Overexpression of calcineurin B subunit (CnB) enhances the oncogenic potential of HEK293 cells

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Calcineurin (CaN) is a Ca^{2+}/calmodulin (CaM)-stimulated protein phosphatase. It is a heterodimeric enzyme consisting of a catalytic A subunit (CnA) and a Ca^{2+}-binding regulatory B subunit (CnB). CaN's key role *in vivo* is well known, while the function of CnB keeps unclear except that it acts as a regulator of CaN. The present paper shows that CnB overexpression promotes proliferation of human embryonic kidney HEK293 cells by comparing with vector control cells in the complete or serum reduced medium. Furthermore, stable CnB transfectants showed dramatically improved growth in soft agar. And the migration ability of CnB overexpressors also was enhanced remarkably. But in the progress of transformation, the activity of CaN remained unchanged between CnB overexpressors and controls. Therefore, CnB, rather than CaN, is involved in the proliferation promotion of HEK293 cells. Subsequently, 11 proteins with different expression levels between CnB transfectants and controls were identified using two-dimensional gel electrophoresis and electrospray ionization time-of-fight mass spectrometry. Therein, the expression of heat shock protein 27 (HSP27) and protein DJ-1 increased along with CnB overexpression. The important role of CnB in cell neoplastic transformation was found and the possible mechanism was analyzed. (*Cancer Sci* 2008; 99: 1100–1108)

CaN, also known as protein phosphatase 2B, the only serine/threorine-specific protein phosphatase regulated by CaM, is a heterodimeric enzyme consisting of a CnA and a CnB.[1,2] Two isoforms of CnB exist in mammalian cells, CnBα and CnBβ. CnBα is ubiquitously expressed as a heterodimer with CnAα or CnAβ, and CnBβ is expressed only in testis and binds to CnAγ (CnBα was employed in the present study). CaN takes part in many physiological and pathological processes as a potentially powerful mediator of intracellular signals. But CnB has always been depicted as a regulatory subunit exclusively for CnA, so little has been known about its independent function. Our previous work has revealed that the expression level of CnB was much higher than that of CnA in some tumor tissues.[3] More interestingly, recombinant human calcineurin B subunit (rhCnB) showed an anticancer potential, and had a positive effect on the phagocytic index and coefficient.[4,5] However, the detailed mechanism of rhCnB's anticancer action has not been well understood yet.

The sequence of CnB shares a high degree of similarity with that of CaM (67%) and CaN homologous proteins (CHP1 [65%], CHP2 [54%]), and all the three proteins play a key role in various intracellular biological processes, such as cell proliferation. The overexpression of CaM[6] and CHP2 (unpublished data from Guodong Li and Xi Zhang) enhanced oncogenic potential of cells while CHP1 overexpression suppressed the proliferation of cells.[7] In some tissues, the molar ratio of CnA/CnB is not equal to one, and sometimes more CnB than CnA or even only CnB exists.[3,8] Recently, Saeki *et al*. found that CnB promoted TNF-α/CHX-induced apoptosis, although the phosphatase activity of CaN was not involved in this progress.[9] All the results suggested that CnB could play other important roles besides the function as a regulatory subunit of CaN.

Due to the elusive mechanism underlying the anticancer potential of rhCnB protein and the multiple biological functions of CHP1, CHP2, and CaM *in vivo*, we hypothesize that CnB could be involved in the regulation of cellular function other than just acting as a regulatory subunit of CaN. In the present study, recombinant vector pEGFP-C1/CnB and control vector pEGFP-C1 were, respectively, transfected into human embryonic kidney HEK293 cells, and G418 was used to select the stable cell clones. Then the alterations in cell behavior following CnB overexpression in HEK293 cells were examined to determine the molecular mechanisms by which CnB exerts function on its own. The results obtained from this experiment reveal a novel independent function of CnB, and also suggest one of the mechanisms underlying the anticancer potential of the rhCnB protein.

Materials and Methods

Antibodies. Anti-GFP (rabbit IgG) and antiβ-actin (rabbit IgG) were purchased from ProteinTech (Chicago, IL, USA). Anti-hsp27 (goat IgG) was purchased from Zhongshan Golden Bridge Biotechnology (Beijing, China). Anti-CnB (mouse IgG) was prepared in our laboratory.

Construction of CnB expression vector. PEGFP-C1/CnB expression vector was constructed as follows. The cDNA sequence covering the full open reading frame of CnB was obtained with PCR from PET21a/CnB, which was constructed previously in our laboratory. The cDNA was digested with *Sal*I-*BamH*I and inserted into the *Sal*I-*BamH*I site of pEGFP-C1 (Clontech, USA) to obtain pEGFP-C1/CnB. Similarly, pcDNA3.1/CnB was constructed with the cDNA inserted into the *BamH*I-*Xho*I site of pcDNA3.1 (Invitrogen, Carlsbad, CA, USA).

Cell culture and transfection. Human embryonic kidney HEK293 cells and human liver LO2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL; Life Technologies, USA) and Jurkat cells in RPMI-1640 (Gibco-BRL), supplemented with 10% heat-inactivated fetal bovine serum (FBS). HEK293 and LO2 cells were transfected using calcium phosphate precipitation as described previously,[10] or using Fu GENHD (Roche, Basel, Switzerland) reagent according to the manufacturer's instructions. Jurkat cells were transfected by eletroporation with a Gene Pulser (Bio-Rad, USA) set at 140 V, 1000 uF according to the manufacturer's instruction. HEK293 stable transfectants were obtained by selection in G418 (0.5 mg/mL) (Amresco, USA), and subcloned by limiting dilution. The expression level of CnB was then determined by reverse transcription–PCR (RT-PCR) and Western blotting.

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Abbreviations: BSA, bovine serum albumin protein; CaN, calcineurin; CaM, calmodulin; CBB, coomassie Brilliant Blue; CnA, calcineurin A subunit; CnB, calcineurin B subunit; 2-DE, two-dimensional gel electrophoresis; ESI-TOF, electrospray ionization time-of-fight; FBS, fetal bovine serum; Hsp27, heat shock protein 27; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; rhCnB, recombinant human calcineurin B subunit; RT-PCR, reverse transcription–polymerase chain reaction.

