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PROTEASOMES REACTIVATOR REG GAMMA ENCHANCES ONCOGENICITY OF MDA-MB-231 CELL LINE VIA PROMOTING CELL PROLIFERATION AND INHIBITING APOPTOSIS

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Abstract – To investigate the effect of proteasomes reactivator REG gamma (γ) on cell cycle and apoptosis in vitro and in vivo. In vitro, we first constructed recombinant plasmid of PcDNA3.1-REGγ and then transfected REGγ into MDA-MB-231 cell line. We confirmed the transfection efficiency by Western blot. Subsequently, we observed cell growth, cycle and colony formation. Specific proliferative molecule proliferating cell nuclear antigen (PCNA) and apoptosis related signal molecule Caspase-3 was assayed by immmunohistochemistry and absorption spectrometry, respectively. In vivo, we successfully established transplantation tumor nude mice model. We determined REGγ mRNA level in the transplantation tumor tissue. Then, FCM was used to determine cell cycle, apoptosis and CD16. Finally, we employed immunohistochemistry to determine P21 positive expression. However, the cells transfected with REGγ grew more rapidly compared with non-transfected ones. Increased cells were observed in S+G2+M phase and S phase in the REGγ transfected group. PCNA expression level in the transfected cells was higher than that in non-transfected ones. In vivo, we observed the similar phenomenon including more rapid tumor growth, higher REGγ mRNA expression, decreased cells number in G_0/G_1 phase and G_2/M phase, increased cells in S phase and decreased apoptosis in the transfected group. In the study of related molecules, we also found related molecules P21 and CD16 positive expression rate were obviously lower than non-infected ones. In present study, we found oncogenicity of MDA-MB-231 cell transfected with REGγ was enhanced, which might be realized via REGγ promoting cell growth, inhibiting cell apoptosis, degrading P21 and suppressing activation of NK, suggesting REGγ promoting tumor growth is a process involving multiple factor mechanisms.

Key words: Proteasomes reactivator, REG gamma, cell cycle, proliferation, apoptosis

INTRODUCTION

Proteasomes participate in most protein degradation in eukaryotic cell cytoplasm and nucleus. However, fundamental cell biological processes such as cell cycle, transcription, signal, death and immune response are regulated by removing impaired or mistaken folded proteins via proteasomes degradation pathway (8).

Up to now, three members-REGα, REGβ and REGγ have been found in REG family that is also called 11S or PA28 family of proteasome reactivators. They have 35% amino acids similarity and can enhance catalytic activity of proteasomes in vitro. In these, REGγ, a 200ku molecule, forms a homologous heptamer complex and can appear alone or along with 20S proteasomes (19). REGγ is a conservative nucleoprotein that is present in insect, worm more developed forms of animals, whereas it is not seen in on yeast or plant. However, REGα/β was only found in vertebrate. Moreover, the affinity of REGγ to proteasome is stronger than REGα or REGβ(13).

Now, the mechanism for REGγ activating 20S proteasomes has been revealed preliminarily. Binding of reactivator leads to the opening of proteasome entrance and exit, which enable peptides substrates to diffuse into the environment of proteasomes(7). We can explain

Abbreviations: **PCNA**, proliferating cell nuclear antigen; **FCM**, flow cytometer; **TEM**, transmission electron microscope; **RT-PCR**, reverse transcriptase polymerase chain reaction; **RNAi**, RNA interfere; **NK**, natural killer (cell); **SPF**, specific-pathogen-free; **RPMI**, Roswell Park Memorial Institute; **SDS-PAGE**, sodium dodecyl sulphate polyacrylamide gel electrophoresis; **MTT**, methyl thiazolyl tetrazolium; **TIL**, tumor infiltrating lymphocyte; **EcoR**, restriction endonuclease from E. coli; **FITC**, fluorescein isothiocyanate.

the effect of reactivator on proteasomes activation although the conformational change may be present on the catalytic site for peptides hydrolization(9).

It has been suggested that REGγ pathway may be involved in protein degradation (5, 20). REGγ, in particular, can degrade non-structural protein. There are over 100 proteins with lysine deletion in eukaryotic proteins database. And, the target protein is lack of lysine residue in REGγ pathway. Therefore, an ubiquitin non-dependent pathway might contribute to the degradation of a lysine deletion permitting protein. For example, the core protein of lysine deleted hepatitis C virus (HCV) is not only a structural virus but a morbigenous factor. However, REGγ can bind to it specifically, which is essential to core protein toxicity (14).

Evidence indicated ubiquitin-proteasomes system had a direct effect on transcription (10, 18). Another possible mechanism is that REGγ can bind to the apoptosisphilic factor and arrest its activity. In addition, it was confirmed that cofactor REGγ could promote P53 ubiquitin and MDM2-dependent proteasomes degradation, suppress P53 accumulation after DNA impairment and weaken its inhibitory effect on apoptosis (25). P53 degradation effect mediated by MDM2 could be eliminated by knockouting endogenous REGγ from human cancer cells (25).

However, oncogenicity of REGγ in breast cancer has not been confirmed thoroughly. In view of this, we investigated cell growth, cycle, apoptosis and associated molecules of MDA-MB-231 cell line transfected with REGγ in vitro. Meanwhile, we successfully established transplantation tumor model to observe the transplantation tumor growth in vivo. In summary, we managed to reveal the role of REGγ in the genesis and development of breast cancer.

MATERIALS AND METHODS

Cell, bacterial line and plasmid

Breast cancer cell line MDA-MB-231, *Escherichia coli* JM109 and PcDNA3.1 plasmid were obtained from Institute of Basic Medical Sciences of Chongqing Medical University(Chongqing, China).

