EGFP lentiviral vector that was transfected into Jurkat cells and observed its effects on biological phenotype and functions of acute T lymphocytic leukemia cells.

MATERIALS AND METHODS

Design

Control experiments.

Time and setting

The experiment was conducted in the Immunology Laboratory of Qingdao University, China from August 2013 to June 2014.

Materials

Cells

Jurkat cells were saved in the Department of Immunology, Medical College of Qingdao University.

Main reagents and instruments for Cbp transfection of Jurkat cells:

Reagents and instruments	Source
PRMI-1640 culture, fetal bovine serum	Hyclone, USA
Anti-lymphocyte-specific protein tyrosine kinase (LCK) rabbit polyclonal antibody	Immunoway ,USA
Anti-CSK rabbit polyclonal antibody	ABGENT, USA
Goat-anti-mouse second antibody	Sangon Biotech, China
SuperSignal West Pico Trial Kit	Thermo Scientific, USA
RevertAid First Strand cDNA Synthesis Kit	Roche, Switzerland
Guava Nexin Reagent, Guava EasyCyte™ Mini	Merck Millipore , Germany
Fluoview FV1000 laser confocal microscope	Olympus, Japan

Methods

Cell culture

Jurkat cells were cultured in RPMI-1640 medium (containing 10% fetal bovine serum and 1% penicillin-streptomycin mixture) at 37 ℃ and 5% CO_2. Cells at the logarithmic phase, with good growth condition, were used for experimental research.

Cell transfections

Cbp-enhanced green fluorescent protein (EGFP) lentivirus and empty vector lentivirus particles were constructed by Shanghai Genechem Company. According to the instruction of Lentiviral Vector Particle, wild-type Jurkat cells at the logarithmic phase were taken and counted. Then 100 μL of cells suspension containing 5 000 cells per well were implanted to 96-well plates. Lentivirus was added, and 12 hours later, the supernatant was removed and RPMI-1640 medium (containing 10% fetal bovine serum and 1% penicillin-streptomycin mixture) was added for further culture. After 96 hours, the transfection rate of virus vector was observed under laser confocal microscope.

Immunofluorescence staining

Immunofluorescence staining was used to detect the transfection rate and locate green fluorescence. Slide was covered by 1×poly-L-lysine. About 5 μL cell suspension was moved to the slide and dried. Then the cells were fixed with 4% paraformaldehyde for 15 minutes and washed by 1×PBS for three times. Then when it was dried, 75% glycerol was used to seal the slide that was immediately observed under confocal microscope.

Real-time quantitative PCR

Trizol was used to extract total RNA from cells of each group. Retrovirus kit (Roche) was used for cDNA synthesis. According to the instruction of Faststart Essential DNA Green Master fluorescence quantitative kit, real-time quantitative PCR was performed. Housekeeping gene GAPDH was used to regulate the amount of total RNA. Primers were as follows: Cbp, forward 5'-TCA GCC TGA GAG GAG GAA AT-3', reverse 5'-GCT CCT GCT ACT TGG GAG TC-3'; GAPDH, forward 5'-GAT GAC CTT GCC CAC AGC CT-3', reverse 5'-ATC TCT GCC CCC TCT GCT GA-3'. Each of RNA samples was repeated three times.

Morphological observation

50 μL cell suspension was removed from each group into 96-well plates. The cells were pipetted for 5–10 times to suspend the cell mass into single and uniform suspended cells. The cells were observed under inverted microscope for morphology, homogeneity and presence of dead cells.

Flow cytometry detection

Apoptotic and necrotic rates were detected. Flow cytometry was used to test cell apoptosis in each group. Cell density was adjusted into 10^6 cells/mL per hole. And then cells of each hole were collected into 1.5 mL EP tube. The solution was centrifuged for supernatant. Cells of each tube were re-suspended into 100 μL complete medium. 80 μL Guava Nexin Reagent was added. Then the cells were incubated at room temperature in dark for 20 minutes and then detected on Guava EasyCyte™ Mini.

Western blot assay

Cells were washed with PBS three times, and then solubilzed in 500 μL of lysis buffer and 5 μL of phenylmethylsulfonyl fluoride (beyotime). Samples were centrifuged at 12 000 r/min for 5minutes at 4 ℃ to separate the membrane fraction from the cytosolic fraction. After boiling for 5 minutes, proteins were resolved by 12% SDS-PAGE, electroblotted onto PVDF membrane and

immunoreacted overnight with the primary antibody (CSK: 1:1 000, LCK: 1:1 000), followed by 2 hours incubation with the secondary antibody conjugated with horseradish peroxidase (Dilution ratio=1:2 000). Chemiluminescent signals were generated by SuperSignal West Pico Trial Kit and then detected.