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Cell proliferation analysis. To determine the cells growth in complete medium, CnB stable transfectants and the vector controls were seeded into six-well plates (Corning Costar, USA) in regular growth medium containing 10% FBS. To determine the cells growth under reduced serum conditions, 12 h after plating cells in regular growth medium, the cells were switched to low serum containing medium (0% or 5% FBS). At each time point, cells from three independent wells were collected by trypsinization and counted with a hemocytometer. For proliferation assay by MTT,[11] CnB stable transfectants and vector controls were seeded in sextuple into flat-bottom 96-well plates (Corning Costar), and then MTT assay was performed at each time-point. In addition, transfected cells (HEK293 and LO2) were maintained for 9–14 days after transfection in culture media supplemented with G418. Then the proliferation of the cells was measured with cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) according to the instructions.

Soft-agar colony formation assay. In the soft agar assay,[12] the same number of cells (1.2×10^4) were suspended in 1 mL of 0.3% agarose (Amresco) and then added to one well of a six-well plate with a foundation layer 0.5% agarose in triplicate. Approximately 24 h later, the cells received an additional 1 mL of growth medium before incubation for further 7–12 days. Colonies were counted in 20 fields of 10× magnification.

Transwell migration assay. Transwell migration assay was performed with Millicell inserts (8-μm pore size; Millipore, USA) in a 24-well culture plate (Corning Costar) as described previously.[13] Briefly, CnB stable cells or the vector control cells, and 7×10^4/100 μL DMEM containing 0.2% FBS, were seeded in the insert chamber of transwell as test group or control group, correspondingly. The lower-room contained 500 μL complete medium. After being incubated for 36 h, the cells on the lower surface were fixed and stained with crystal violet. Then the membranes were fixed onto slides and examined by microscope, and the number of migrated cells was counted.

CaN enzymatic activity. Cell lysates were prepared as described elsewhere.[14] The concentration of protein was measured with the Bradford method using BSA (Sigma, USA) as the standard. CaN enzymatic activity was measured with the substrate ^{32}P-labeled RII peptide as described previously.[15,16] Briefly, in the first series of experiments, Ca^{2+} and okadaic acid (OA) (100 nM) (Sigma) were added to the assay, then we got total activity less protein phosphatases 1 and 2A (PP1, PP2A). In the second series of experiments, glycol-bis (2-aminoethylether)-N, N, N′, N′-tetraacetic acid (EGTA) and OA (100 nM) were added to the assay, then we got the activity, but not PP1, PP2A, and CaN. Therefore, CaN activity was determined as phosphate released from RII in the presence of Ca^{2+} and OA minus phosphate released in the presence of OA plus EGTA. Finally, released ^{32}P was separated from RII peptide (Biomol, USA) and quantified with the liquid scintillation spectrometry (Packard 2900TR; Packard Bioscience, USA). The units (U) of protein phosphatase activity were defined as nanomoles of ^{32}P of RII peptide released per milligram lysates per minute.

Pull-down assay. CaM-sepharose pull-down assays were carried out essentially as described previously.[17] CnA was prebound to CaM-sepharose beads (prepared in our laboratory) in the binding buffer (50 mM Tris-HCl [pH 7.4], 5 mM $CaCl_2$). After washing with the same binding buffer five times, the beads with or without CnA were added into the cell lysates or rhCnB, and incubated at 4°C for 2 h with shaking. Then the beads were washed five times with the binding buffer. The bound proteins were eluted with the elution buffer (50 mM Tris-HCl [pH 7.4], 10 mM EGTA). The eluted proteins were separated on sodium dodecyl sulfate (SDS) gels and examined with Western blotting.

Western blotting. Western blotting analysis was done as previously described.[11] Briefly, the cultured cells were harvested, washed with phosphate-buffered saline, and lyzed with cell lysis buffer (Beyotime Biotechnology, Haimen, China). The cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the nitrocellulose membrane (Millipore). The blot was then probed with primary antibody followed by reaction with horseradish peroxidase–conjugated secondary antibody. The signal was detected using enhanced chemiluminescence and recorded on X-ray film.

Two-dimensional gel electrophoresis (2-DE), image analysis, in-gel digestion followed by electrospray ionization time-of-fight (ESI-TOF) mass spectrometry, and database search. 2-DE analysis was performed as detailed previously.[18] The cultured cells were harvested, and lyzed with lysis buffer,[18] (containing 8 M urea, 4% 3-([3-cholamidopropyl] dimethylamonio)-1-propane-sulfonate, 40 mM Tris-HCl, 0.5% pharmalyte, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 5 mM phenylmethylsulfonyl fluoride, and 1% dithiothreitiol) for 1 h on ice, then the mixture was centrifuged at approximately 175 000g to remove insoluble substance. Protein concentration was determined with 2-DE Quant Kit (Amersham Biosciences, Uppsala, Sweden). The image analysis, ESI-TOF mass spectrometry, and database search were done the same way as previously described.[18] Briefly, each sample of 2 mg total protein was loaded on immobilized 18-cm, pH 3–10 linear strip and then rehydration was performed at 30 V for 12 h. The sample focusing condition was as follows: 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 8000 V gradient for 1 h, and 8000 V for 60 000 Vh. The 2-DE separation was performed on 12% SDS-PAGE and the gels were stained with CBB R350.[18] The images of the 2-DE were scanned and analyzed using a MagicScan densitometer in transmission mode and were analyzed with the software ImageMaster 2D Elite. Protein spots of interest were then excised from the gels, destained, digested with trypsin, and underwent mass determination with the Qstar Pulser I Quadruple time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) mounted with NanoESI source. The peptide matching and protein searching of the SWISS-PROT database were done using the MASCOT search engine (http://www.matrixscience.com).

Total RNA extraction and real-time quantitative RT-PCR. Total RNAs were isolated from cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's directions, and then treated with RNase-free DNase I (Takara, Japan). Total RNA were reversely transcribed using reverse transcriptase M-MLV and oligo(dT) 15 primer (Takara). Primers for real-time PCR (Table 1) were designed using Primer Premier 5.0 and synthesized by Invitrogen. Real-time quantitative PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems) using SYBR Green (Applied Biosystems) reagent according to the manufacturer's instructions. Correct PCR products were confirmed by agarose gel electrophoresis and melting curve analysis. The relative expression of mRNA was calculated using the comparative threshold cycle (Ct) method and the following formula: $\text{Ratio} = 2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct(\text{sample}) - \Delta Ct(\text{calibrator})]}$, where ΔCt = Ct of target genes – Ct of endogenous control gene (β-actin).[19]

Statistical analysis. All experiments were performed in triplicate and the results are expressed as the mean ± SD. Statistical significance was determined using the software SPSS 11.0 for Windows (SPSS, Chicago, IL, USA). One-way ANOVA with the Latin Square Design test was performed for multiple comparisons and student's *t*-test was used for paired samples. Differences were deemed significant if the calculated *P*-value was < 0.05.