Experimental animals

The 4 -week-old specific-pathogen-free (SPF), weighing 12-15g, female Balb/c nude mice used in this study were provided by the Experimental Animal Center of Chongqing Medical
University(Chongqing, China). All the experiments were conducted in accordance with the national guidelines for the care and use of laboratory animals.

Main reagents

Plasmid Mini Kit and PCR-Preps DNA Purification System were purchased from OMEGA Company. Rabbit anti-PA28γ (N-term) monoclonal antibody (Batch No. 60807072) was obtained from Invitrogen Co.,Ltd. (Shanghai, China). Anti-mouse CD16 antibody was purchased from Biolegend Co., Ltd.(CA, USA). P21 monoclonal antibody was purchased from CRUZ Co. Ltd. (SANTA, USA). Immunohistochemistry SP kit and Goat anti-rabbit IgG were provided by Zhongshan gold bridge Biotechnology Co., Ltd. (Beijing, China). All the primers used in this study were synthesized by Bioengineering Co., Ltd. (Shanghai, China).

Construction of recombinant plasmid and transduction

Constructed recombinant PcDNA3.1-REGγ, prepared and transferred competent cell DH5α(Takara, Dalian). Colony picking, enzyme digestion assay and lipidosome transfection were performed in accordance with Malumbres et al. (15). DNA sequencing was completed by Viral Hepatitis Institute of Chongqing Medical University(Chongqing, China) and Molecular Biology Key Laboratory of Infectious Diseases of the Ministry of Education(Chongqing, China).

Establishment of stable transfected cell line

Prepared MDA-MB-231 cell suspension. Counted and adjusted it to a moderate concentration of 1×10^4 /ml. Then, the cell suspension was inoculated in a 24-well plate (200μl/ well). Additional 800μl of RPMI1640 medium was added in each well. 10 concentrations gradients(100-1000 mg/L) of G418 (Gibco, USA), also known as Geneticin, were set and triple wells were set for each concentration. The minimum concentration for all cell death was 600 mg/L in 14 d. Therefore, 600 mg/L of G418, was selected as an optimal effective concentration.

Western Blot analysis

Total cellular protein from the three groups was extracted, respectively. The protein was separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis(SDS-PAGE).The proteins were transmembranced to PDVF membrances. The membranes were co-incubated with 5% defatted milk in TBS containing 0.1% Tween 20 (TBST) for 4 h at room temperature. Then, the primary antibody [Rabbit anti-PA28γ(N-term)] (1:120) was added. Overnight at 4℃ for 12 hr followed by the adding of secondary antibody Goat anti-rabbit IgG (1:3000) at 37℃ for 6 hr. The membrane was stained by DAB. Quantity One software (BioRad, USA) was used to analyze the specific straps quantitatively.

Cytotoxicity assay

Cells in log phase were collected to prepare single cell suspension. Cells were inoculated in a 96-well plate (2×10^3 cells/ well) and triple wells were set for each group. Viable cell number was measured by methyl thiazolyl tetrazolium (MTT) at every 24 hr for 7 d. Briefly, 20μl of MTT (5g/L) was added in each well and cocultured for 4 hr followed by the adding of DMSO (200μl). Shaked for 15min and absorbance was measured at 570 nm by using a microplate reader.

Cell cycle assay

Briefly, cells in log phase in groups were digested by trypsogen (0.25%) and collected respectively. Washed twice with cold PBS. Centrifugated at 1000× g for 10 min and removed the supernatant. 1ml of ethanol (70%) was added and then overnight at 4°C. Centrifuged at 1000× g for another 10 min. Washed twice with cold PBS followed by the adding of PI staining solution. Cocultured for 30 min without light. FCM (BD Co., CA, USA) was employed to assay cellular distribution proportion in different cell phases in groups. Excitation and emission wave length was set at 488nm and 630nm, respectively.

Soft agar colony formation

Prepared agar medium (0.5%) on culture capsule (D=3.5cm). After coagulation, cells in log phase, 20% calf serum and RPMI1640 medium containing 0.3% agaric were mixed and inoculated on each bottom-layer agar (1×10^3 cells). Cells in each group were inoculated on three culture capsules. Then, were placed into incubator and cultured at 37°C for 14 d. Microcolony (50-200 cell clones) and macrocolony (>200 cell clones) were taken into consideration.

Immunohistochemistry of PCNA positive expression

Creep slides of the three group cells were prepared conventionally in a 24-well plate. 5 wells were set for each group. Creep slides were picked out when the cellular convergence degree reached 70-80%. They were washed twice with PBS and immersed into 4% paraform to fix for 15-30 min at room temperature. Washed twice with PBS and immersed into 3% H_2O_2 for 10min. Washed twice with PBS and immersed into TritonX-100 (0.2%) for 30 min. Antigen was recovered at 90°C for 10 min overnight with rabbit anti-human PCNA monoclonal antibody (1:200) at 4°C and incubated with goat anti-rabbit IgG (1:1000) for 1 h at room temperature. The sections were stained by DAB at room temperature. Pictures of each tissue sample for PNCA were collected and assayed by Image Analysis Software (Leica, Germany).

Assay of Caspase-3 activity

Bradford method was employed to assay Caspase-3 activity in MDA-MB-231 cells under different transfection concentrations of REGγ (0, 25, 50, 100, 150, 200, 250 and 300 μg/ml). Briefly, prepared eight standard solutions (1 ml each) containing 0, 25, 50, 100, 150, 200, 250 and 300 μg/ml bovine serum albumin (BSA). Added 5 ml of Coomassie Brilliant Blue G-250 and co-incubated for 5 min. Absorbance of each sample under a concentration was read at 595 nm by using a microplate reader.Prepared a standard curve of absorbance of different BSA concentrations
. Determined concentrations of original samples from the standard curve.

