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Nuclear Matrix Protein, Prohibitin, Was Down-Regulated and Translocated From Nucleus to Cytoplasm During the Differentiation of Osteosarcoma MG-63 Cells Induced by Ginsenoside Rg1, Cinnamic acid, and Tanshinone IIA (RCT)

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ABSTRACT

Ginsenoside Rg1, cinnamic acid, and tanshinone IIA (RCT) are effective anticancer and antioxidant constituents of traditional Chinese herbal medicines of Ginseng, Xuanseng, and Danseng. The molecular mechanisms of anticancer effects of those constituents and their targets are unknown. Prohibitin, an inner membrane-bound chaperone in mitochondrion involved in the regulation of cell growth, proliferation, differentiation, aging, and apoptosis, was chosen as a candidate molecular target because of its frequent up-regulation in various cancer cells. We demonstrated that prohibitin existed in the filaments of the nuclear matrix of the MG-63 cell and its expression was down-regulated by the treatment of RCT using proteomic methodologies and Western blot analysis. Immunogold electro-microscopy also found that prohibitin was localized on nuclear matrix intermediate filaments (NM-IF) that had undergone restorational changes after RCT treatment. Prohibitin may function as a molecular chaperone that might interact with multiple oncogenes and tumor suppressor genes. We found that oncogenes c-myc and c-fos and tumor suppressor genes P53 and Rb were regulated by RCT as well and that these gene products co-localized with prohibitin. Our study identified prohibitin as a molecular target of the effective anticancer constituents of Ginseng, Xuanseng, and Danseng that down-regulated prohibitin in nuclear matrix, changed prohibtin trafficking from nucleolus to cytoplasm, and regulated several oncogenes and tumor suppressor genes. Prohibitin downregulation and cellular trafficking from nucleolus to cytoplasm indicated RCT protective roles in cancer prevention and treatment. J. Cell. Biochem. 108: 926–934, 2009.

KEY WORDS: CELL DIFFERENTIATION; HUAMAN OSTEOSARCOMA MG-63 CELLS; NUCLEAR MATRIX; PROHIBITIN

Ginsenoside Rg1, cinnamic acid, and tanshinone IIA (RCT) are effective constituents of Ginseng, Xuanshen, and Danshen, respectively. They were valued in Chinese traditional medicine for maintaining youth, promoting longevity, and balancing whole body ying and yang to prevent diseases. Previous studies showed that RCT prevents tumor growth through reversal of gene expression of transformed cells by antioxidant, anti-inflammatory, and apoptotic mechanisms [Yun, 2001; Helms, 2004].

Prohibitin (PHB) is an tumor-suppressive protein that is expressed in various cells [Sato et al., 1992; Snedden and Fromm, 1997; Loukas and Maizels, 1998]. There are two members of prohibitin family, PHB1 and PHB2. They were reported as integral

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mitochondrial inner membrane proteins that depend on each other for their functional stability. PHB1 and PHB2 act not only as an inner membrane-bound chaperone [Nijtmans et al., 2000] in mitochondrion, but are also located in nuclei acting as tumor suppressors. Prohibitins play important roles in the regulation of cell growth, proliferation, differentiation, aging, and apoptosis [Nijtmans et al., 2002; Piper et al., 2002; Fusaro et al., 2003; Sanz et al., 2003]. The over-expression of prohibitin was found in various tumor cells [Jang et al., 2004; Qi et al., 2005; Tsai et al., 2006]. This seems to be contradictive with its tumor suppressive role. So far, prohibitin's subcellular localization, cellular trafficking, and regulation are not clearly shown in cell proliferation and differentiation.

In our previous study about the differentiation of human osteosarcoma MG-63 cells, we found the existence of prohibitins in nuclear matrix. We would like to know whether prohibitins are involved in molecular mechanisms of anti-cancer effects of the combination of ginsenoside Rg1, cinnamic acid and tanshinone IIA (RCT)-induced differentiation of human osteosarcoma MG-63 cells. We reported previously that osteosacoma MG-63 cells could be induced to differentiation by RCT [Li et al., 2009]. In this study, we demonstrated that prohibitins were localized in nuclear matrix, its expression was down-regulated, and its subcellular localization was shifted from nucleus to cytoplasm by treatment with RCT in MG-63 cells. We further studied the relationship of prohibitins and relative products of oncogenes and tumor suppressor genes. This study provided scientific evidence for the function of prohibitins during cell differentiation as a molecular antitumor target for Ginseng, Xuanshen, and Danshen effective components.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENT

The osteosarcoma MG-63 cells, provided by the China Center for Type Culture Collection (CCTCC), were maintained in RPMI-1640 medium supplemented with 15% heat-inactivated fetal calf serum, 100 U ml^{-1} penicillin, 100 $\mu g\,ml^{-1}$ streptomycin, and 50 $\mu g\,ml^{-1}$ kanamycin at 37°C, 5% CO_2. After being seeded for 24 h, MG-63 cells were treated with culture medium containing a combination of 33 $\mu g\,ml^{-1}$ ginsenoside Rg1, 2 mmol L^{-1} cinnamic acid and 0.3 $\mu g\,ml^{-1}$ tanshinone IIA (abbreviated as RCT) for 7 days to induce differentiation. Meanwhile, MG-63 cells were cultured in RPMI-1640 medium as the control group. The components of RCT were purchased from National Institute of the Control for the Pharmaceutical and Biological Products (NCPBP). The concentration of RCT was determined by previous work that evaluated the effects of terminal differentiation on MG-63 cells treated with these components and their combination.