Results

Generation of CnB+ clones of HEK293 by transfection with an EGFP/CnB fusion protein expression vector. The Hek293 cell line was selected as the recipient because of its lower levels of endogenous CnB (Fig. 1a). After transfection of HEK293 cells with the pEGFP-C1/CnB expression vector (pEGFP-C1/CnB) and control vector (pEGFP-C1), nine stable CnB overexpressors

Table 1. Primer sequences and their melting temperature (°C), and the product sizes of for RT-PCR

Gene	Primer sequences (5′-3′)	Tm† (°C)	Product size (bp)
HSP27	5-CCCAAGTTTCCTCCTCCCTGTC-3 (forward) 5- GCGGCAGTCTCATCGGATTTT-3 (reverse)	60	160
DJ-1	5′GGAGCAGAGGAAATGGAGACG3′ (forward) 5′TCAAGGCTGGCATCAGGACAA3′ (reverse)	60	140
CnA	5′GAGACGCTACAACATCAAACTG3′ (forward) 5′CGCCGAATCTGCTCCATA3′ (reverse)	60	130
β-actin	5-GTGACAGCAGTCGGTTGGAG-3 (forward) 5-AGTGGGGTGGCTTTTAGGAT-3 (reverse)	60	149

†Tm, melting temperature.

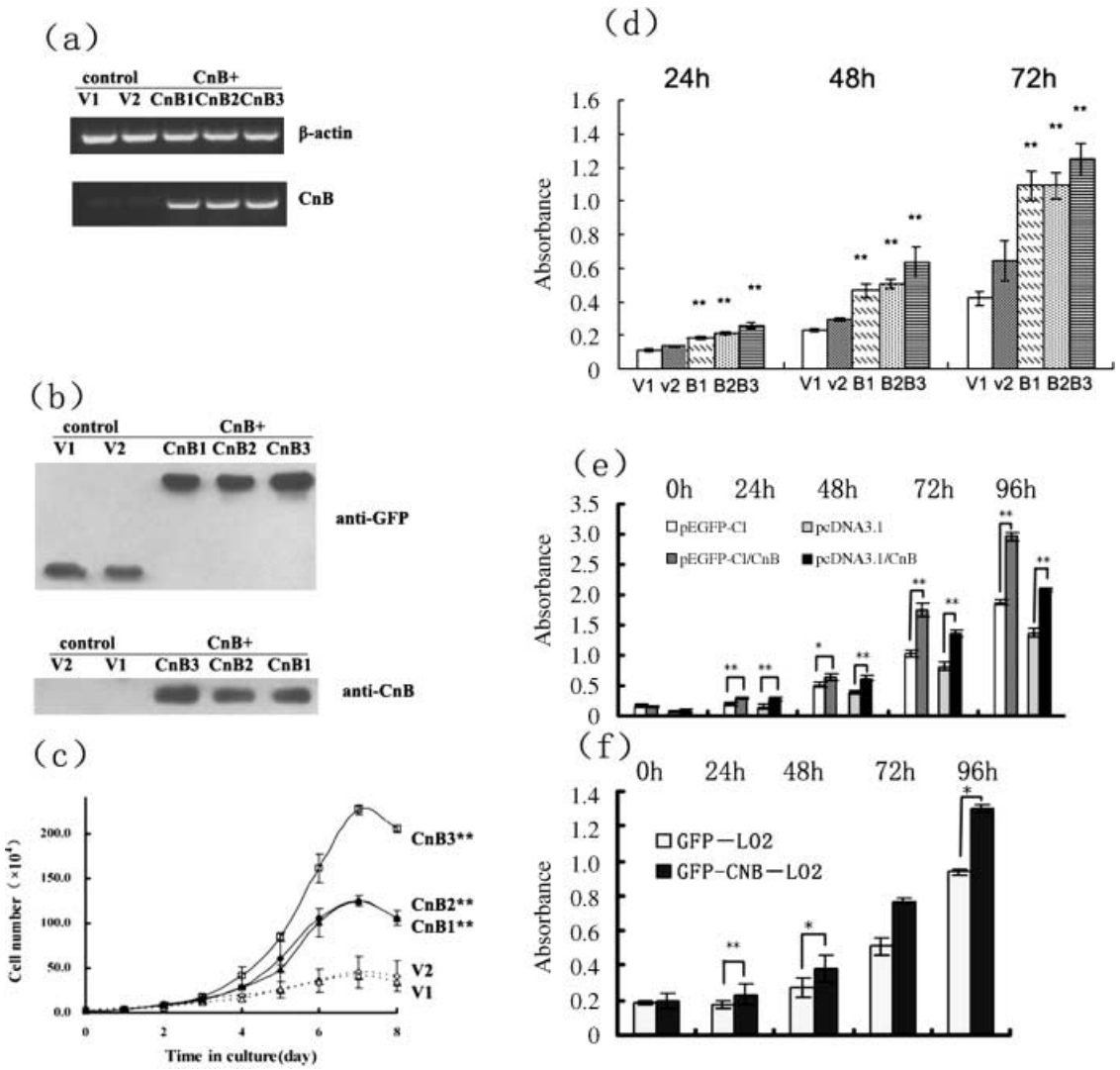

Fig. 1. Expression analysis of calcineurin B subunit (CnB) in stable transfectants and accelerated proliferation of CnB-transfected cells. After transfection of HEK293 cells with pEGFP-C1/CnB or pEGFP-C1, three CnB transfectant clones (CnB1, CnB2, CnB3) and two vector controls (V1 and V2) were established. CnB expression in HEK293 stable transfectants was determined by (a) reverse transcription–polymerase chain reaction (RT-PCR) and (b) Western blotting. In Western blotting assays, total cell lysates were subjected to immunoblotting with anti-GFP or anti-CnB antibodies. In this result, the expression of endogenous CnB could not be detected, because the expression level of endogenous CnB was very low while the expression level of GFP-CnB was much higher. (c) CnB transfectants and vector controls were cultured for 8 days, and cell counts were obtained every day. Data shown are averages of two independent experiments (**$P < 0.001$, compared to the average absorbance of V1 and V2 cells, respectively). (d) Proliferation of CnB transfectants and vector controls analysis with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. CnB+ cells and control cells (2×10^3) were seeded into 96-well plates. The MTT assay was performed at 24 h, 48 h, and 72 h, respectively (**$P < 0.001$, compared to the average absorbance of V1 and V2 cells, respectively). (e) Effect of pEGFP-C1/CnB and pCDNA3.1/CnB on the growth of HEK293 cells. Cell counting kit-8 (CCK-8) assays were performed 9 days after transfection and subsequent incubation with media containing G418 (**$P < 0.001$, *$P < 0.01$). (f) Effect of CnB-overexpression on the growth of LO2 cells. CCK-8 assays were performed 9 days after transfection and subsequent incubation with media containing G418 (**$P < 0.001$, *$P < 0.01$).

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and four vector control clones were established, wherein three CnB stable clones (CnB1, CnB2, CnB3) and two vector control clones (V1, V2) (Fig. 1a,b) were selected to study the effect of CnB overexpression on cell proliferation, migration, and clone formation in soft agar.