Cell apoptosis determination

The process was performed according to the manufactuer's instructions of Annexin V-FITC cell apoptosis assay kit (Haimen, China). Briefly, collected cell suspension and centrifuged at 1000 rpm for 5 min. Removed the supernatant and re-suspended the precipitate. Counted 50,000
-100,000 cells and centrifuged at 1000× g for 5 min. Removed the supernatant and added 195 μl of Annexin V-FITC binding solution (1X) to re-suspended the cells. Added 5μl of Annexin V-FITC and mixed slightly. Cocultured at 20-25°C for 10 min without light. Removed solution and added 190 μl of Annexin V-FITC binding solution (1X). Then, 10 ul of propidium lodide staining solution was added. Apoptotic cells were quantified by FCM using a laser emission wave at 488 nm (BD Biosciences).

TEM observation for the cells ultrastructural change

MDA-MB-231 cells in log phase in the three groups were selected. Primarily fixed in 2.5% glutaraldehyde in a buffer (0.1M, pH 7.4) for 4 hr at 4°C. Washed with 0.1M buffer (pH 7.4) for 2 hr at 4℃. Secondary fixation was performed in 1% osmium tetroxide in distilled water for 1 hr at room temperature. Washed twice in distilled water with 5 min each time. Next, the samples were dehydrated as following: 50% ethanol for 15 min, 70% ethanol for 15 min, 90% ethanol for 15 min, 95% ethanol for 15 min, 100% ethanol for 15 min, 100% ethanol for 15 min, 100% ethanol for 15 min and 100% ethanol for 15 min. Propylene oxide treatment was done twice with 10 min each time. Embedded in mould and polymerise in oven at 60°C for 24 hr. Prepared semithin sections (50-70 nm) using the slicer instrument (Leica, Germany). The sections were observed under CM120 TEM (Philips, Netherlands).

Establishment of breast cancer bearing nude mice model

36 4-week-old nude mice were randomly divided into 3 groups (n=12). Cells transfected with REGγ in log phase in the 3 groups were digested by 0.25% trypsogen and prepared single cell suspension. Trypan blue assay was used to confirm cell vitality. 1×10^7/ml of the cell (0.2ml for each animal) was inoculated subcutaneously respectively. The subcutaneous tumor nodus diameters (≧0.5 cm) were thought to be a standard for tumorigenesis. Length and width of tumors were measured every 7 d and volumes of the tumors were recorded. The nude mice were sacrificed at 5 w after the inoculation and tumors were picked out for weighing. Non-transfected group was set as a control to evaluate tumor control rate.

RT-PCR of REGγ mRNA expression

To extract RNA, 200 mg of the nude mice tumor tissues were picked out from liquid nitrogen. Washed twice with PBS. Then cut into pieces and ground on ice. Total RNA was
prepared according to the manufacturer's instructions. After DNase I treatment, 2 μg of RNA was reverse transcribed with AMV reverse transcriptase. A master mix containing the reaction buffer, dNTPs, Taq polymerase and 2 μl cDNA in 25 μl reaction mixture was transferred to different PCR tubes. The following primers were: mouse REGγ (780bp), forward (5'-CACCATGGCCTCGTTG
CTGAAGG-3') and reverse (5'-TCAGTACAGTGTCTCTGCATTGCTG-3'); and mouse β-actin (455-bp product), forward (5′-CCC TGT ATG CCTCTGGTC-3′) and reverse (5′-TTT ACG GAT GTC AAC G-3′).

These reactions were re-performed for 30 cycles. The annealing temperature was maintained at 66 °C for 30 s; the rest of the conditions included denaturation at 94 °C for 1 min followed by extension at 72 °C for 3 min. The PCR products were determined using 1.0% agarose gel electrophoresis and ethidium bromide staining. Images of the gels were analyzed using the Quantity One software (Bio-Rad, CA, USA), which compares the relative density of objective straps and β-actin.

Analysis of CD16 expression, cell cycle and apoptosis by FCM

Part of the tumor tissue was prepared single cell suspension. 100% and 75% of lymphocyte separation media were used for discontinuous density gradient centrifuge. Transplantation tumor tissular lymphocytes, tumor infiltrating lymphocyte (TIL) layer and tumor cell layer was removed respectively. Then washed with PBS and made into single cell suspension. 0.1ml of CD16 monoclonal antibody working solution was added into TIL single cell suspension with cell density 1×10^6 /ml. 100 μL of FITC-lgG second antibody working solution was added. Incubated for 30 min. PBS took the place of primary and second antibodies as a negative control. Only secondary antibody adding was regarded as a positive control. CD16 positive percent was analyzed quantitatively. In addition, part of the tumor tissue was fixed by 80% cold ethanol and made into single cell suspension. Then PI was added for 30 min without light. Cell cycle change and apoptosis were analyzed by FCM.

Immunohistochemistry of P21 positive expression

The tumor tissue was fixed by 4% neutral formalin followed by conventional paraffin imbedding, slice, deparaffinage and hydration. Then the process was performed according to manufacturer's instructions. 10 fields of vision grids were selected randomly under 400 times fields of vision. P21 dyed positive cells in 1,000 transplantation tumor cells were counted and positive percent was calculated.

Statistics and presentation of data

Experimental data is expressed as means ± standard deviation. SPSS12.0 statistics software is employed to perform one-factor analysis of variance. Each experiment was repeated at least twice, and each data point represents the mean of at least 3 parallel samples. A p value of less than 0.05 was considered significant, and one less than 0.01 was considered highly significant.

RESULTS

Identification of PcDNA3.1-REGγ recombinant plasmid

Recombinant was generated by EcoR I and EcoR V enzyme. Figure 1. showed the recombinant was cut into two pieces of fragments PcDNA3.1（5400bp）and REGγ（780bp）respectively. Present eukaryotic expressive recombinant was confirmed to be constructed successfully by sequence analysis.