EXTRACTION OF NUCLEAR MATRIX PROTEINS (NMPs)

For 2-DE analysis, we followed the procedures from Gao et al. [1994], Nickerson et al. [1997], and Michishita et al. [2002] to extract NPMs. Briefly, harvested MG-63 cells were washed twice with ice-cold PBS. The cells were then homogenized and extracted with cytoskeleton (CSK) buffer (100 mmol L^{-1} KCl, 3 mmol L^{-1} MgCl, 5 mmol L^{-1} EGTA, 10 mmol L^{-1} PIPES pH6.8, 300 mmol L^{-1} sucrose, 0.5% triton X-100, 2 mmol L^{-1} PMSF) for 10 min at 0°C. After being centrifuged at 600*g* for 5 min, the pellets were washed with ice-cold PBS to remove soluble cytoplasmic proteins. The pellets were then re-centrifuged and suspended within digestion buffer (same as CSK buffer except with 50 mmol L^{-1} NaCl instead of KCl) containing 400 $\mu g\,ml^{-1}$ DNase I for 30 min at room temperature. Cold ammonium sulfate was added to a final concentration of 0.25 mol L^{-1} to terminate the DNase I activity. After centrifugation at 1,000*g* for 10 min, the pellets were washed with CSK buffer and then dissolved in lysis buffer (7 mol L^{-1} urea, 2 mol L^{-1} thiourea, 4% CHAPS, 1.5% Triton X-100, 1% Pharmalyte (Amersham Biosciences, pH 3–10), 65 mmol L^{-1} DTT, 40 mmol L^{-1} Tris, 5 mg ml^{-1} aprotinin, 1 mg ml^{-1} leupeptin, 1 mg ml^{-1} pepstatin, and 1 mmol L^{-1} PMSF, 3 mmol L^{-1} EDTA). The sample was sonicated at 0°C for 30 min and centrifuged at 15,000*g* for 1 h. The protein concentrations of the control and treated set in the supernatant were measured using the Bradford assay (Beyotime Biotechnology, China) and then diluted to the same concentration (about 5 mg ml^{-1}) with lysis buffer and stored at −70°C until future use.

TWO-DIMENSIONAL GEL ELECTROPHORESIS AND IMAGE ANALYSIS

2-D PAGE was performed with general methods described in the URL (http://proteome.tmig.or.jp/2D/2DE_method.html). Immobilized linear pH 3–10 gradient 18 cm dry strips (Bio-Rad, USA) were used for first-dimension isoelectric focusing. In the second-dimensional run, 12% SDS–polyacrylamide gels were used. The gels were stained with silver nitrate compatible with MS. After image scanning (UMAX PowerLook III), quantification analysis of three repeated sets of silver-stained 2-D gels for each sample was performed using PDQuest 8.0 software (Bio-Rad). The intensity of each protein spot was normalized to the total intensity of the entire gel. The spots of protein whose intensity changed twofold were defined as differentially expressed nuclear matrix proteins.

MALDI-TOF-MS ANALYSIS AND PROTEIN IDENTIFICATION

The spots of differentially expressed proteins were cut out from the gels. After performing a series of steps including silver removal, reduction with DL-Dithiothreitol, alkylation with iodacetamide, and in-gel digestion with modified trypsin (sequencing grade; Promega), the peptide mass finger maps (PMF) were generated by an autoflex MALDI-TOF mass spectrometry (Bruker Daltonics Co., Germany). All spectra data were obtained with a positive-ion reflector and analyzed using FlexAnalysis (Bruker Daltonics Co.) software to calibrate and remove polluted peaks. The peptide fragments (842.509, 2211.104) obtained by trypsin auto-catalytic hydrolysis were used as inner control for calibration. The resulting PMF were then searched against NCBInr and Swiss-Prot protein databases by the program MASCOT in NCBI.

WESTERN BLOTTING

The proteins were separated by SDS–PAGE (12%) and transferred onto polyvinylidene fluoride (PVDF) membranes using an electro-blotting apparatus. Nonspecific reactivity was blocked with 4% BSA in 20 mM Tris–HCl pH 8,150 mM NaCl and 0.5% Tween-20 (TBST) overnight and subsequently incubated with mouse anti-Prohibitin

(Ab-1, clone II-14-10; NeoMarkers, USA) monoclonal antibody (1:1,000) and rabbit anti-β-actin monoclonal antibody (Bioss, China) at room temperature for 2 h. After being washed three times with TBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG (1:10,000) in 4% BSA in TBST for 1.5 h at room temperature. The membrane was washed three times in TBST and immunoactivity was detected using enhanced chemiluminescence (ECL) detection system (Pierce, USA). The quantitative analysis of each protein band was performed by gel analysis software Quantity One (Bio-Rad).