CnB overexpression accelerates proliferation of HEK293 cells. The difference in proliferation between the CnB+ cells and V+ cells was measured with cell enumeration and MTT assay. As shown in Figure 1c, the CnB stable transfectants (CnB1, CnB2, and CnB3) constantly produced more cells than the vector controls at each time point. A faster proliferation rate of the CnB3 cell line was observed along with a higher expression of CnB, so the proliferative activity of the test cells seemed to have a positive correlation with the expression level of CnB. According to the MTT assay, the overexpression of CnB could enhance the proliferation of HEK293 cells remarkably (Fig. 1d). It is worth noting that this phenomenon is not unique to either the HEK293 cell line or some specific clones, because the proliferation-promoting effect of CnB was also observed in the LO2 cells in bulk cultures (Fig. 1e,f). In addition, similar proliferation-promoting results were observed in HEK293 cells transiently transfected with another construct, pcDNA3.1/CnB (Fig. 1e). All of the results suggested that CnB overexpression could accelerate the proliferation of cells, and that the proliferation-promoting potential was not restricted to a specific cell line or vector.

Oncogenic activity of CnB to HEK293 cells. Since it is widely believed that the alteration in growth control is a critical phenotype of neoplastic transformation, we also examined other characteristics of tumorigenesis in CnB overexpressors, such as anchorage-independent growth, serum-independent growth, and motility.[20,21] In the soft agar assay, CnB-overexpressed cells and vector-transfected control cells were seeded into soft agar, and the results from a representative experiment are shown in Figure 2a. The data of three independent experiments are shown in Figure 2b. As can be seen in the soft agar plate, the cell lines overexpressing CnB formed many more colonies, and they were larger in shape compared with the vector control cells. Furthermore, when examined under the light microscope, CnB overexpression cells appeared to be more densely packed than the vector control cells (Fig. 2a). In other words, the anchorage-independent growth of HEK293 cells was promoted by CnB overexpression. In the serum dependent growth assay, CnB+ and V+ cells were placed in medium containing 0%, 5%, and 10% FBS, and the cell numbers were determined in triplicate at 36 h, 60 h, and 84 h. Statistical analysis of the results (shown in Fig. 2c) demonstrated a significant increase ($P = 0.001$) in cell numbers of CnB+ at all time-points when compared with those of V+ cells at three different concentrations of FBS. Since enhanced motility is another characteristic of transformation, the migration abilities of CnB overexpressors were determined.