Main outcome measures

Morphology, apoptosis and protein expression of transfected cells.

Statistical analysis

The paired-samples test was conducted for statistical analysis. SPSS 17.0 software was used for analysis. A value of $P < 0.05$ was considered significant.

RESULTS

Transfection rate and distribution of Cbp in Jurkat cells

Jurkat cells were transfected by Cbp-EGFP lentivirus vector and empty lentiviral vector (multiplicity of infection=100) (**Figure 1A**), and were detected under the confocal microscope after 96 hours. As shown in **Figure 1B**, there was no green fluorescence in Jurkat cells untransfected by lentivirus vector. Meanwhile, there was a lot of green fluorescence sustainable expression in negative control and Cbp-EGFP groups (**Figures 1C−D**). Under five-fold magnification, the transfection efficiency of lentivirus vector was calculated to be greater than 95%. After further amplification, green fluorescence of the negative control group evenly distributed in the cytoplasm (**Figure 1E**), and in the Cbp-EGFP group, green fluorescence focused on the cell membrane on which there was a bit tufted gathering area (**Figure 1F**), suggesting it is possibly lipid rafts.

Cbp mRNA expression

The amplification curve and melting curve of negative control and Cbp-EGFP groups showed that gene amplification was a single peak, consistent with the annealing temperature, and no nonspecific amplification (**Figure 2**). Cbp mRNA expression was significantly increased in the Cbp-EGFP group, which was 3.5 times as much as the negative control group (**Table 1**). It suggested that the mRNA expression of Cbp was indeed at a high level after transfection.

Table 1 Transcriptional expression of Cbp gene in negative control and Cbp-EGFP groups ($2^{-\Delta Ct}$)

Group	Cbp mRNA expression
Negative control	4.117×10^{-6}
Cbp-EGFP	1.474×10^{-5}

Cell growth

Jurkat cells is an acute T leukemia cell line and can be immortalized. The growth mode is suspended growth[16]. As shown in **Figure 3A**, the cell state was mellow and bright, liked eggs, and cell morphology was relatively uniform. There were almost no pyknotic cells in the visual field. By comparing **Figure 3A** and **Figure 3B**, we found that the cell morphology and size in the negative control and untransfected groups were identical. A small amount of pyknotic cells were seen within the field of vision. But in **Figure 3C**, the cell growth rate was not as fast as the other groups, the cell size was heterogeneous, and a lot of small cells could be seen. We could easily see the shrinkage of cells and debris under the microscope.

Cell apoptosis

As showed in **Figure 4**, the cells were concentrated and had the uniform size in the untransfected group; and the same as negative control cells. But there were also a certain amount of small cells in the negative control group which was compared to the untransfected group. In the Cbp-EGFP group, the small-head cells accounted for a large proportion in the whole cells, and the cell size was large. Rates of cell apoptosis and necrosis increased in proper order (untransfected group < negative control group < Cbp-EGFP group) (**Figure 4**). This suggests that viral vectors are toxic to cells, and Cbp proteins promote apoptosis or necrosis.

Csk and Lck expression

Western blot showed free Lck expression in TCR activated signal pathway was increased, while free inhibitory tyrosine Csk expression was obviously decreased (**Figure 5**). The statistical results showed the content of Lck increased and Csk protein decreased were both significantly different from those in the Cbp group and negative control group (**Figure 5**). These suggest that high-expression CBP combined with CSK may inhibit the activity of Src family kinase and play a certain inhibitory role in cell proliferation, which may induce cell apoptosis or cell death.

DISCUSSION

Adapter protein Cbp is widely expressed in human. Its mRNA can be detected in most of the tissues (heart, lungs, brain, liver, spleen and peripheral lymphocytes)[6]. In T cells at resting state, Cbp has a strong background phosphorylation, and a large number of Csk are recruited into lipid rafts, to inhibit the activity of Src kinase[15-16]. At present, most of studies aim at the biological function of Cbp in signal transduction, namely Cbp as a negative feedback regulating factor of receptor signal[17]. So, the number and the localization of Cbp can change the signal transduction. Researches show that Cbp is significantly down-regulated in non-small cell lung cancer tissue[18], and at the same time Cbps in B lymphocyte and T lymphocyte both negatively regulate proximal signal transduction[14, 19]. Recently, Saitou *et al*[20] used mathematical modeling and *in vitro* experiments to confirm that Cbp plays an important role in regulation on space of Src family kinase. In the early stage of the experiment, we have confirmed that Cbp plays a role in T lymphocyte proliferation and activation. And when the plasmid is interfered by restructured Cbp and proper stimulation is given, Jurkat cell activation and proliferation