SAMPLE PREPARATION FOR NUCLEAR MATRIX INTERMEDIATE FILAMENTS SYSTEM

The cells were selectively extracted as described below [Capco et al., 1982; Li et al., 2008]. The cells were seeded on cover slip strips and then treated with or without RCT. The cells were rinsed with D-Hank's solution twice at 37°C, and extracted by high ionic strength extraction solution (10 mmol L^{-1} PIPES, pH 6.8, 250 mmol L^{-1} $(NH4)_2SO_4$, 300 mmol L^{-1} sucrose, 3 mmol L^{-1} $MgCl_2$, 1.2 mmol L^{-1} PMSF, 0.5% Triton X-100) at 4°C for 3 min. The extracted cells were then rinsed in non-enzyme digestion solution (same as extraction solution except with 50 mM NaCl instead of 250 mmol L^{-1} $(NH4)_2SO_4$), and digested in digestion solution containing DNase I (400 mg L^{-1}) and RNase A (400 mg L^{-1}) for 20 min at 23°C. The extracted samples were then placed in high ionic strength extraction solution at 23°C for 5 min. Only the nuclear matrix-intermediate filament (NM-IF) structure remained intact. Intermediate filament is attached to nuclear lamina and thus during chemical extraction NM-IF separates as one entity.

SAMPLE PREPARATION FOR LIGHT MICROSCOPY

The cells seeded on the cover slip strips were selectively extracted. The NM-IF samples were prefixed in 2% glutaraldehyde (made in a nonenzyme digestion solution) at 4°C for 30 min. Then after being washed with PBS (pH 7.4), they were stained with 0.2% Coomassie Brilliant Blue G-250. Finally, the NM-IF system was observed under a light microscope after the procedures of washing with water, airing, vitrification with xylene, and enveloping with resin.

SAMPLE PREPARATION FOR FLUORESCENCE MICROSCOPY

The NM-IF samples on the cover slip strips were prefixed in 4% paraformalclehyde at 4°C for 10 min, blocked by 5% BSA at room temperature for 1 h, incubated with mouse anti-Prohibitin (Ab-1, clone II-14-10; NeoMarkers) monoclonal antibody (1:300) and then with secondary antibody (1:1,000) that which was labeled with fluorescence dye Cy3 (red), washed with water, and dried by airing. Next, they were enveloped with 90% glycerol and then observed under fluorescence microscopy. The whole process after incubation with secondary antibody was performed in the dark.

IMMUNOGOLD STAINING SAMPLE PREPARATION

The cells were seeded on cover slip strips on which there were some nickel grids covered with formvar, coated with carbon, and covered with polylysine. After extraction, the NM-IF samples on the nickel grids were blocked by 5% BSA at 37°C for 30 min and incubated with mouse anti-Prohibitin monoclonal antibody (1:100) at room temperature for 1.5 h. They were then incubated with 5% BSA for 30 min, washed with PBS (pH 8.2), and incubated with the secondary antibody (1:30) which were labeled with colloidal gold (10–15 nm) for 2 h. Next, they were washed with PBS, incubated with 3% paraformaldehyde and 2% glutaraldehyde for 10 min, postfixed in 1% O_SO_4 at 4°C for 5 min, dehydrated in ethanol series, replaced in isoamyl acetate, and dried at the CO_2 critical point. Finally, the immunogold staining samples were observed under transmission electron microscope (TEM).

SAMPLE PREPARATION FOR LASER SCANNING CONFOCAL MICROSCOPE (LSCM)

The cells on the cover slip strip were submerged in PBS or TBS (including 0.5% Triton X-100) at 37°C for 30 min. After being washed with PBS, the cells were fixed in 4% paraformaldehyde for 10 min, blocked by 5% BSA at room temperature for 1 h, and then incubated with dual primary antibodies at room temperature for 30 min and then at 4°C overnight. The dual primary antibody sets were prohibitin/c-fos, prohibitin/c-myc, prohibitin/Rb, and prohibitin/p53. After being washed with TTBS three times in 30 min, the cells were incubated with different secondary antibodies sets that which were labeled with Cy3 or FITC (green), washed with TTBS for 4 min × 10 min and with water for 2 × 5 min, enveloped with 90% glycerol after airing, and then observed under LSCM.

RESULTS

PHB1 DOWN-REGULATION SHOWN BY 2-D PAGE AND IMAGE ANALYSIS

The nuclear matrix proteins extracted from MG-63 cells and the cells treated with RCT were subjected to 2-D PAGE (Fig. 1). Quantification analysis of images was performed using PD Quest 8.0 software (Bio-Rad). Quantitative analysis and identification by MALDI-TOF found that prohibitin is one of the differential expressed proteins in the nuclear matrix (Fig. 2). The intensity of prohibitin at protein levels was reduced dramatically in nuclear matrix to 14-fold in RCT-treated MG-63 cells in comparison with the untreated control cells (Fig. 3).

PHB1 DOWN-REGULATION SHOWN BY MALDI-TOF-MS ANALYSIS AND DATABASE SEARCH

The protein spot from 2-DE gels with altered expression in RCT treatment were identified as PHB1 after MALDI-TOF-MS analysis and Swiss Prot database search (Table I).

PHB1 DOWN-REGULATION SHOWN BY WESTERN BLOTTING

The family of prohibitin genes has two highly homologous members including PHB1 and PHB2. The product of PHB1 is a 32-kDa protein, and that of PHB2 is 37-kDa. The results of Western blot showed that the dominating strip is located on the position of 32-kDa. The intensity of prohibitin immunoband with a molecular weight of 32 kDa was decreased significantly by 15-fold in NM-IF samples prepared from RCT-treated cells in comparison with control cells normalized with beta-actin immunoband as an endogenous control (Fig. 4). This alteration is in accord with the results of quantitative analysis of 2-D PAGE.