Identification of REGγ protein expression by Western Blot

Total cellular proteins of the three groups were extracted respectively. Western Blot was employed to detect intracellular REGγ protein content. The straps were analyzed quantitatively by Quantity One software of BioRad company. The result showed REGγ protein expression production in REGγ transfected group was significantly higher than that in the other two groups Fig. 2.

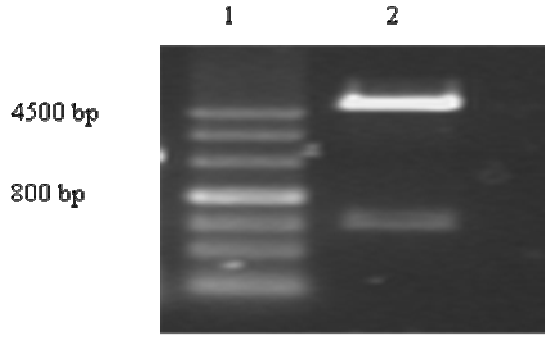

Figure 1. Restrict enzyme digestion analysis of the recombinant PcDNA3.1-REGγ

The recombinant PcDNA3.1-REGγ was digested by restrict enzymes EcoR I and EcoR V specifically. The products including two fragments PcDNA3.1 (5400 bp) and REGγ (780 bp) could be seen on the gel clearly. It suggested the successful construction of recombinant PcDNA3.1-REGγ.

1: DNA Marker; 2: Recombinant digested by EcoR I and EcoR V: PcDNA3.1 (5400 bp) and REGγ (780 bp)

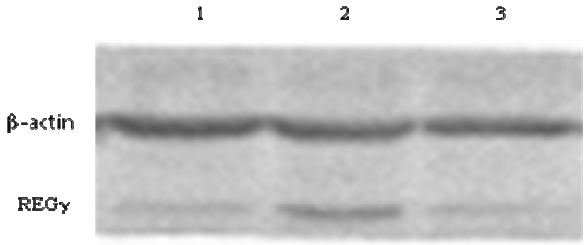

Figure 2. Expression of REGγ detemined by Western Blot

The brightness of strap in MDA-MB-231/PcDNA3.1- REGγ group was stronger than non-transfected groups, which confirmed the successful transduction of REGγ into MDA-MB-231 cell line.

1: MDA-MB-231; 2: MDA-MB-231/PcDNA3.1- REGγ; 3: MDA-MB-231/PcDNA3.1

Effect of REGγ on cell growth

The cells transfected REGγ grew more rapidly and the absorbance value increased significantly ($p<0.05$). Fig. 3

The cell cycle analysis by FCM indicated the cellular percent in G_0/G_1, G_2/M and S phase in the control, empty vehicle and REGγ transfected group was 55.91%, 21.49% and 22.60%; 56.31%, 20.48% and 23.21% ; 44.09%, 21.21% and 34.70%, respectively, suggesting the number of the cells transfected REGγ in S+G2+M multiplication period was obviously higher than that of the non-transfected cells. Furthermore, the cells in S phase increased, suggesting REGγ promoted MDA-MB-231 cell to go into S phase.

Respectively, 3.67%, 4.06% and 10.23% colony forming efficiency could be seen in the control, empty vehicle and REGγ transfected group in soft agar colony formation experiment (p<0.05). Moreover, macrocolonies of the two formers were smaller than the latter. Fig. 4.

Comparatively stronger brown particles were in 90% REGγ transfected cells, which was higher than non-transfected REGγ ones. The PCNA dyeing grays cale value of the control, empty vehicle and REGγ transfected cell was 98.59±11.25, 107.15±8.42 and 183.47±17.62, respectively Fig. 5. The value of the transfected cells was significantly higher than that of non-transfected ones (p<0.01). Meanwhile, PCNA expression level of cells transfected with REGγ increased remarkably compared with the control group (p<0.05).

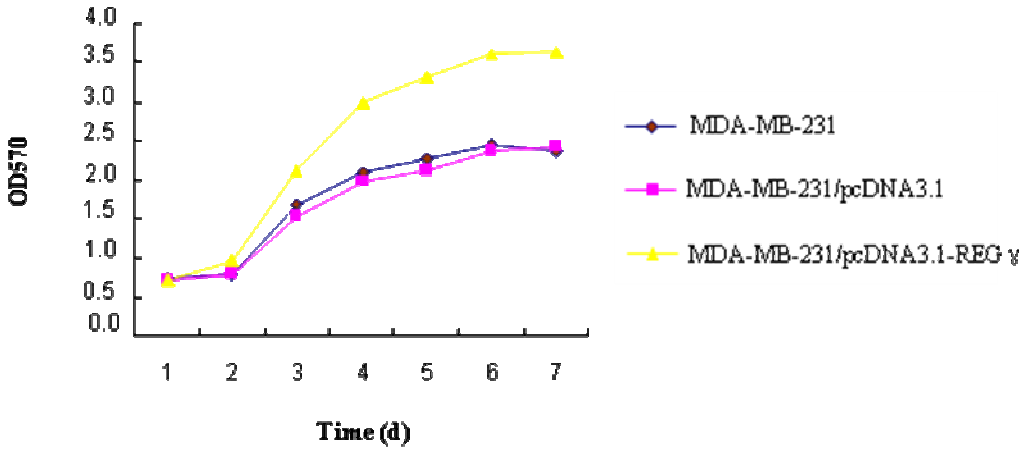

Figure 3. Proliferation of MDA-MB-231, MDA-MB-231/PcDNA3.1 and MDA-MB-231 /PcDNA3.1-REGγ in 7 d
During the first two days, there was no significant difference in cell proliferation rate among the three groups. However, the MDA-MB-231 cell line transfected with REG γ began to grow more rapidly by the 3rd day. The growth of the MDA-MB-231 cell line transfected with REG γ reached a peak on the 6th day. There were significant differences in proliferation between the REG γ transfected group and the other two groups from 4-7 d.
1: MDA-MB-231/PcDNA3.1-REG γ ; 2: MDA-MB-231/PcDNA3.1; 3: MDA-MB-231