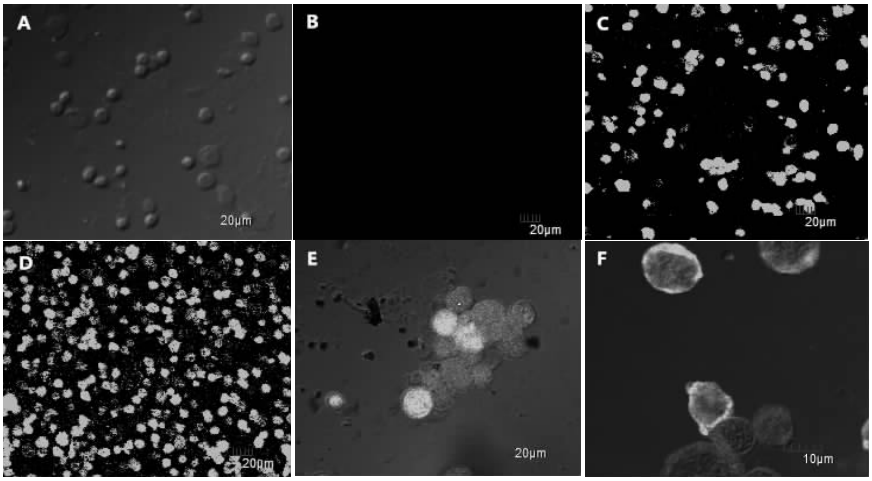

Figure 1 The transfection efficiency and distribution of green fluorescence under confocal microscope
Note: (A) Jurkat cells with no luminescence; (B) Jurkat cells; (C, E) Jurkat cells transfected with EGFP virus only; (D, F) Jurkat cells transfected with Cbp-EGFP virus; (B–F) all cells were stimulated by 488 nm luminescence.

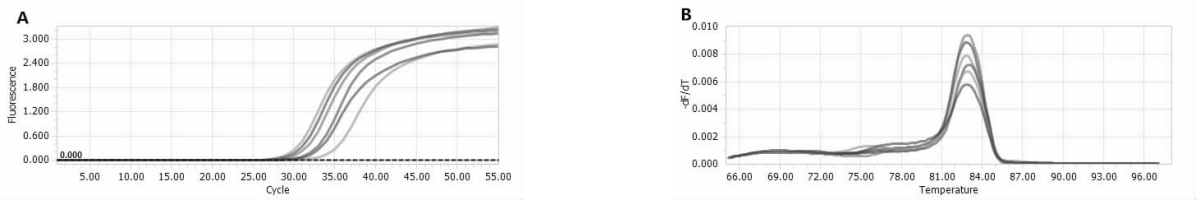

Figure 2 Amplification curves and melting peaks of Cbp gene expression in negative control and Cbp-EGFP groups
Note: (A) Amplification curves; (B) melting peaks.

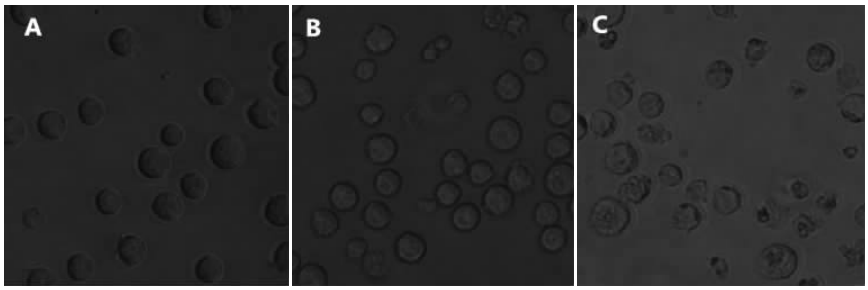

Figure 3 Morphological changes of Jurkat cells after Cbp transfection (×400)
Note: (A) Untransfected Jurkat cells; (B) negative control group; (C) Cbp-EGFP group. Arrows refer to the shrinkage of cells and debris.

