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The Effect of Overexpressed Daxx in Liver Tumor Cells on The Apoptosis Induced by Oxidative Stress*

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Abstract In order to study the effects and the possible mechanisms of Daxx overexpressed in $HepG_2$ to hydrogen peroxide treatment, and to search new targets for cancer chemotherapy, $HepG_2$ cells were transfected using lipofectamine 2000, and selected by treatment with G418. Stable cell lines were confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) targeting vector gene. Experiments include the following groups: (1) control group (non-transfected cells); (2) transfected with empty vector ($HepG_2$/GFP cells); and (3) transfected with pEGFP-C1-Daxx ($HepG_2$/GFP-Daxx cells). After incubation with hydrogen peroxide (H_2O_2) for 24 h, cellular viability was analyzed by MTT, and cellular apoptosis was measured by flow cytometric analysis. Gene expression at protein level was detected by Western blot. The RT-PCR results showed that Daxx RNA in cells transfected with pEGFP-C1-Daxx was increased significantly compared with that in the $HepG_2$/GFP cells. Fluorescence microscopy revealed that Daxx protein was localized in the nuclei. Hydrogen peroxide was used to induce apoptosis of $HepG_2$ cells and observed that the hydrogen peroxide decreased the viability of $HepG_2$ cells in concentration-dependent pattern. The IC_{50} values in three groups (Normal cells, $HepG_2$/GFP cells and $HepG_2$/GFP-Daxx cells) were 0.72, 0.76, and 0.49 mmol/L respectively. The apoptotic ratio was significantly higher in $HepG_2$/GFP-Daxx cells as compared to the other two groups. $HepG_2$/GFP-Daxx cell incubated with hydrogen peroxide, showed a significant increase in the activation of caspase-3 and JNK as compare with the other groups. Over-expression of Daxx facilitated $HepG_2$ cells apoptosis induced by hydrogen peroxide. Furthermore, there may be a synergetic relation with apoptosis and increase of JNK activity.

Key words Daxx, apoptosis, $HepG_2$ cells, oxidative stress

Liver cancer is the third leading type among all cancers because of its common threat to health and mortality rate[1]. Relatively low therapeutic selectivity and high drug resistance are two major issues in liver cancer chemotherapy[2]. Recent evidence demonstrated that drugs could target different pathways to promote apoptosis, taking the advantage that apoptosis between cancer cells and normal cells is different. An increased number of studies[3] suggests that cancer cells, compared to normal cells, are increased with oncogenes expression and activity, which can prevent tumor cells from apoptosis and lower sensitivity to drugs therapy. Reconstruction of apoptosis signal transmitting system or promotion of death gene expression in tumor cells are reasonable targets that should be an aim in the research of cancer therapeutic drugs.

Though cell apoptosis has many different signaling pathways, the important death signals are Fas (factor associated suicide，also termed Apo or CD95) and TNF (Tumor necrosis factor), which activate intrinsic apoptotic suicide program if bound with ligand[4]. Death domain-associated protein (Daxx) was first identified in yeast two-hybrid screens for cDNAs

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encoding proteins capable of binding the cytosolic domain of Fas [5]. Daxx links this receptor to an apoptosis pathway involving activation of Jun N-terminal kinase (JNK)[6, 7]. JNK, also termed stress-activated protein kinase (SAPK), activates a pro-apoptotic event resulting in activation of pro-apoptotic members of caspase family and cytochrome c release from mitochondria. Daxx not only mediates apoptosis through Fas-Daxx-JNK pathway but also serves as tumor growth factor (TGF-beta) receptor to promote apoptosis[8, 9]. However, the impact of Daxx on the liver tumor cell line $HepG_2$ has not been reported. This experiment is to study the effects and possible mechanisms of Daxx overexpressed in $HepG_2$ exposed to hydrogen peroxide, and to search for new target for cancer chemotherapy.

1 Methods

1.1 Materials

Modified Eagle medium (MEM) and fetal bovine serum were purchased from GIBCO/BRL. Antibodies (Santa Cruz) against active caspase3 or JNK were used to detect the activation of caspase3 or JNK, β-actin antibody (Santa Cruz) was used to assay the expression of total protein. The plasmids of pEGFP-C1/Daxx and pEGFP-C1 were provided by Dr. WAN Yan-Ping laboratory. The pEGFP-C1/Daxx contains a full-length cDNA of hDaxx in pEGFP-C1 vector. All reagents were of analysis grade.