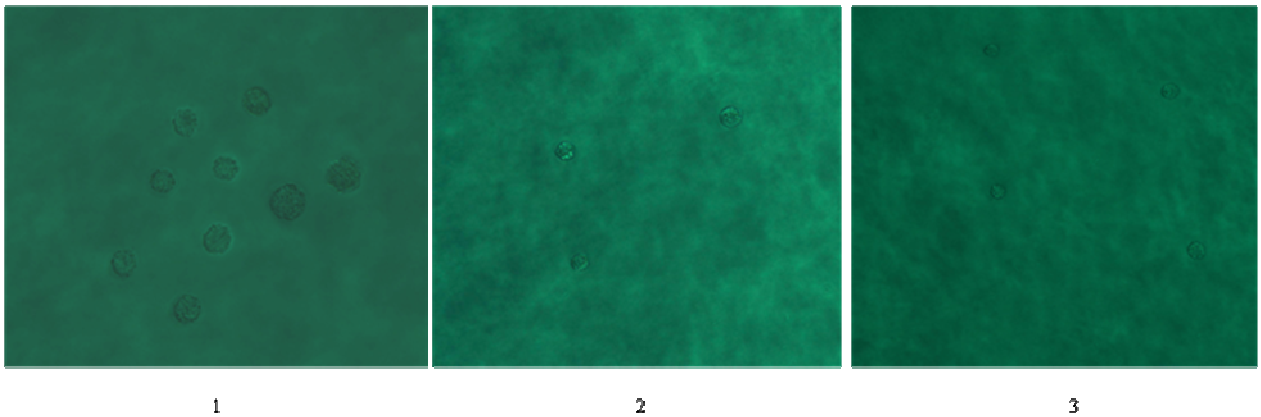

Figure 4. Agar colonies
Comparative higher colony forming efficiency could be seen in REGγ transfected group in soft agar colony formation experiment. Moreover, macrocolonies of the two others was smaller than that of REGγ transfected group, respectively.
1: MDA-MB-231/PcDNA3.1-REGγ; 2: MDA-MB-231/PcDNA3.1; 3: MDA-MB-231

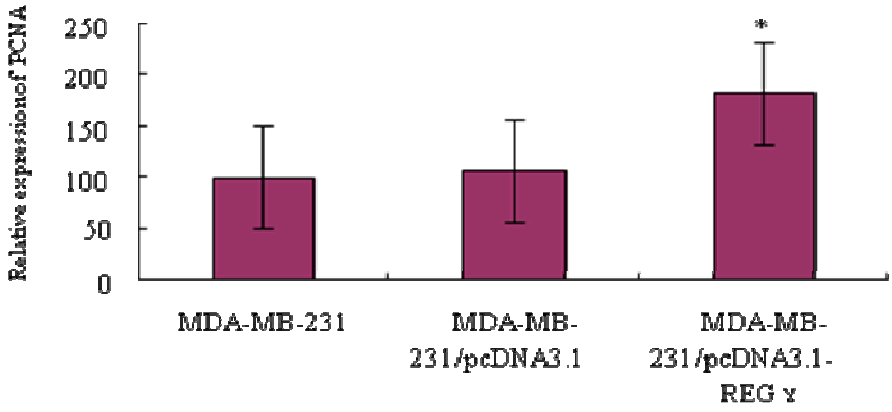

Figure 5. Determination of PCNA positive expression
Relative positive expression level of PCNA in cells transfected with REGγ was significantly higher than the other two groups ($p<0.05$). Meanwhile, there was no statistical difference between the other two groups ($p>0.05$).

Effect of REGγ on apoptosis in vitro

The apoptosis rate of the control, empty vehicle group, early and middle period of experimental group was 1.38%, 1.79% and 0.11%, respectively ; Proportion of late apoptotic and necrotic cells was 0.64%, 0.48% and 0.04%, respectively, suggesting cell apoptosis of REGγ group was obviously higher than the control group.

Absorbance value of related molecule Caspase-3 in REGγ transfected cells was lower than the other non-transfected ones at the same concentration condition. However, the Caspase-3 activity in REGγ transfected group did not increase significantly as well as the other two groups although the transfected concentration of REGγ was enhanced remarkably. Meanwhile, there was no statistical significance in absorbance value between the other two groups. (Fig. 6)

Ultrastructural observation

Cellular ultramicrostructure in REGγ transfected group displayed generally hypertrophic nucleolus, increased or expanded mitochondria and Golgi's body, no apoptotic body and nuclear bag formation in some areas (Fig. 7). Apoptotic body could be seen in the control group occasionally.