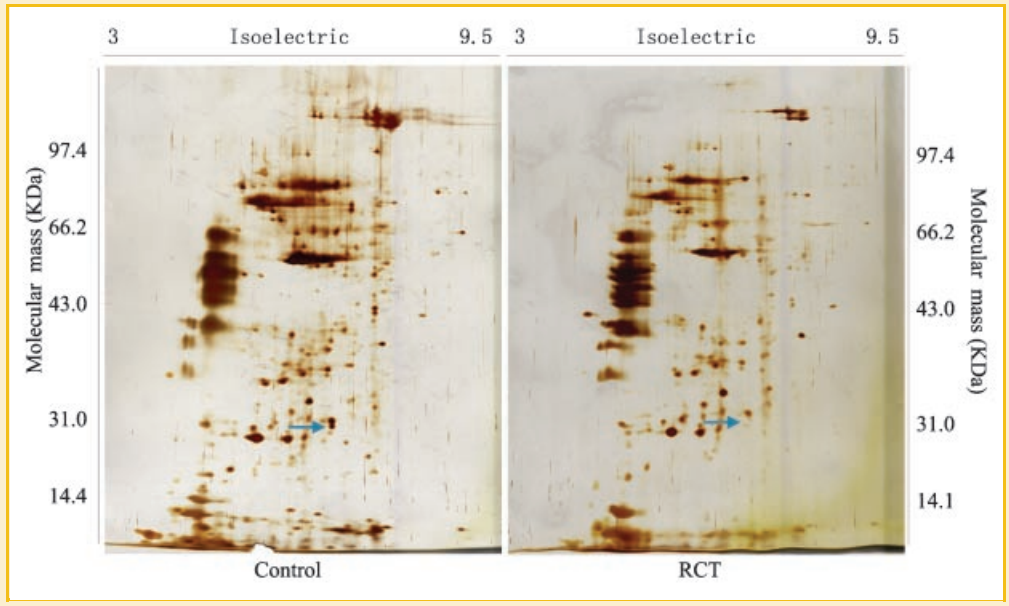

Fig. 1. 2-D protein profiles from nuclear matrix of human osteosarcoma MG-63 cells. Proteins were separated on the basis of pI (*X*-axis) and molecular mass (*Y*-axis) and visualized by silver staining. The arrows indicate indicated the spots of PHB (control: the nuclear matrix protein sample of MG-63 cell; RCT: the nuclear matrix protein sample of MG-63 cells treated by RCT). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

PHB1 DOWN-REGULATION AND TRANSLOCATION IN NM-IF SHOWN BY FLUORESCENCE MICROSCOPY

Light microscopy observation revealed that the intermediate filaments in MG-63 were sparse and arranged irregularly. The thick layer of lamina was prominently stained and appeared uneven. Multiple residual nucleoli were observed within the nucleus of untreated MG-63 cells (Fig. 5A). In the RCT-treated MG-63 cells, the karyoplasmic ratio decreased, the whole framework became more widespread, and the NM-IF system showed characteristics of uniform distribution of untransformed cells. The intermediate filaments, stained uniformly, spread from the region around the nucleus to the cellular edge and formed a well-distributed and regular network throughout the cytoplasm region. The appearance of lamina became thinner and more evenly distributed. The nuclear matrix filaments were abundant and evenly distributed (Fig. 5B).

The immunofluorescence staining of PHB1 revealed that it was mainly distributed in the residual nuclear and cytoplasm regions of MG-63 cells and the intensity of the fluorescence signal was very high in the nucleus (Fig. 5C). After treatment with RCT, the distribution of PHB1 in the NM-IF system was altered significantly. The holistic intensity of the immunofluorescence signal was lowered. Especially in the nuclear matrix region, the PHB1 immunofluorescence signal was distributed mainly in the regions of lamina and nuclear membrane and was weakened dramatically in the center of the NM. In contrast, PHB1 immunofluerescence signal in the punctuate granule regions was increased markedly. PHB1

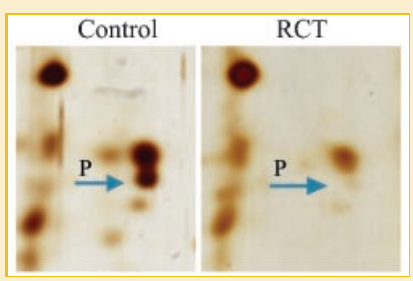

Fig. 2. Enlarged portions of prohibitin protein spots from 2-DE gels. Arrows indicate prohibitin protein spots. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

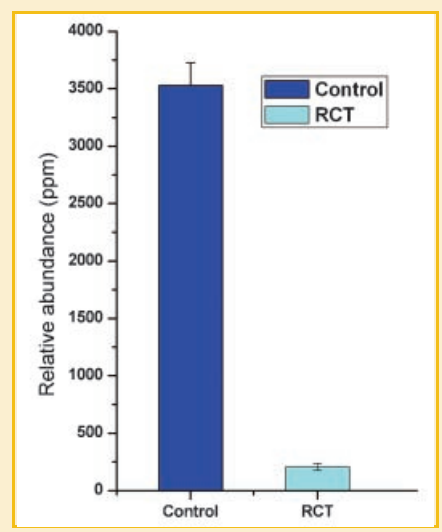

Fig. 3. Optical density changes of identified prohibitin protein spots in control- and RCT-treated MG-63 cells. Three repeated sets of silver-stained 2-D gels for each sample were performed using PD Quest 8.0 software ($P < 0.02$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. The Nuclear Matrix Proteins Identified by PMF Searching Against SwissProt Database