1.2 Cell culture and transfections

Hepatocyte line, $HepG_2$ cells, was obtained from Zhongshan University (Guangzhou, China). These cells were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FCS) and penicillin/streptomycin, at 37℃ in a humidified incubator containing 5% CO_2. Cells were seeded at a density of 1×10^5 cells/well in 24-well plate and cultured for 24 h to 60% ～ 80% confluence. To obtain stable transfectants, $HepG_2$ cells were transfected with the pEGFP-C1/Daxx or pEGFP-C1 plasmid using Lipofect 2000 Plus reagent (Invitrogen) in serum-free medium for 4 h at 37℃, according to the manufacturer's recommendations. The transfection medium was removed, and fresh complete growth medium was added. After 24 h post-transfection, the cells in two wells were split into 10-cm dishes in medium containing 500 mg/L geneticin (G418, Amresco, Solon, USA), and the medium was changed every 3 days until G418-resistant colonies were clearly evident. Individual colonies were picked into 6-well plates to continue incubation with G418 selection medium. Fluorescence microscopy was used to evaluate individual colonies for Daxx expression as described previously [10]. A monoclonal line was used for all experiments successively.

1.3 Reverse transcription-PCR

Total RNA was extracted from the cells using Trizol reagent (Gibco BRL) according to the manufacturer's protocol. Three micrograms of total RNA were used for reverse transcription in a total volume of 20 μl with the SuperScript preamplification system (Promega, Madison, MI). Aliquots of 2 μl cDNA were subsequently amplified in a total volume of 25 μl using the GeneAmp PCR kit (Promega) following conditions recommended by the manufacturer. The sense and antisense primer for Daxx were 5′ TGGCGCTCTATGTGGCAGAGATC 3′ and 5′ CTGCATCTGTTCCAGATCCTCCT 3′ (829 bp); the sense and antisense primers for actin that were used as an internal control were 5′ GGTGGCACCT-GTGGTCCACCT 3′ and 5′ CTTCACTTGTGGCC-CAGATAG 3′ (420 bp), respectively. The cycling conditions: 94℃ for 5 min, followed by 28 cycles of 94℃ for 30 s, 58℃ for 30 s, and 72℃ for 1 min, and a final extension of 72℃ for 10 min. PCR products were separated on the 1.5 % agarose gel viewed by ethidium bromide (EB) staining. These data were acquired with Alpha Imager 2200 software.

1.4 Cell viability assay

Cells were seeded in 96-well plates with density of 4×10^4/well. When nearly 80% confluent, the cells were treated with hydrogen peroxide. After 24 h, cells in 96-well plates were washed twice with PBS and MTT (final concentration of 500 g/L) was added to each well. Cells were incubated at 37℃ for 4 h and then 100 ml of DMSO was added to dissolve formed crystals. The absorbance measured at 570 nm was used to calculate the relative cell viability ratio. Fifty percent inhibitory concentrations were calculated as previously described[10].

1.5 Flow cytometric analysis

Cells were inoculated in 6-well plates with 2×10^5/ml density. When nearly 80% confluent, the cells were treated with hydrogen peroxide. Following trypsinization, detached cells were collected, washed twice with ice-cold PBS, and fixed in 70% cold ethanol. After fixation, cells were washed one more

time with PBS and stained in a PBS buffer containing propidium iodide (50 g/L), RNase A (100 g/L) and 0.05% Triton X-100 for 30 min. DNA content of the cells was analyzed by fluorescent activated cell sorting (FACS). At least 10 000 cells were analyzed. The percentage of cells in the sub-G1 population was calculated.