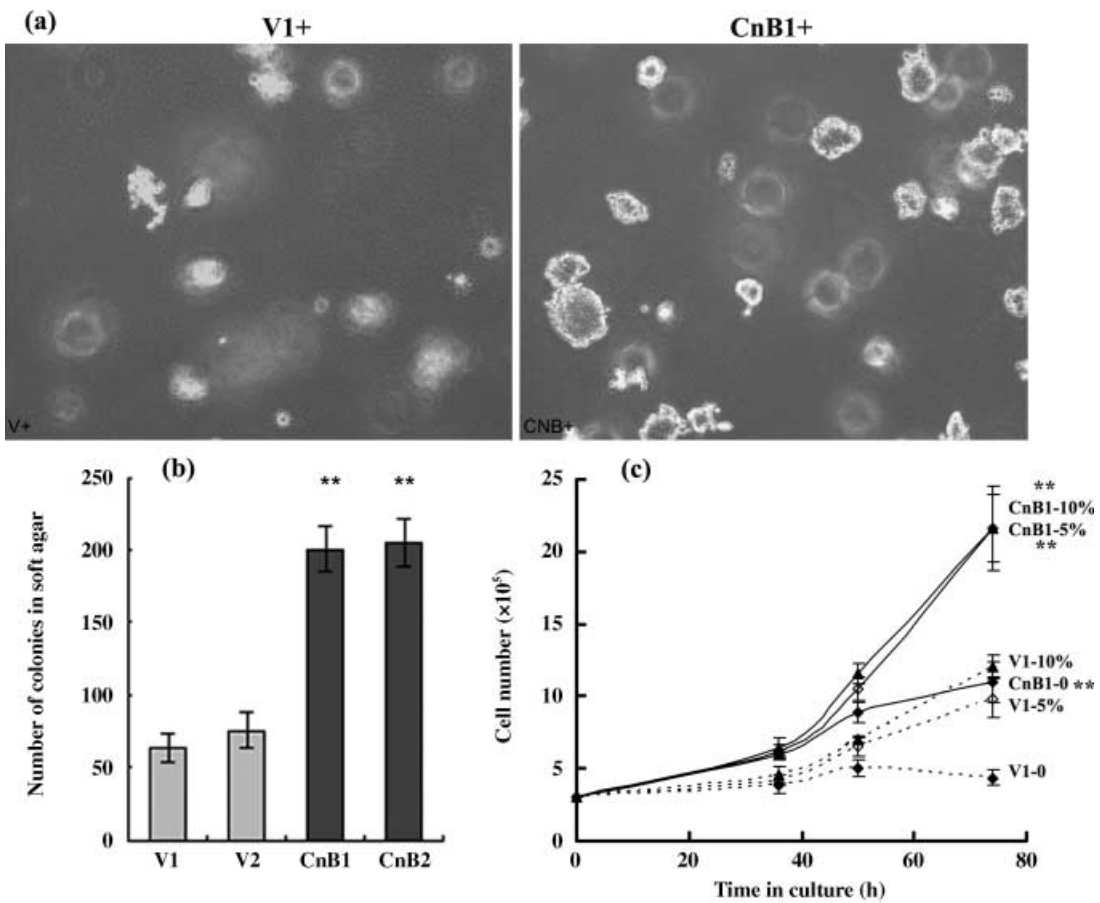

Fig. 2. Accelerated anchorage-independent and serum-independent growth of calcineurin B subunit (CnB)–transfected cells. (a) Images of colony growth in soft agar assays. (b) Graph of colony number formed by CnB+ cells and V+ cells. The figure shows the average and SD of two independent assays. (**$P < 0.001$. compared with the average number of colonies of V1 and V2 cells, respectively). (c) *In vitro* growth in reduced serum. Growth rates were determined by plating cells at 3×10^4 cells/well in six-well tissue culture plates in 10%, 5%, and 0% serum. Triplicate wells of each clone were harvested at the indicated times, and the number of viable cells was counted. Data are expressed as mean ± SD (**$P < 0.001$. CnB1-10% *vs* V1-10%, CnB1-5% *vs* V1-5%, and CnB1-0% *vs* V1-0%, respectively). CnB+ cells showed an ability to grow in reduced serum, while the growth of V+ cells was inhibited. The experiments were repeated on three separate occasions with essentially similar results.

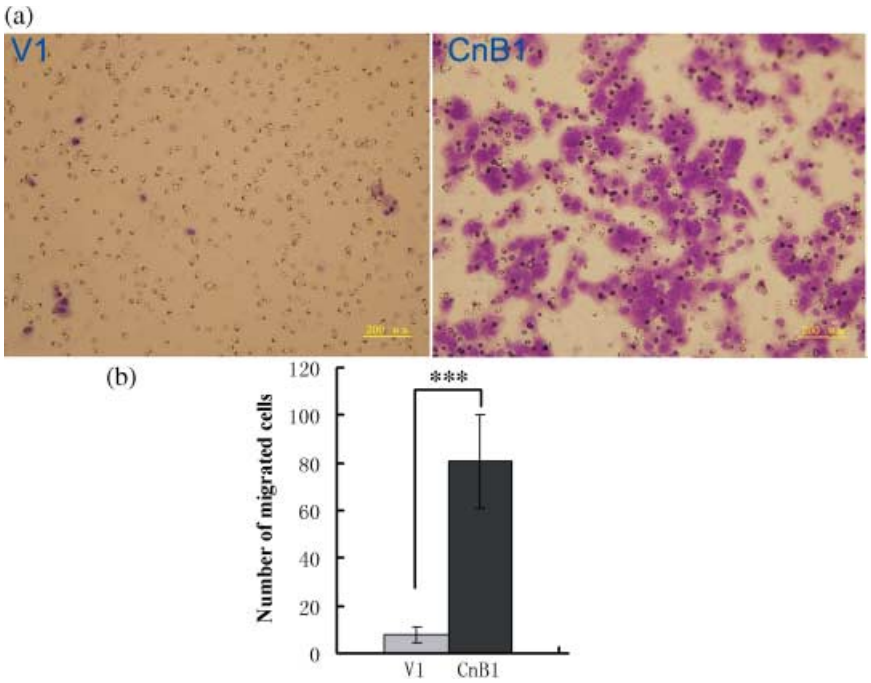

Fig. 3. Accelerated migration ability of calcineurin B subunit (CnB)–transfected cells. Image of cells migrated through pored membrane in the CnB+ cells and V+ cells. CnB transfectants and vector controls (7×10^4/100 μL) in Dulbecco's modified Eagle's medium containing 0.2% fetal bovine serum were seeded into the insert chamber of the transwell. The lower-room contained 500 μL complete medium. After being incubated for 36 h, the cells on the upper surface were removed, cells on the lower surface were fixed and stained with crystal violet, and then membranes were fixed on slides to be examined with microscope. (a) Images of migrated cells on transwell membrane. (b) The graph of the number of migrated cells (**$P < 0.001$).