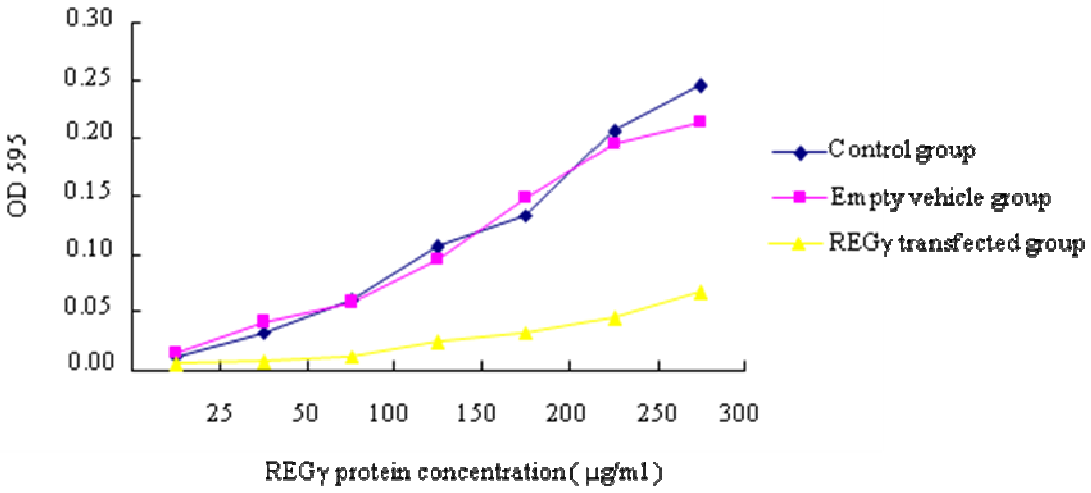

Figure 6. Active degree assay of Caspase-3
Absorbance value of Caspase-3 in REGγ transfected cells was lower than the other non-transfected ones at the same concentration condition. The Caspase-3 activity in REGγ transfected group did not increased significantly as well as the other two groups although the transfected concentration of REGγ was enhanced. Meanwhile, there was no obvious significance between absorbance values between the other two groups.

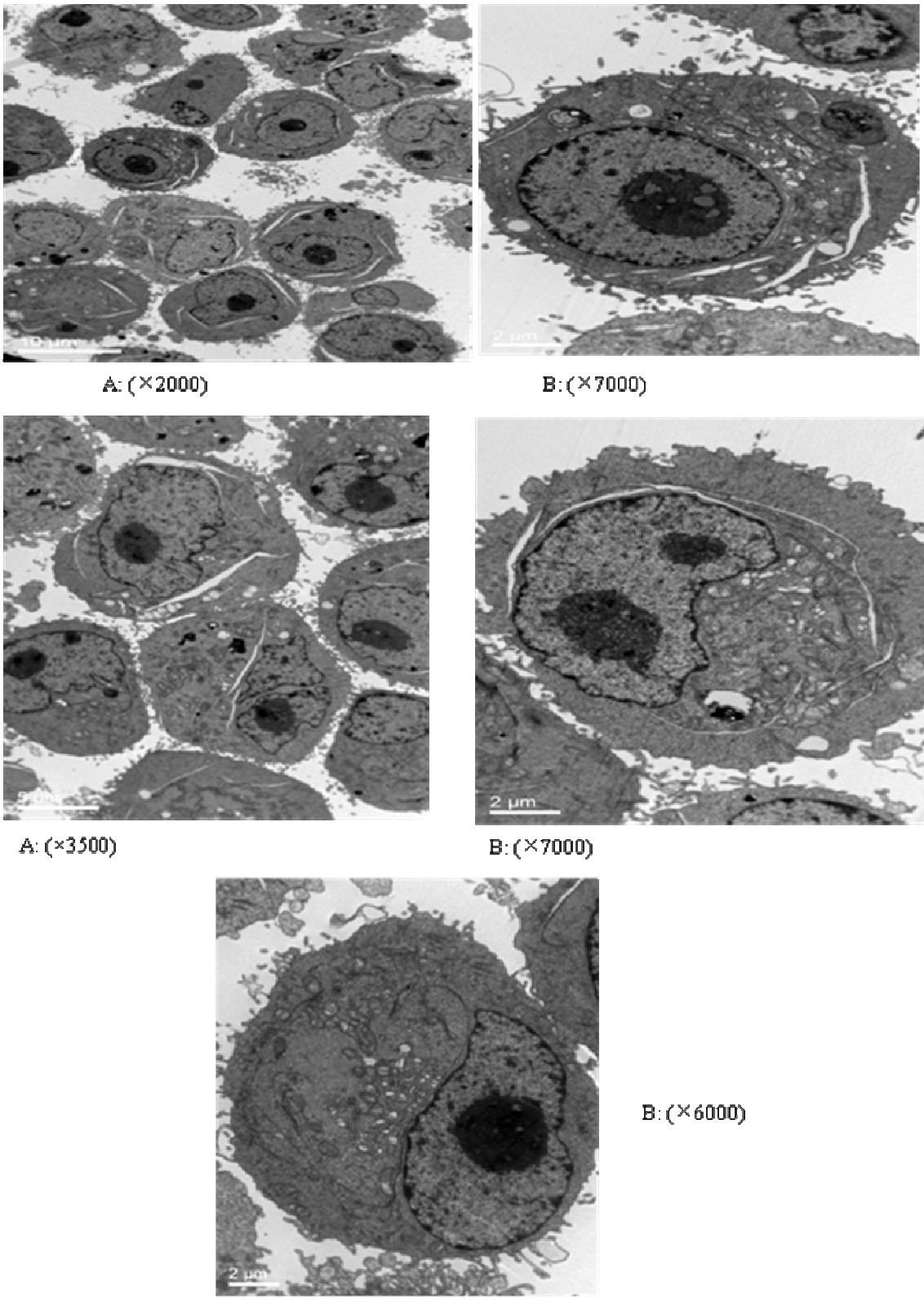

Figure 7. Ultrastructural observation of cells transfected with REGγ gene
Cellular ultramicrostructure in REGγ transfected group showed generally hypertrophic nucleolus, increased or expanded mitochondria and Golgi's body, no apoptotic body and nuclear bag formation in some areas. Meanwhile, Apoptotic body could be seen in the control group occasionally.
A: REGγ transfected group; B: Control group

Tumorigenesis effect of transfected cells in nude mouse body

Tumor nodus in the three groups cells were formed at 1 w after the inoculation. Tumorigenesis rate was 100% and the tumor grew progressively. However, the growth rate in REGγ transfected group was more rapid than those in the other two groups (Fig. 8). The tumor body was peeled off and weighed, suggesting the weight of tumor body in REGγ transfected group (0.81±0.02 g) increased significantly compared with the empty vehicle group(0.46±0.02 g) and the control group (0.49±0.01g) (p<0.05). There was a statistical significance in tumor inhibition rate between REGγ transfected group and empty vehicle group (p<0.01).