1.6 Western blotting analysis

The $HepG_2$ cells were washed with PBS, and 0.5 ml of TME lysis buffer (10 mmol/L Tris, pH 7.5, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 25 mmol/L NaF) containing fresh 100 μmol/L Na_3VO_4, 20 mg/L leupeptin, 1 mg/L pepstatin A, 4 mg/L aprotinin, and 1 mmol/L DTT were added. Cell lysates were prepared by freezing, thawing on ice, scraping, sonicating for 30 s, and centrifuging for 30 min at 15 000 *g*. Protein concentration in the supernatant was determined by bicinchoninic acid (BCA) protein assay kit, and the samples were stored at –80℃. For Western blot analysis, 20 μg of protein was subjected to SDS-PAGE under reducing conditions, and proteins were then transferred to polyvinylidene difluoride membrane as described previously. The membrane was blocked for 2 h at room temperature with a commercial blocking buffer from Life Technologies, Inc. The blots were incubated for 1 h at room temperature with the primary antibodies, 1 ∶ 1000 dilution, and followed by 1 h incubation with secondary antibody (horseradish peroxidase-conjugated). The presence of target proteins was revealed by a chemiluminescent assay (Amersham-Pharmacia Biotech).

1.7 Statistical analysis

All data were presented as $\bar{x} \pm s$. The differences between groups were assessed using student's t test for each trial. $P < 0.05$ was considered statistically significant.

2 Results

2.1 Location and expression of Daxx in $HepG_2$ cells

To examine the function role of Daxx in hepatic cells, $HepG_2$ cells were stably transfected with a Daxx-containing vector (pEGFP-C1-Daxx) or with the respective Daxx-negative "mock" vector (pEGFP-C1). Transfected cells were selected by G418 resistance. Majority of the colonies had fluorescence, only few could not be detected fluorescence[11]. Daxx was mostly located in the nuclear of $HepG_2$ (Figure 1a, b, c). To confirm the stable integration of the vector, RT-PCR was carried out with primers as specified in materials and methods. We observed that Daxx mRNA expression was increased significantly in $HepG_2$/GFP-Daxx cells compared to $HepG_2$/GFP and control cells (Figure 1d).

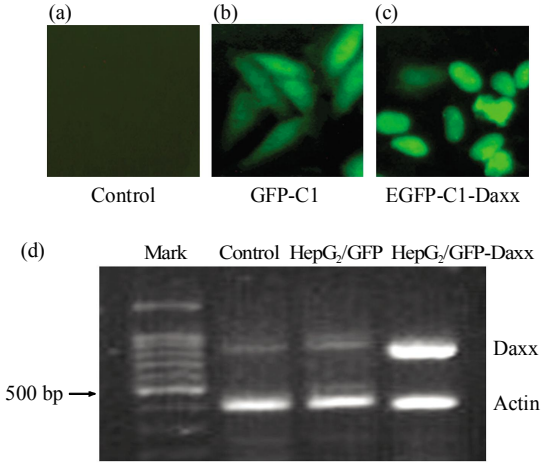

Fig. 1 The overexpression of Daxx in $HepG_2$ cells

(a), (b), (c) Images show the location and expression of Daxx in $HepG_2$ cells (400× magnitude). (d) RT-PCR of Daxx reveals mRNA expression has a significant increase in $HepG_2$/GFP-Daxx cells. Control: Untransfected cells; $HepG_2$/GFP: Cells transfected with pEGFP-C1 vectors; $HepG_2$/GFP-Daxx: Cell transfected with pEGFP-C1-Daxx vectors.

2.2 Overexpression of Daxx decreases $HepG_2$ viability injured by hydrogen peroxide

Table 1 shows hydrogen peroxide treatment for 24 h reduced viability of $HepG_2$ cells in a concentration dependent manner. Daxx overexpressing cells were more significantly sensitive to injury induced by hydrogen peroxide than control cells. Fifty percent inhibitory concentrations (IC_{50}) of hydrogen peroxide were 0.72, 0.76, 0.49 mmol/L in control, $HepG_2$/GFP, $HepG_2$/GFP-Daxx cells respectively (Figure 2a).