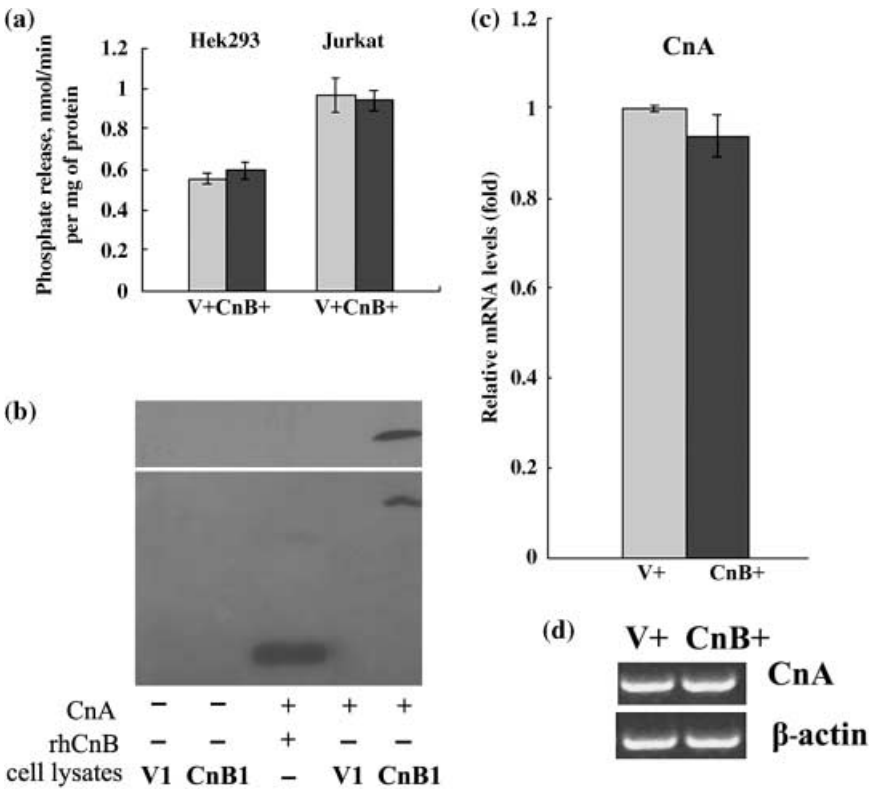

Fig. 4. Expression analysis of calcineurin A subunit (CnA), the binding of GFP-calcineurin B subunit (CnB) with CnA, and enzymatic activity of calcineurin (CaN) in CnB transfectants and vector control cells. (a) No significant difference in calcineurin enzymatic activity between CnB overexpressors and vector controls. CaN enzymatic activity was determined with ^{32}P-labeled RII peptide using stably transfected HEK293 cells and transiently transfected Jurkat cells. Phosphatase activity is expressed as nmol phosphate released per min per mg of cell protein. (b) GFP-CnB could bind with CnA. CaM-sepharose pull-down assays were performed to determine the binding. The CaM-sepharose without CnA could not pull down CnB, while the CaM-sepharose with CnA could pull down the rhCnB and GFP-CnB of the cell lysastes. (c) Real-time quantitative polymerase chain reaction (PCR) analysis of the expression of CnA. Relative mRNA expression ratios of CnA mRNA expression in CnB+ and V+ cell line. β-actin was used as an internal control. The data are shown as means of three independent experiments ± SD. (d) The expression of CnA mRNA in transiently transfected Jurkat cells. Jurkat cells were transfected with CnB or empty vectors by eletroporation. Total RNA were isolated 48 h after transfection and reverse transcription–PCR was performed with *β-actin* gene as the inner control.

As shown in Figure 3, the number of CnB+ cells migrating through the pored membrane into the transwell cell culture chamber was increased remarkably when compared with V+ cells. In conclusion, all of the results above revealed that CnB overexpression promoted neoplastic transformation of HEk293 cells.

CaN enzymatic activity remained unchanged. Because several lines of evidences suggested that CaN may be involved in tumorigenesis,[22–24] we examined the enzymatic activity of CaN in these CnB overexpressors using the ^{32}P-RII assay,[14,15] as shown in Figure 4a,b; although GFP-tagged CnB has the ability to bind with CnA, CaN enzymatic activity remained unchanged in CnB-overexpressed HEk293 cells and Jurkat cells when compared with vector control cells. In addition, the same mRNA expression level of CnA in all CnB overexpressors was observed using real-time quantitative PCR and RT-PCR (Fig. 4c,d).

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Table 2. List of proteins identified as being differentially expressed

Spot No.	ProteinAC[†]	Protein name	Mr[‡] (Da)	pI[§]	Sequence coverage
C1	Q9UI95	Mitotic spindle assembly checkpoint protein MAD2B	24 334	6.05	EVYPVGIFQK
C2	Q86UN2	Reticulon-4 receptor-like 1	49 065	9.25	TLAPETFQGLVK
C3	Q14961	Putative nitric-oxide synthase IIC	8432	10.68	ILVRPGTGIAPFHSFUQQR
C4	Q71RG4	Transmembrane and ubiquitin-like domain-containing protein 2	33 788	4.92	QAGAGSSSPEAPLR
C5	P42212	Green fluorescent protein	26 886	5.67	SAMPEGYVQER
C6	Q8IYX4	Dead end protein homolog 1	38 687	9.74	LMMTFSGLNR
D1	O14558	Heat shock protein beta-6	17 136	5.95	MEIPVPVQPSWLR
D2	Q92551	Inositol hexaphosphate kinase 1	50 236	6.81	FLLLENVVHHFK
D3	P04792	Heat shock protein beta-1	22 783	5.98	VSLDVNHFAPDELTVK
D4	P52907	F-actin-capping protein subunit alpha-1	32 923	5.45	LLLNNDNLLR
D5	Q99497	Protein DJ-1	19 891	6.33	GPGTSFEFALAIVEALNGK

[†]SWISS-PROT accession number of each protein. [‡]Theoretical molecular weight of the matching protein in Da. [§]Theoretical isoelectric point of the matching protein.

Thus, the higher CaN enzymatic activity as a candidate to enhance the proliferation of CnB+ cells can be excluded. In fact, we also looked into the reason why CaN activity remained unchanged. We believe that, when endogenous CnA has been bound and saturated by CnB, it can not be further activated by extra overexpressed GFP-tagged CnB (data not shown).

Fifteen proteins were differentially expressed in CnB+ cells and V+ cells. To further investigate the mechanism underlying CnB-overexpression induced neoplastic transformation, the protein expression profiles from these stable transfectants were examined using 2-DE SDS-PAGE gel. Analysis of total lysates from these cell lines revealed 15 protein spots, whose expression between CnB+ cells and V+ cells showed significant differences. Then these 15 candidates were chosen for further ESI-TOF mass spectrometry analysis. Eleven of these spots were identified with good peptide coverage (Table 2). Two of them, HSP27 and DJ-1, have an increased expression in CnB+ cells (Fig. 5). To our surprise, HSP27 and DJ-1 have been found to have a higher expression level in many tumors,[25–29] such as renal cell carcinoma, pancreas carcinoma, breast cancer, and leukemia, as well as non-small-cell lung carcinoma, and they have been considered to play important roles in tumorigenesis. However, the question of whether the increased expression of HSP27 and DJ-1 were induced directly by CnB overexpression or by the subsequent transformation remains unclear to us. We hope that further investigation into this issue can shed light on the mechanism of CnB-induced transformation.

Real-time quantitative PCR and Western blotting revealed the same trends in 2-DE results for HSP27 and DJ-1. To validate the identified proteins, Western blotting analysis and real-time quantitative RT-PCR were performed to compare the levels of HSP27 and DJ-1 between the CnB+ and V+ cells. Both of these results suggested the expression of HSP27 and DJ-1 increased approximately one-fold in CnB+ cells compared with that in control cells (Fig. 6a,b). The enhancement of their expression in real-time PCR was not as obvious as that in 2-DE and may be due to the regulation of translational and post-translational modifications and other processes. The discordance between protein and mRNA levels has been observed previously in mammalian cells.[18,30,31] Furthermore, there are two forms of HSP27 in the cell, phosphorylated and non-phosphorylated, and the two forms of HSP27 could be separated in 2-DE assay due to their different isoelectric points.[32,33] Indeed, we detected these two forms in our electrophoresis and the expression of the non-phosphorylated remained unchanged. However, the antibody we used in Western blotting assay could not distinguish these two forms of HSP27, thus the results were not as significant as for the 2-DE. In addition, like stable transfectants, the expression of HSP27 and DJ-1 also increased approximately one-fold in CnB-transient transfected cells, while no significant difference was observed in the expression levels of these genes before and after transfection.