Protein description	Accession no.	MW/calc (kDa)	pI/calc	Score[a]/sequence coverage	Matching peptides/number of queries
Down-regulated proteins					
Prohibitin	P35232-00-00-00	30.0	5.57	101/44%	8
HnRNP A2/B1	P22626-01-00-00	36.0	8.67	105/27%	10
Nucleophosmin	P06748	32.7	4.64	58/28%	6
Centromere protein F (Mitosin)	P49454	36.7	5.03	62/7%	17
TGF-β receptor interacting protein 1	S60335	36.9	5.38	105/24%	8
Up-regulated proteins					
Msh3	P20585	128.3	8.30	55/7%	6
Protein mago nashi homolog 2	Q96A72	17.3	5.95	83/72%	8
Vimentin	P08670	53.5	5.06	96/18%	11

[a]Scores greater than 54 are significant ($P < 0.05$).

displayed a tendency of translocation from NM regions to lamina and cytoplasm regions (Fig. 5D).

PHB1 DOWN-REGULATION AND TRANSLOCATION IN NM-IF SHOWN BY TEM

Using the transmission electron microscope, we observed that the framework of nuclear matrix, lamina, and intermediate filaments were joined together to form an integrated NM-IF network system in control MG-63 cells. The filaments were relatively sparse and scattered and arranged irregularly. The nuclear lamina in the untreated MG-63 cells was unevenly thick and compact. The inner nuclear lamina was connected to thick nuclear matrix filament bundles and the outer nuclear lamina was connected to thin and short intermediate filaments. The intermediate filaments with a few single filaments were chiefly in thick bundles and arranged irregularly (Fig. 5E).

In contrast to the structure of NM in the control MG-63 cells, we observed abundant and increased single filament content with a slender and evenly distributed appearance in the nuclear matrix filaments of RCT-induced MG-63 cells. The nuclear lamina in the RCT-treated cells appeared in a thin and uniform fibroid structure. The configuration of NM-IF systems in RCT-treated cells had the appearance of untransformed cells in which the nuclear matrix, lamina, and intermediate filaments are interconnected in a compact and regular network (Fig. 5F).

We also used immunogold staining to study the PHB1 localization in nuclear matrix. The black high-density immunogold granules occurred fascicularly in the nuclear matrix filaments, indicating that PHB1 was localized in the nuclear matrix filaments of untreated MG-63 cells (Fig. 5G). After RCT treatment, there were less immunogold granules that appeared singly and fascicularly in the RCT-treated MG-63 cells (Fig. 5H). The transmission electronic microscopy of immunogold staining clearly demonstrated the localization of PHB1 in the nuclear matrix filaments system and its regulation and location in nuclear matrix were altered by RCT-treatment in MG-63 cells.

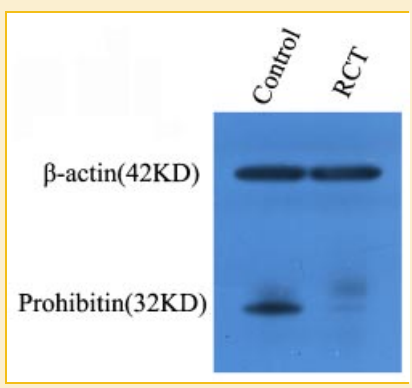

Fig. 4. Confirmation of the differential expression of PHB1 by Western blot. The selectively extracted nuclear matrix proteins (15 μg/band) were applied to an SDS–polyacrylamide gel, separated by electrophoresis, and transferred to PVDF membrane by electroblotting. PHB1 immunoband was then detected using mouse monoclonal antibody. β-actin was also detected as endogenous control using rabbit monoclonal antibody. Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG were used as secondary antibodies for PHB1 and β-actin, respectively. The bands were detected by enhanced chemiluminescence (ECL) detection system. (Control: the nuclear matrix protein sample of untreated MG-63 cell; RCT: the nuclear matrix protein sample of MG-63 cells treated by RCT.) [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CO-LOCALIZATION OF PHB1 WITH ONCOGENES DURING RCT-INDUCED DIFFERENTIATION BY LSCM

We used double immunostaining of PHB1 and several prominent oncogenes to test RCT effects on PHB1 cellular co-localization with other oncogenes. The co-localization fluorescence was yellow or orange when the PHB1 signal was overlapped with other oncogenes (Fig. 6). In control MG-63 cells, the red fluorescence of PHB1 was distributed mainly in the nuclear, cytoplasmic, and peripheral regions of the cells (Fig. 6A,G,M,S). After treatment with RCT, PHB1 fluorescence became weaker in nuclei and scattered in cytoplasm (Fig. 6D,J,P,V).

CO-LOCALIZATION BETWEEN PHB1 AND C-FOS IN MG-63 CELLS

In control MG-63 cells, the highly intensified fluorescence of c-fos was distributed mainly in the nucleolus region (Fig. 6B). The yellow overlapped fluorescence indicated co-localization between PHB1 and c-fos, especially in nucleolus region (Fig. 6C). In MG-63 cells treated with RCT, the holistic intensities of PHB1 and c-fos fluorescence signals were all weakened (Fig. 6D,E). The nucleoli co-localization of PHB1 and c-fos disappeared but the co-localized fluorescence signals in lamina region became intensified (Fig. 6F). The PHB1 signal was translocated from nucleolus to nucleoplasm.