Table 1 The effect of H_2O_2 on $HepG_2$ cells viability

$c(H_2O_2)$/(mmol•L^{-1})	Cell viability ratio (%)		
	Control	$HepG_2$/ GFP	$HepG_2$/ GFP-Daxx
0	100.0±8.04	98.7±7.61	102.8±12.65
0.05	108.5±10.56	113.3±5.47	109.2±15.21
0.1	92.3±8.88	94.6±9.52	82.5±9.83[1)]
0.2	83.6±5.48	81.1±7.89	61.6±2.58[2)]
0.5	69.1±6.90	71.1±13.91	52.3±10.21[2)]
1.0	50.2±18.26	52.1±7.85	43.6±7.72[2)]

$n = 5$, ($\bar{x} \pm s$). [1)] $P < 0.05$ *vs* control or $HepG_2$/GFP group, [2)] $P < 0.01$ *vs* control or $HepG_2$/GFP group.

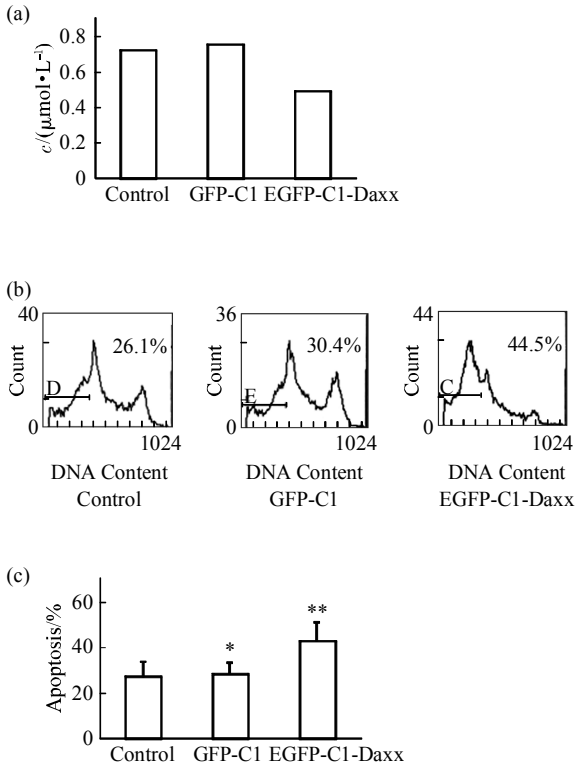

Fig. 2 Effects of Daxx overexpression on H_2O_2-induced apoptosis in $HepG_2$ cells

(a) The effects of Daxx overexpression on H_2O_2 IC_{50}. (b, c) The cells were stained with PI, and the apoptosis rate was determined using a flow cytometer. Data were expressed as ($\bar{x} \pm s$) of three independent experiments. *$P > 0.05$ *vs* control , **$P < 0.05$ *vs* control or $HepG_2$/GFP group.

2.3 Daxx accelerates $HepG_2$ apoptosis induced by hydrogen peroxide

$HepG_2$/GFP and $HepG_2$/GFP-Daxx cells had no difference when compared with control cells in spontaneous apoptosis and cellular cycle (data not show). Daxx did not directly affect cell viability. When treated with 0.02 mmol/L hydrogen peroxide for 24 h, $HepG_2$/GFP-Daxx cells had apoptosis significantly more than $HepG_2$/GFP or control cells ((42.9 ± 8.42)% *vs* (27.3 ± 6.38)% or (28.5 ± 4.71)%, Figure 2b, c). Western blotting data (Figure 3a) further illustrated an increase of caspase-3 activation in $HepG_2$/GFP-Daxx cells induced by hydrogen peroxide. Activating caspase-3 is an executive step in the apoptosis process. As shown in Figure 3b, the activation of caspase-3 is about doubled in $HepG_2$/GFP-Daxx cells than control, or $HepG_2$/GFP cells.

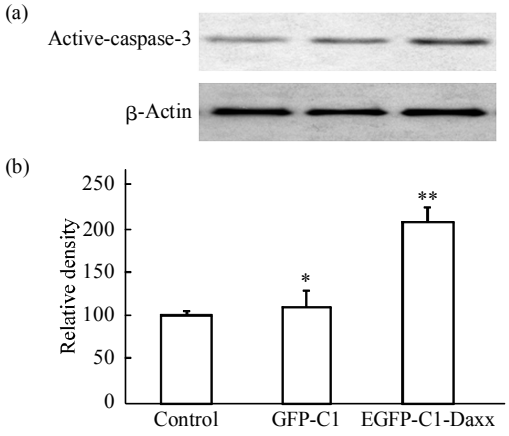

Fig. 3 Effect of Daxx overexpression on H_2O_2-induced activation of caspase-3

(a) Western blot analysis of the cell lysates shows that caspase-3 activation in Daxx-overexpressed cells is increased compared to control following H_2O_2 exposure. (b) Quantification of the relative densities of active-caspase-3 (19 ku) levels by densitometric analysis. Data are the ($\bar{x} \pm s$) of three independent experiments. *$P > 0.05$ *vs* control, **$P < 0.05$ *vs* control or $HepG_2$/ GFP group.