Discussion

The key role of CnB in stimulating CaN phosphatase activity as a regulatory subunit in a Ca^{2+}-dependent manner has been well documented. However, the independent intracellular function of CnB remains unclear. In addition to the fact that the molar ratio of CnB/CnA can exceed 1 in some tissues, recent findings have indicated that CnB had a high expression in mitochondria,[8] and some tumors,[3] in the absence of detectable CnA. Furthermore, the rhCnB protein had been proved to possess a remarkable anticancer activity *in vivo* but a clear mechanism has not been determined.[4,5] In this paper, the gene of CnB was enforced into the HEK293 cells to explore its independent function in cells and analyze the mechanism underlying the anticancer potential of rhCnB protein. Data from this study demonstrated that CnB had an oncogenic potential.

Although previous studies on *D. discoideum* have revealed that a 30-fold overexpression of single *CnBα* gene resulted in moderately accelerated multicellular development with recombinant populations completing morphogenesis about 2–3 h earlier than wild-type cells,[34] the function of CnB in proliferation of mammalian cells has not been tested. In this study, CnB overexpression remarkably promoted the proliferation of HEK293 cells and LO2 cells *in vitro*. In addition, CnB+ stable cells formed larger and denser clones within 1 week based on soft agar assay, and the number of colonies in CnB+ stable cells was approximately three-times of that of vector controls. CnB overexpressors proliferated faster under reduced serum conditions and displayed more enhanced migration ability than control cells. Since the ability to form colonies in soft agar and the growth independent of serum were reflective of tumorigenicity in nude mice,[35,36] all of the phenotypes of CnB overexpressors suggested that CnB played a role in inducing the neoplastic transformation of HEK293 cells. In addition, since CnB is a critical regulator to CaN function, the RNA interference (RNAi)–induced silence of *CnBα* gene could also reduce the enzymatic activity of CaN, which cannot exclusively reflect the independent function of CnB. That was why we did not performed RNAi assay towards CnB in our studies. Indeed, some studies also revealed that the effect of RNAi of CnB was similar to that of CnA,[37] and after the CnA gene was silenced, the

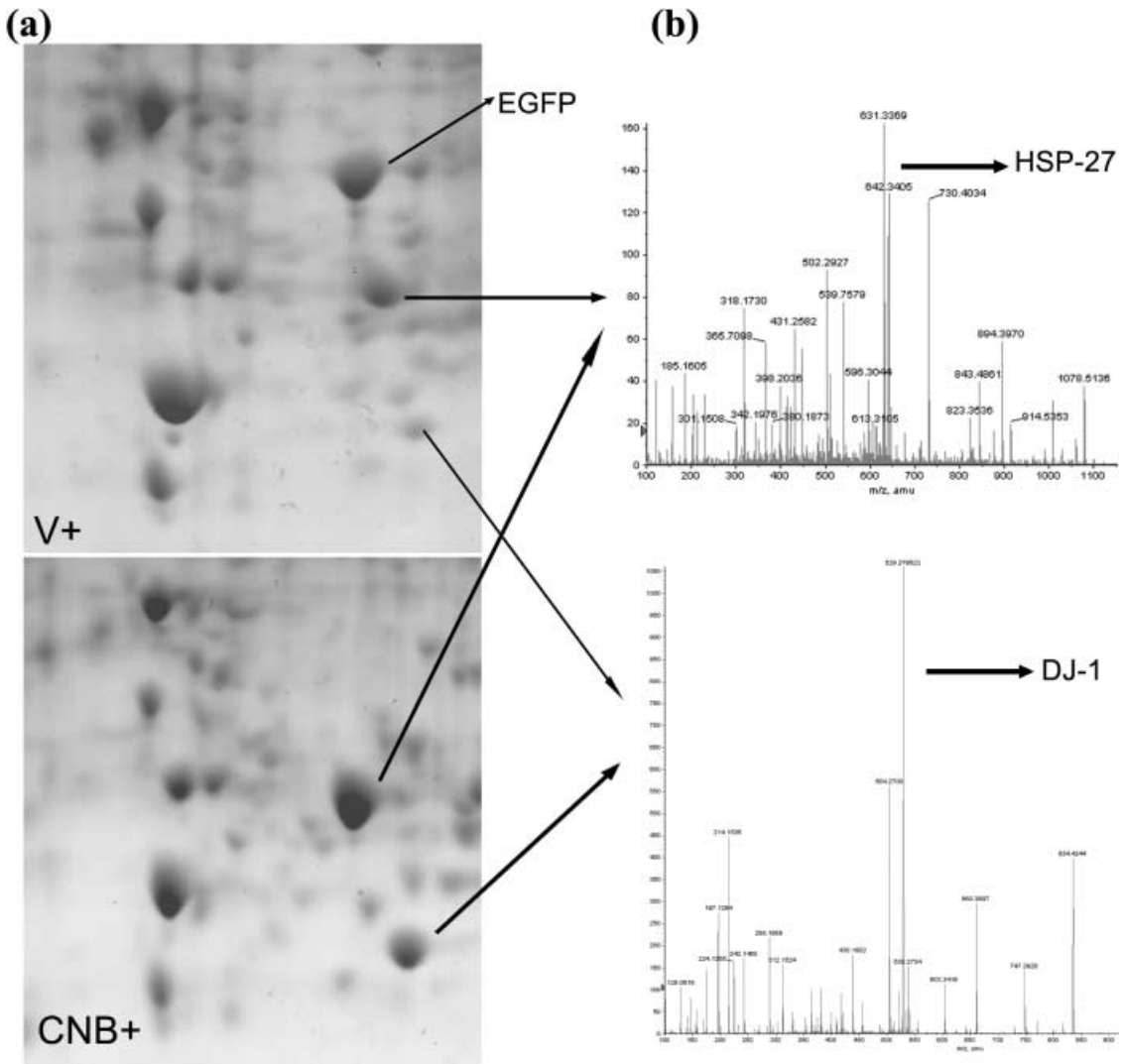

Fig. 5. (a) Partial enlarged view of coomassie Brilliant Blue (CBB) R350-stained 2-D electrophoresis (2-DE) patterns of the CnB+ cells and V+ cells. For each, 2 mg protein samples were separated on linear immobilized pH gradient strips, followed by 12.0% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). (b) A representative mass spectrometry spectrum. Mass determination used the Qstar Pulser I Quadruple time-of-flight mass spectrometer mounted with NanoESI source. The peptide matching and protein searching of the SWISS-PROT database were done using the MASCOT search engine (http://www.matrixscience.com).