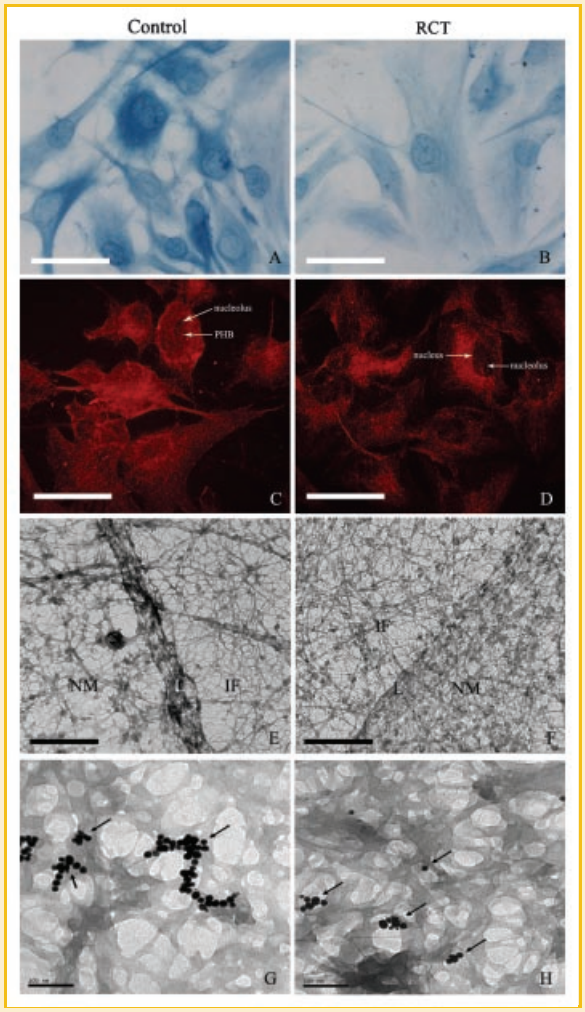

Fig. 5. Light microscope and TEM study of the NM-IF system and PHB1 regulation and translocation in MG-63 cells induced by RCT. A,B: Observation of NM-IF system in MG-63 cells (A) and RCT-treated cells (B) by light microscopy (Dyed by CBB G-250, Bar = 30 μm); C,D: Distribution of PHB1 in the NM-IF system of MG-63 (C) and RCT-treated cells (D) by fluorescence microscopy (labeled with fluorescence dye CY3, Bar = 30 μm). E,F: Observation of NM-IF system in MG-63 cells (E) and treated cells (F) by TEM (Bar = 0.5 μm). G,H: Distribution of PHB1 in the NM-IF system of MG-63 cells (G) and RCT-treated cells (H) by TEM. (The arrows indicate indicated the immunogold granule of PHB, Bar = 100 nm; NM, nuclear matrix; L, lamina; IF, intermediate filaments.) [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CO-LOCALIZATION BETWEEN PHB1 AND C-MYC IN MG-63 CELLS

In control MG-63 cells, the strong green fluorescence particles of c-myc were distributed mainly in nucleolus and its peripheral regions (Fig. 6H). The overlapped fluorescence of PHB1 and c-myc indicated co-localization in karyon and inner nuclear membrane (Fig. 6I). In MG-63 cells induced by RCT, PHB1 became weaker in nuclei and scattered in cytoplasm (Fig. 6J). C-myc was down-regulated as well in nuclear and cytoplasmic regions (Fig. 6K). The overlapped fluorescence was not observed in RTC-induced MG-63 cells (Fig. 6L).

CO-LOCALIZATION BETWEEN PHB1 AND MTP53 IN MG-63 CELLS

In control MG-63 cells, the strong mtP53 green fluorescence was lumped in karyon and faint fluorescence could be observed in cytoplasm around the nuclei (Fig. 6N). The PHB1 and P53 overlapped fluorescent signals indicated the co-localization distributed mainly in the region of nuclear membrane and its periphery (Fig. 6O). In the treated MG-63 cells, PHB1 was weakened and dispersed inside of nuclear membrane (Fig. 6P) and mtP53 became weaker and concentrated mainly in nuclei (Fig. 6Q). The overlapped fluorescence indicated the co-localization in cytoplasm and lamina regions between PHB1 and mtP53 and the co-localization fluorescence signals became much weaker after the treatment with RCT (Fig. 6R).

CO-LOCALIZATION BETWEEN PHB1 AND RB IN MG-63 CELLS

Most Rb of MG-63 cells distributed unevenly in the nucleus and the intensity of fluorescence was faint in cytoplasm (Fig. 6T). The overlapped fluorescence signals of PHB1 and Rb were concentrated in lamina regions (Fig. 6U). After treatment with RCT, the subcellular distribution of PHB1 changed. The fluorescence of PBH1 increased in nuclear lamina but decreased in nucleolar regions. It represented a trend of trafficking of PHB1 from nucleolus to karyoplasm and nuclear membrane (Fig. 6V). The fluorescence intensity of Rb increased markedly after RCT treatment (Fig. 6W). The overlapped fluorescence signals of PHB and Rb was higher in the RCT-treated cells (Fig. 6X).