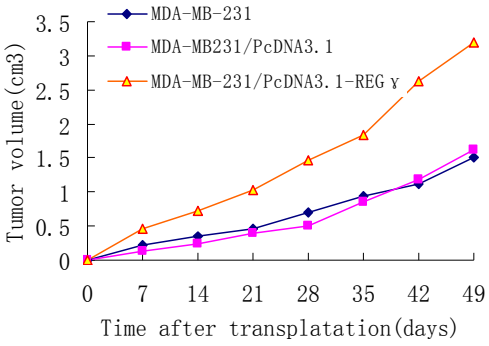

Figure 8. Tumor cells growth in nude mice after the inoculation

The transplanted tumor in the REGγ transfected group grew more rapidly than in non-transfected group. There was significant difference between the REGγ transfected group and the other two groups, respectively. However, there was no statistical difference in tumor growth rate between the other non-transfected groups.

REGγ mRNA expression in transplantation tumor tissue

Total RNA was extracted from the transplantation tumor in the three groups respectively. RT-PCR amplification result indicated REGγ mRNA expression in REGγ transfected group was significantly stronger than those in the other two groups (Fig. 9).

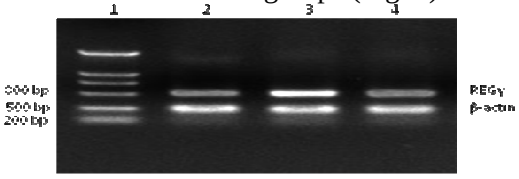

Figure 9. Expression of REGγ in transplanted tumor

The products containing REGγ (780 bp) and β-actin (455bp) in different groups could be seen in gel. The REGγ mRNA expression level in REGγ transfected group was higher than the other two groups.

1: DNA Marker; 2: MDA-MB-231/PcDNA3.1; 3: MDA-MB-231/PcDNA3.1-REGγ; 4: MDA-MB-231

CD16, cell cycle and apoptosis in transplantation tumor tissue

FCM indicated positive rate of CD16 in REGγ transfected, empty vehicle and the control group was (9.74±1.32) %, (24.41±1.96) % and (25.68±2.17) % respectively, and the positive rate of the latter was significantly lower than those in the two groups. Cell cycle showed cells in G_0/G_1 and G_2/M phase in REGγ transfected group decreased and cells number in S phase increased significantly compared with the other two groups. The apoptosis rate of the tumor cell decreased significantly (p<0.05). (Fig. 10A and B).

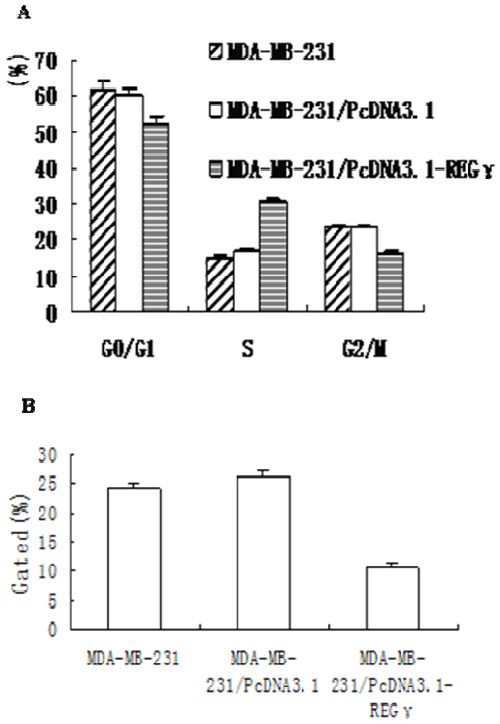

Figure 10. Cell cycle and apoptosis in transplanted tumor tissue

Cell cycle showed cells in G_0/G_1 and G_2/M phase in REGγ transfected group decreased and cell numbers in S phase increased significantly compared with the other two groups. The apoptosis rate of the tumor cell decreased significantly

1: DNA Marker; 2: MDA-MB-231/PcDNA3.1; 3: MDA-MB-231/PcDNA3.1-REGγ; 4: MDA-MB-231

Immunohistochemistry of P21 positive expression

Positive cellular percentage of P21 in transplantation tumor tissue of the control, empty vehicle group, REGγ transfected group was 36.82±5.17%, 32.34±6.28% and 17.40±4.64% respectively, suggesting positive percentage of P21 in REGγ transfected group was significantly lower than those in the other two groups(p<0.05) (Fig. 11).