decreased malignant potential of cells was observed. Furthermore, cyclosporine A (an inhibitor of CaN) had an antiproliferative effects in a wide variety of mammalian cells, including adenocarcinoma cell lines, lymphoma and leukemia cell lines, keratinocytes, fibroblasts, and smooth muscle cells.[38–41] Therefore, the silence of CnB may suppress the transformation potential of cells.

In this study, the activity of CaN was measured in these CnB overexpressors, vector controls in HEK293, and Jurkat cells, as some researchers had claimed that the activity of CaN can also contribute to the carcinogenesis and metastasis of some cells.[22–24] However, the phosphatase activity of CaN remained unchanged between CnB and empty vector transfectants, The unchanged activity of CaN was due to the unchanged expression of CnA. Although GFP-tagged CnB could bind to CnA, the extra CnB could not promote the activity of CaN after CnA had been saturated. Moreover, the addition of the rhCnB protein to HEk293 and Jurkat cell lysates could not promote the CaN enzymatic activity either (data not shown). Thus, it was proved to be CnB, rather than CaN, that is involved in the neoplastic transformation of CnB+ cells.

Except for the rhCnB protein's positive effect on the phagocytic index and coefficient,[4,5] the detailed mechanism of the tumor-suppressive function of rhCnB protein has not been understood. Based on the much higher expression level of CnB than CnA in some tumor tissues;[3] higher levels of CaN in human colorectal adenocarcinomas, cervical, and neuronal tumors;[22–24] as well as CnB's oncogenic potential, we hypothesized that the rhCnB protein could activate T-cell immunity as a tumor-associated antigen; and the potential of the rhCnB protein in activation of T-cells may be another key mechanism underlying its anticancer effect. It is generally accepted that the fragments of antigenic proteins are recognized by Th1 lymphocytes when they are bound to major histocompatibility complex class II (MHC-II). The possibility of the rhCnB protein binding to MHC-II was also predicted using the ProPred server (http://www.imtech.res.in/raghava/propred/), as described previously.[42] Analysis of the complete sequence showed that rhCnB could bind to 49 (96%) of the total 51 HLA-DR alleles included in the ProPred database (data was not shown), which revealed the promiscuous nature of rhCnB for presentation to T-cells through the MHC class-II processing pathway. Experimental

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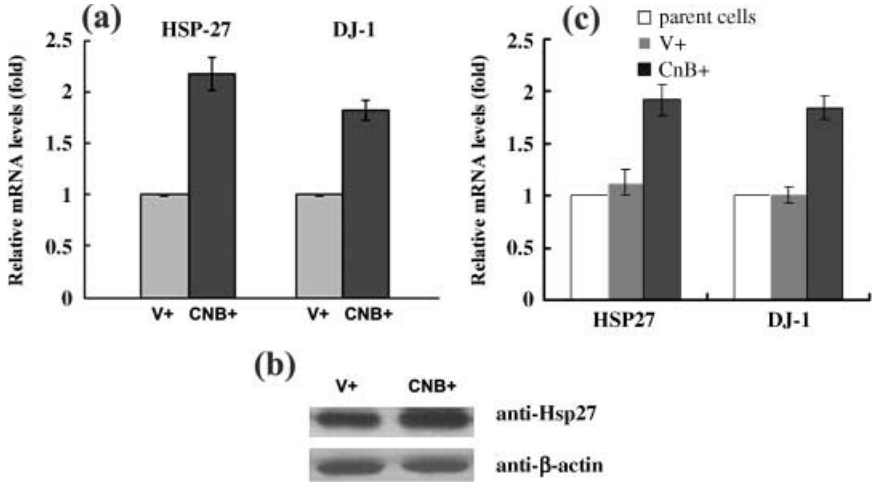

Fig. 6. Real-time quantitative polymerase chain reaction and Western blotting analysis of the expression of HSP27 and DJ-1. (a) Relative mRNA expression ratios of HSP27 and DJ-1 mRNA expression in the CnB+ and V+ cell line. β-actin was used as an internal control. The data are shown as means of three independent experiments ± SD. (b) Validation of the differential expression of HSP27 protein between the CnB+ and V+ cell line. β-actin was used as an internal control for equal protein loading. (c) The expression levels of HSP27 and DJ-1 were compared with transiently transfected cells. Similar results with stable cells were obtained between CnB overexpressors and vector controls, while no significant difference was observed between vector control cells and parental cells.

validation of this prediction is presently being undertaken and it is expected to identify some more effective fragments of CnB as anticancer agent.

The precise mechanism of the CnB-inducing transformation remains to be determined. Neoplastic cell transformation was hypothesized to be the result of a multistep process including the activation of oncogenes and the inactivation of tumor-suppressor genes.[43] The 2-DE gel and ESI-TOF mass spectrometry were performed to identify differentially expressed proteins between overexpressors and control cells. The expression of HSP27 and protein DJ-1 was up-regulated in CnB+ stable cells, which may provide some insight into the mechanism underlying the CnB-induced transformation. In addition, Wei *et al*. believed that this function of CnB was involved in a manner similar to that played by CaM.[8] Therefore, the manner of CaM in cell-cycle progression could act as a reference for the study of CnB. Recently, we have been trying to identify the proteins interacting with CnB and fill in the blanks in the signal transduction circuit between HSP27 and DJ-1 and CnB. Our final goal is to elucidate the precise mechanism of CnB's oncogenic potential.

In conclusion, a new function of CnB was found as a regulator in neoplastic cell transformation other than in stimulating CaN. The results of our experiment provided the guidance to find the more effective anticancer fragments of CnB. Insights into relationship between CnB and cancer require further investigation.

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