DISCUSSION

REGULATION AND SUBCELLULAR LOCALIZATION CHANGES OF PHB1 IN THE NUCLEAR MATRIX INDUCED BY RCT

We used several approaches of proteomic and immunocytochemical methodologies to demonstrate that prohibitin was a nuclear matrix protein. Prohibitin was down-regulated markedly in nuclear matrix and it is translocated from nuclei to the regions of nuclear membrane and cytoplasm after RCT treatment. Immunogold transmission electron microscopy further confirmed the existence of prohibitin in nuclear matrix filament. These results indicated that prohibitin existed not only in mitochondria of the cytoplasm but also in the nuclear matrix of MG-63 cells and its nuclear expression and localization is associated with canceration and reversion. The down-regulation and translocation of prohibitin from cytoplasm to nucleus might be one of the molecular mechanisms associated with the effectiveness of RCT cancer prevention ability. There were conflicting experimental results regarding prohibitin regulation in relationship with cell proliferation and differentiation. Dixit et al. [2003] reported that prohibitin decreased markedly in proliferating cells while it increased markedly in differentiated cells. Sun et al. [2004] found that increasing prohibitin could repress muscle differentiation. In contrast, our data clearly showed the down-regulation of prohibitin in RCT-induced cancer reversion of MG-63 cells. We postulated that the different regulation of prohibitin in different cell lines and differentiation stages might indicate bi-directional regulation, that is, prohibitin could control the over-proliferation of cells as well as maintain cell survival. The drastic

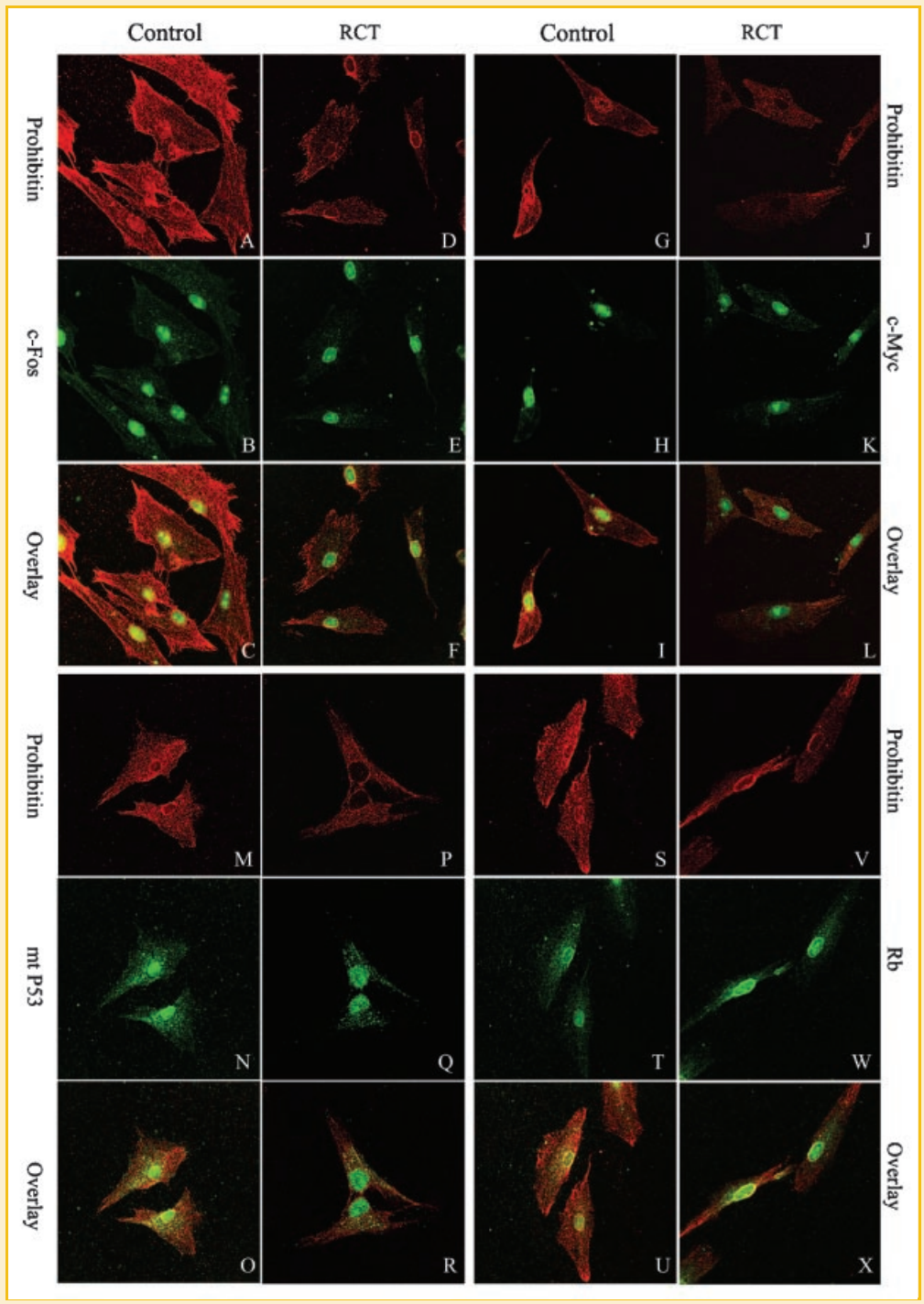

Fig. 6. The cellular co-localization assays of several prominent oncogenes and PHB1. PHB1 was localized mainly in cytoplasm and karyon in untreated MG-63 cells; it decreased in nucleoplasm and translocated to nuclear lamina and cytoplasm in RCT-treated MG-63 cells. The overlay showed the co-localization of immunofluorescence (yellow or orange) of PHB1 and other oncogenes (A–F) the expression and the co-localization between PHB1 and c-fos in MG-63 cells without and with RCT treatment. G–L: The expression and the co-localization between PHB1 and c-myc in MG-63 cells without and with RCT treatment. M–R: The expression and the co-localization between PHB1 and P53 in MG-63 cells without and with RCT treatment. S–X: The expression and the co-localization between PHB1 and Rb in MG-63 cells without and with RCT treatment. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