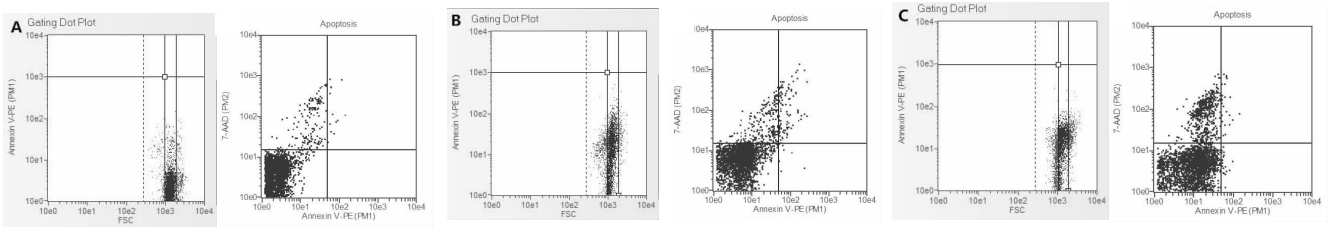

Figure 4 Effect of Cbp transfection on apoptosis of Jurkat cells
Note: (A) Untransfected Jurkat cells; (B) negative control; (C) Cbp-EGFP.

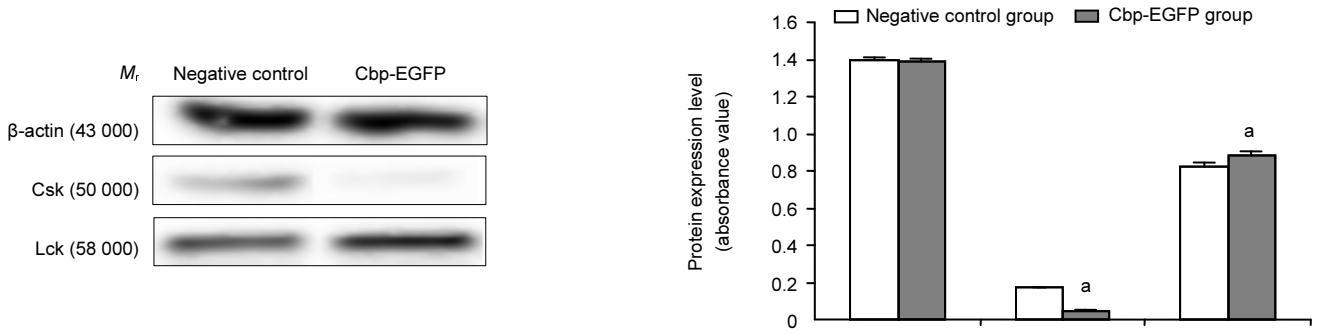

Figure 5 Effect of Cbp transfection on expression of Csk and Lck in Jurkat cells
Note: [a]$P < 0.05$, *vs*. negative control group.

are enhanced[21].With Jurkat cells transfected by Cbp-EGFP as the research objects, the effects of Cbp on Jurkat cells were illustrated from apparent and biological filed. Under the confocal microscope, Cbp was orientated on the glycolipid-enriched lipid rafts area on cell membrane. Cell physical parameters under optical microscopy and flow cytometry showed that growth condition of cells transfected with Cbp-EGFP was worse than that of negative control group. The cells were shrunk, ruptured with poor uniformity. The number of necrotic and apoptotic cells in the Cbp group was greater than that in the negative control group, and western blot showed free LCK (58 000) expression in TCR activated signal pathway was increased, while free inhibitorytyrosine Csk (50 000) expression was obviously decreased. These suggest that the high-expression Cbp combined with Csk may inhibit the activity of Src family kinase and play a certain inhibitory role in cell proliferation, which may induce cell apoptosis or cell death. Through changes of cell morphology and protein molecule in signal pathways, it is verified that Cbp plays a negative feedback role in T lymphocytes. Overexpression of Cbp molecules has a stronger inhibition by inhibiting the activity of Src family kinase, but the exact mechanism is unclear.