2.4 Effect of Daxx on phosphorylation of JNK

JNK will be activated in Fas mediated apoptosis pathway [12]. Hydrogen peroxide stimulated the phosphorylated of JNK in $HepG_2$ cells. At the same time, β-actin was used as the intrinsic reference of phosphor-JNK. Daxx increased activation of JNK nearly 3 times in HepG2/GFP-Daxx cells than control or $HepG_2$/GFP cells ((279.45 ± 15.67)% *vs* (100 ± 15.67)%, (95.9 ± 26.31)%, $P < 0.05$)(Figure 4).

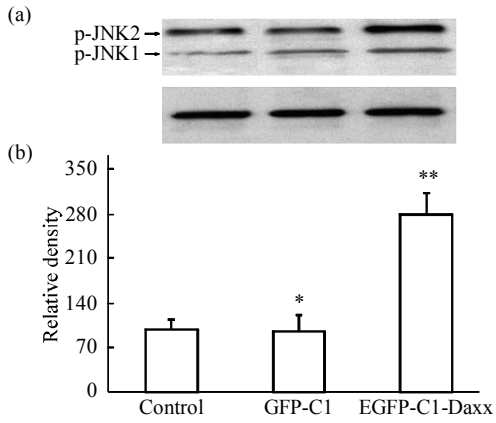

Fig. 4 Effect of Daxx overexpression on H_2O_2-induced activation of JNK

(a) Western blot analysis of the cell lysates shows that JNK activation in Daxx-overexpressed cells is increased compared to control following H_2O_2 exposure. (b) Quantification of the relative densities of phosphor-JNK levels by densitometric analysis. Data are the ($\bar{x} \pm s$) of three independent experiments. *$P > 0.05$ *vs* control, **$P < 0.05$ *vs* control or $HepG_2$/ GFP group.

3 Discussion

Many reports [13, 14] have implicated that Daxx sensitized apoptosis in tumor cells. Daxx, initially identified as interacting with the Fas receptor death domain, was shown to enhance Fas-mediated apoptosis when overexpressed [5]. Fas (also termed CD95 or APO-1) is a cell surface transmembrane receptor of the tumor necrosis factor receptor family. Its association with the Fas-associated death domain (FADD) efficiently activates death pathway of Fas-FADD-JNK-caspase and induces apoptosis [15]. Daxx was proposed to function in a pathway dependent of FADD and to mediate apoptosis through activation of caspase family[16].

We detected Daxx in the nucleus of $HepG_2$. As reported[17, 18], Daxx was present exclusively in nucleus and localized into promyelocytic leukemia protein nuclear body (PML-NB), and Daxx had no effect on survival and cellular cycle of $HepG_2$ cells by itself. However, our data from MTT assay confirmed lower viabilities in Daxx overexpression cells compared to control cells when injured with hydrogen peroxide. Consistent with these data, flow cytometric analysis demonstrated that Daxx overexpression accelerated $HepG_2$ apoptosis induced by hydrogen peroxide.

Several anticancer agents currently used for cancer treatment have been shown to cause increased cellular ROS (reactive oxygen species) generation. ROS generation play an important role in mediating apoptosis induced by therapeutic agents such as cisplatin, bleomycin and anthracyclines [19, 20]. ROS are broadly defined as oxygen-containing chemical species with reactive chemical properties, and includes free radicals such as superoxide and hydroxyl radicals, and non-radical molecules such as hydrogen peroxide. ROS can cause cardiolipin peroxidation and alteration in membrane permeability, leading to caspase-3 activation and apoptosis[21]. Caspase-3 is a major player in caspase family. Caspase families are cysteine proteases that cleave target proteins at specific separate residues to execute apoptosis. In the present study, we further observed that Daxx overexpression accelerated caspase-3 activation when induced with hydrogen peroxide, implying Daxx can increase liver tumor sensitivity to drugs.