decrease of prohibitin in RCT-induced MG-63 cells could release its interaction with tumor suppression genes that are indirectly activated by RCT. The change of phohibitin expression and subcellular localization by RCT suggested that prohibitin might be a target protein of RCT and participated in the differential regulation of MG-63 cells.

PHB1 is a molecular chaperone and its subcellular trafficking and interaction with other cancer-related genes are also important

mechanisms for cell differentiation. The most prominent down-regulation of PHB1 was observed in nuclear matrix that maintains nuclear structure and support chromatin structure in order for normal DNA synthesis and RNA transcription. The identification of PHB1 in the filaments of nuclear matrix and altered expression of PHB1 in the nuclear matrix indicate that PHB1 might function as a chaperone in the nuclear matrix, interact with other oncogenes, and interfere with DNA synthesis and RNA transcription.

POSSIBLE ALTERATION OF CO-LOCALIZATION AND INTERACTION BETWEEN PROHIBITIN AND OTHER ONCOGENE AND TUMOR SUPPRESSOR GENES IN THE RCT-INDUCED MG-63 CELLS

The results of immunofluorescence microscopy and laser scanning confocal microscopy revealed that there were co-localization between prohibitin and the products of c-fos, c-myc, p53, and Rb genes in MG-63 cell and that were altered by RCT treatment.

As a product of a potential tumor suppressor gene, prohibitin participates in the regulation of cell growth, proliferation, and differentiation and this process could involve c-fos and c-myc genes whose expressions were up-regulated frequently in osteosarcoma. Our research indicated prohibitin was co-localized with c-fos, c-myc respectively in the nucleus of MG-63 cells. When MG-63 cells were induced into differentiation, there was distinct co-localization between prohibitin and c-fos in the regions of lamina and nuclear membrane but there was no co-localizational relationship between prohibitin and c-myc in the nucleus, only some faint co-localizational immufluorescence could be found around the nuclear membrane. The differential interaction of prohibitin with c-fos and c-myc could indicate that the protein-protein interaction could also be targets of RCT.

Previous studies showed that prohibitin and c-fos were regulated by Rb, and the expression of prohibitin and c-fos were increased in the cells in which Rb was over-expressed [Buchmann et al., 1998] and in the c-myc over-expressed cells as well [Menssen and Hermeking, 2002; Haggerty et al., 2003]. Recent research found that there was a co-localization between prohibitin and RB, the product of tumor suppressor gene, in the nucleus [Wang et al., 2002a]. Prohibitin could bind to Rb and E2F and regulate the genes that are activated by transcription factor E2F to control proliferation of cells [Fusaro et al., 2002; Wang et al., 2002b]. Our study found that the co-localization of prohibitin and Rb in the region of nuclear lamina was enhanced after treatment with RCT. Obviously, the alteration is correlated with the proliferation and differentiation of MG-63 cells because the region of nuclear lamina is an active site of DNA replication and transcription. The alteration of prohibitin trafficking and its enhanced combination with Rb in the region of lamina may repress the ability of E2F to activate the expression of its downstream genes sequentially control cell proliferation and promote differentiation. The congregation of Rb gene to nucleus and decrease of c-myc expression in the nucleus after RCT treatment could all contribute drastically to down-regulation of prohibitin. Both Rb and c-myc are transcription factors and Rb might negatively and c-myc might positively regulate prohibitin gene expression.

P53 and prohibitin were co-localized in nucleus and nuclear membrane and prohibitin immunofluorescence was were translocated from nucleus to cytoplasm near the nucleus after RCT treatment. Fusaro et al. [2003] found that prohibitin interacted with p53 and E2F1 in the nucleus of breast cancer cells. They postulated that prohibitin could activate the transcription mediated by P53 and enhance the binding affinity of P53 to gene promoters. They also found that apoptosis could cause the P53-prohibitin complex translocation from nucleus to cytoplasm in the vicinity of nucleus [Fusaro et al., 2003; Rastogi et al., 2006]. In accordance with previous studies, we suggest that prohibitin could interact directly with P53 and participate in the transcriptional regulation of p53 in MG-63 cells. After RCT-induced differentiation, prohibitin and p53 were trafficking out of the nucleus. This synergistic translocation of prohibitin and p53 may influence their expression in the MG-63 cells for p53 and its downstream genes, resulting in cancer cell reversion.

A novel definition of nuclear matrix prohibitin was proposed to serve as a RCT target by this study. We postulate that RCT could regulate the prohibitin chaperone activity in relationships with other oncogenes and tumor suppressor genes. Although RCT acts on multiple molecular targets that are associated with cell transformation and reversion, chaperones serve as master drug target that regulate many cellular processes and pathways.

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