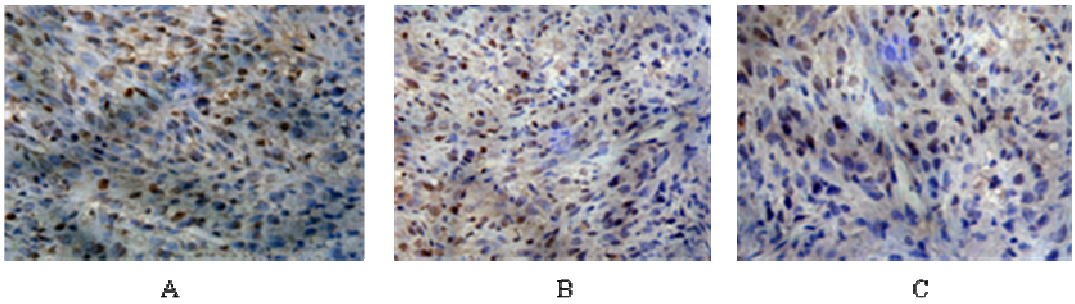

Figure 11. The positive expression of P21 in transplanted tumor
36.82±5.17%, 32.34±6.28% and 17.40±4.64% positive cellular percentage of P21 was in transplantation tumor tissue of the control, empty vehicle group, REGγ transfected group, respectively. It suggested that positive percentage of P21 in REGγ transfected group was significantly lower than those in the other two groups.
A: MDA-MB-231; B: MDA-MB-231/PcDNA3.1; C: MDA-MB-231/PcDNA3.1-REGγ

DISCUSSION

Tumor is known to be a cellular cyclic disease (21), and regulatory imbalance of cell cycle is one of the main causes due to malignant tumors proliferation. Whether cell cycle can initiate and cause proliferation relies on whether it can pass G1/S check point or not. Once past the check point, cell cycle can be accomplished under the driver of cyclins and CDK even though the stimulation from growth factor is absent (23). Therefore, G_1/S check point plays an important role in the genesis and development of tumors (3, 15).

Studies have suggested REGγ was associated with the physiological regulation of cell proliferation and growth. REGγ-/- mouse was born normally but it grew more slowly than wild type (WT) (16). Barton et al. (1) also confirmed that REGγ-/- embryo fibroblasts proportion increased in G_1 phase, whereas G_1/S phase was arrested, which led to about a 3-fold increased apoptosis. Cell proliferation was inhibited in G_1 phase when REGγ in drosophila cell was knockout (11, 17). In addition, a DNA replication related promoter element was just found to locate on upstream of REGγ gene in drosophila (13). Meanwhile, overexpressive REGγ could promote mitosis and REGγ deletion (24).

In the present study, we determined the distribution of MDA-MB-231 cell in cell cycle before and after REGγ transfection. We found the cells decreased in G_0/G_1 phase and increased in S phase significantly, indicating REGγ could facilitate cell cycle process in G_1/S phase. Meanwhile, cloning efficiency of cells transfected with REGγ gene was higher than that in the control group in soft agar colony forming experiment. Furthermore, volume of the clone was comparatively bigger and survival time was comparatively longer, suggesting REGγ could enhance clonality of the transfected cells. We also found that cells transfected with REGγ grew more rapidly compared with non-transfected ones in MTT experiment.

Following, we assayed proliferation associated molecule PCNA using immunohistochemistry in vitro. PCNA is a nuclear protein which expressed particularly during the proliferative phase. It involves duplication and repair of DNA backbone as a helper factor of DNA pclymerase δ. Bianchi et al. (2) found more or less PCNA positive cells could reflect tumor cell growth. In the present study, we found that PCNA positive expression in MDA-MB-231 cell line transfected with REGγ gene increased significantly compared with the control group, suggesting REGγ enhanced cell proliferative activity, which suggested REGγ gene transfection had a remarkable promotion effect on cell growth.

To further investigate proliferation promotive effect of REGγ on breast cancer cell line MDA-MB-231 and related mechanism in vivo, we established breast cancer bearing nude mice model. And, we obtained similar results as in vitro: more rapid growth of tumor tissue, higher REGγ mRNA expression level, the decreased cells in G_0/G_1 phase and increased cells in S phase in REGγ group. Subsequently, related molecules P21, CD16 and Caspase-3 were investigated.

Recently, some scholars found REGγ could independently degrade cyclin-dependent kinase inhibitor P21 (8, 22). Zhong et al. (12) found the proliferative rate of cells transfected with P21 was significantly lower than that of

non-transfected cells after P21 gene was transfected into breast cancer MCF-7 cell line, which suggested REGγ promoting cell growth might be related to its degradative effect on P21. REGγ was also confirmed to control abundances of three important cyclin P21, P16 and P19 and cell death via decreasing REGγ expression (6). In our study, positive percentage of P21 in REGγ transfected group was significantly lower than those in the other two groups. We supposed more degradation of P21 contributed to the more rapid growth of MDA-MB-231 cell line transfected with REGγ.

CD16 is commonly used for NK activity evaluation. Activated NK plays an important role in tumor immunology. Infiltrative and activated NK is closely related to prognosis of tumor (4). Our results indicated high REGγ expression had an inhibitory effect on NK activation. REGγ could promote cell cycle and suppress cell apoptosis. It might be due to REGγ promoting genesis and development of tumors.

Caspase family plays an important role in apoptosis. However, activation of Caspase enzyme is an early event of apoptosis. Inactive Caspase-3 is activated when apoptosis occurs, and then it initiates the following apoptosis events. In present study, we found activity of Caspase-3 in cells transfected with REGγ gene was generally weaker than that in non-transfected ones, which displayed a negative relationship between REGγ and Caspase-3 activation. In late apoptosis event, apoptotic body formation is characterized. In present study, no apoptotic body was seen in cells transfected with REGγ, and whereas it appeared in the control group occasionally. Furthermore, the cell transfected with REGγ gene showed active cellular proliferative characteristics including generally hypertrophic nucleolus, abundant mitochondria, increased or expanded Golgi's body and nuclear bag in some areas. It also confirmed the anti-apoptosis role of REGγ from morphological view.

In summary, oncogenicity of MDA-MB-231 cell transfected with REGγ was enhanced. Maybe this is related to REGγ promoting cell cycle, accelerating cell growth, inhibiting cell apoptosis, degrading P21 particularly and suppressing activation of NK, suggesting REGγ promoting tumor growth is a process involving multiple mechanisms. Therefore, it is worthy of investigating REGγ proteasomes pathway in depth, which might provide an effective approach to the treatment of REGγ related tumors.

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