Daxx can promote Fas-mediated apoptosis through activating signal-regulating kinase-1 (ASK-1)/ JNK pathway [7]. JNK is a member of the mitogen-activated protein kinase family, and is also activated in response to oxidative stress. Recent data showed that Daxx mediates cell apoptosis induced by oxidative stress [22]. Khelifi *et al.* [23] showed that Daxx was essential for stress-induced death of human fibroblasts and was able to regulate the JNK pathway in physiological settings. Consistent with our data, overexpression Daxx increased phosphorylation of JNK induced by hydrogen peroxide in $HepG_2$ cell.

In conclusion, overexpression Daxx increased activation of JNK and promoted $HepG_2$ apoptosis induced by hydrogen peroxide.

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Daxx 过表达对氧化应激诱导的肝肿瘤细胞凋亡的影响 *

度勤慧[1)**]　熊国祚[2)**]　朱炳阳[1)]　曹建国[3)]　廖端芳[1)***]

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摘要　死亡结构域相关蛋白 Daxx 可以敏化多种肿瘤细胞的凋亡过程，但对于肝肿瘤细胞株 $HepG_2$ 的影响未见报道．为了研究 Daxx 增加肝 $HepG_2$ 细胞对药物敏感性的影响及机制，为开发药物新的药理作用提供理论依据，分别转染 pEGFP-C1 和 pEGFP-C1-Daxx 这两个载体到 $HepG_2$ 细胞．实验分组如下：(1)正常对照组(未转染细胞组)；(2)pEGFP-C1 空载体转染组($HepG_2$/ GFP 细胞)；(3)pEGFP-C1-Daxx 表达载体转染组($HepG_2$/ GFP-Daxx 细胞)．筛选稳定细胞株，用逆转录聚合酶链反应检测 mRNA 的表达；用过氧化氢孵育 24 h 诱导细胞凋亡，采用 MTT 法和流式细胞术检测细胞凋亡率，Western blot 检测蛋白质的表达．经 G418 筛选稳定的细胞运用 RT-PCR 技术分析其 mRNA，结果显示，转染绿色荧光蛋白 Daxx 表达载体的细胞 Daxx 的 mRNA 明显上调；用荧光显微镜观察到 Daxx 蛋白主要定位于细胞核．用过氧化氢诱导 $HepG_2$ 细胞凋亡，观察到过氧化氢呈浓度依赖性地抑制 $HepG_2$ 细胞活性．正常对照细胞、$HepG_2$/ GFP、$HepG_2$/ GFP-Daxx 3 组细胞的 IC_{50} 值分别是 0.72、0.76、0.49 mmol/L．并且运用流式细胞仪检测到 $HepG_2$/ GFP-Daxx 组细胞凋亡率明显高于转染空载体质粒组与未转染组((42.9±8.42) *vs* (27.3±6.38) or (28.5±4.71))．提示 $HepG_2$/ GFP-Daxx 细胞对过氧化氢的反应性较未转染细胞和 $HepG_2$/ GFP 敏感．还运用 Western-blot 检测到活化的 caspase3 在 Daxx 转染组细胞表达最强，达到(204.66±19.68)%，而未转染和 $HepG_2$/ GFP 组细胞分别是(100±3.1)%、(107.39±20.1)%，进一步说明了 Daxx 可以增加 $HepG_2$ 细胞对于过氧化氢的敏感性．同时，观察到过氧化氢处理 24 h 后，Daxx 转染组细胞磷酸化的 JNK 表达明显高于空载体转染组和未转染细胞组．上述结果表明：a．Daxx 可以增加肝 $HepG_2$ 细胞对过氧化氢诱导的细胞凋亡敏感性；b．Daxx 蛋白敏化过氧化氢诱导的 $HepG_2$ 细胞凋亡可能与协同增加 JNK 活性有关．

关键词　Daxx，$HepG_2$ 细胞，细胞凋亡，过氧化氢
学科分类号　Q25

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