In this study, after transfection by Cbp-EGFP, cell morphology changed a lot and the number of death cells was increased, suggesting it may be due to apoptosis or autophagy, or both. Cell apoptosis, commonly called programmed cell death, means the cells receive a signal or stimulated by certain factors, which is an active death process in order to maintain a stable internal environment[22-23]. It appears in the normal development of the individual, and also appears in the abnormal physiological condition or disease. In addition to apoptosis, the forms of autophagy and necrosis may also be programmed, and they constitute the basis for cell death in isolated or combined with apoptosis[24]. There are multiple mechanisms such as caspase dependent apoptosis[25] and non-Caspase dependent apoptosis[26]. In recent years, an autophagic death in many organisms has been found. Autophagy is that the cytosol and organelles are sequestered into double membrane vesicles, which are transported to lysosomes/vacuoles to degrade, and make a formation of macromolecular recycling[27]. Autophagy death becomes a new way to promote tumor death[28]. In Riji lymphoma cell death induced by arsenic trioxide, cell apoptosis and autophagy death both exist at the same time[29]; autophagy inhibitor 3-methyl adenine (3-MA) can increase the apoptosis of Jurkat cells in acute T lymphocytic leukemia induced by arsenic trioxide[30]. Experimental findings from this study confirm that Cbp can inhibit the signal transduction of T cells, resulting in apoptosis, but the specific mechanism needs to be further improved. Further morphological detection is needed to distinguish autophagy and apoptosis. Apoptosis and autophagy pathway related proteins are also needed to be detected so as to confirm the role of Cbp in signal transduction pathways of acute T lymphocytic leukemia and provide better prospects for gene targeting treatment.

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高表达衔接蛋白 Src 羧基端激酶结合蛋白对 Jurkat 细胞形态及功能的影响

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文章亮点：
实验以病毒转基因技术建立了高表达 Src 羧基端激酶结合蛋白 Jurkat 细胞的细胞株，研究 Src 羧基端激酶结合蛋白基因在 T 系淋巴信号传导中所起的重要作用，高表达 Src 羧基端激酶结合蛋白对 T 系白血病作用的研究在国内和国际均较少。作者以“Cbp 衔接蛋白；均一性；免疫荧光；实时定量 PCR；细胞凋亡；Csk；Lck；信号转导”为检索词，检索 1990 至 2014 年中国知网期刊全文数据库、中国医学科普文献数据库、PubMed 数据库、中国期刊全文数据库等，最终认定实验具先进性。

关键词：
组织构建；组织工程；衔接蛋白；Src 羧基端激酶结合蛋白；细胞凋亡；Src 羧基端激酶；酪氨酸蛋白激酶；信号转导；国家自然科学基金
主题词：
细胞凋亡；T 淋巴细胞；Jurkat 细胞；衔接蛋白质类；信号转导
基金资助：
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摘要
背景：衔接蛋白分子的联动和协同有效调控 T 细胞的信号转导，形成级联放大或限制，最终实现 T 细胞复杂的免疫功能。蛋白分子 Src 羧基端激酶结合蛋白正是衔接蛋白的一种，其主要是通过负反馈调节 Src 家族激酶活性。而这种负反馈调节作用依赖于 Src 羧基端激酶结合蛋白的 Y317，可能涉及 Src 羧基端激酶的 SH2 结构域。
目的：探索衔接蛋白 Cbp 对 Jurkat 细胞形态及功能的作用。
方法：构建仅表达增强绿色荧光蛋白的融合蛋白和 Src 羧基端激酶结合蛋白-增强绿色荧光蛋白的融合蛋白的病毒颗粒。将 Jurkat 细胞分为未转染组、阴性对照组和 Src 羧基端激酶结合蛋白组，后 2 组转染仅表达增强绿色荧光蛋白的病毒和转染 Src 羧基端激酶结合蛋白-增强绿色荧光蛋白的病毒。
结果与结论：细胞转染效率大于 95%，Src 羧基端激酶结合蛋白定位于细胞膜上。转染 Src 羧基端激酶结合蛋白-增强绿色荧光蛋白病毒的 Jurkat 细胞，皱缩细胞增多，大小均一性较差，细胞坏死凋亡的数量增加，细胞中 Src 羧基端激酶结合蛋白基因表达上调，Src 羧基端激酶表达下降，而酪氨酸蛋白激酶表达增加。提示 Jurkat 细胞转染 Src 羧基端激酶结合蛋白后，可使细胞存活率下降，细胞信号转导功能减弱。

作者贡献：实验设计为通讯作者，实验实施为第一作者，辅助实验为第三、四、五作者，实验评估为通讯作者。第一作者成文，通讯作者审校，第一作者对文章负责。
利益冲突：文章及内容不涉及相关利益冲突。
伦理要求：实验过程中未涉及到动物实验，不存在伦理道德问题。
学术术语：Src 羧基端激酶结合蛋白-又称富含糖脂的脂膜微区相关的磷蛋白，是 2000 年 Brdicka 和 Kawabuchi 分别独立发现的 1 个一次穿膜蛋白，广泛表达于多种细胞膜表面，包括 T 淋巴细胞。它的发现补了 Src 羧基端激酶对 Src 家族激酶负反馈调节这一经典循环机制的空白。
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