The membrane molecule RCAS1 induces immune cell apoptosis via the RCAS1-RCAS1R pathway

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Abstract. Tumor-associated antigen receptor-binding cancer antigen expressed on SiSo cells (RCAS1) has been identified as an estrogen-responsive gene and reportedly acts as a ligand for a putative receptor present in a variety of human cell lines and peripheral lymphocytes, thus leading them to apoptosis. In this study, we investigated the biological function of RCAS1 *in vitro* in inducing the apoptosis of immune cells. We detected the expression of the RCAS1 receptor (RCAS1R) in the cell lines, and investigated the mechanisms behind the apoptosis induced by RCAS1. HeLa cells were transfected with recombinant adenovirus Ad-RCAS1. RCAS1 induced the apoptosis of activated T cells, K562 cells and phytohemagglutinin (PHA)-activated Jurkat cells via the RCAS1-RCAS1R pathway. The expression of RCAS1R was induced. The intracellular overexpression of RCAS1 inhibited the growth of Jurkat and K562 cells. The expression of RCAS1 negatively correlated with the expression of glycogen synthase kinase 3β (GSK3β), but positively correlated with the expression of phosphorylated GSK3β (phGSK3β). RCAS1 expression was identified as a brown staining pattern in the breast cancer specimens. These findings may provide insight into the mechanisms through which tumor cells escape from immune surveillance.

Introduction

Tumor-associated antigen receptor-binding cancer antigen expressed on SiSo cells (RCAS1) is a type-II membrane protein, expressed in estrogen target organs as well as several other tissues such as the brain, liver and kidney tissues (1).

Previous studies have shown that RCAS1 is expressed in a variety of malignancies, such as breast, ovarian, prostate and hepatocellular carcinoma (2-5). The RCAS1 protein acts as a ligand for a putative receptor present in various human cells, including normal peripheral lymphocytes, such as T, B and natural killer (NK) cells. RCAS1 inhibits the growth of receptor-expressing cells both *in vitro* and *in vivo* and induces apoptotic cell death through the activation of caspase-3 and the collapse of mitochondrial transmembrane potential (6,7). Therefore, it has been generally accepted that RCAS1 is involved in the immune escape of tumor cells. Immunohistochemical studies of tissue samples have revealed that RCAS1 expression significantly correlates with poor overall survival in patients with a variety of malignancies (8-10). However, the possible mechanism behind the RCAS1-induced cell apoptosis has not yet been elucidated.

In our previous studies, we induced the expression and purification of glutathione S-transferase (GST)-RCAS1 fusion protein (11), and reported that RCAS1 promoted the tumor growth and metastasis of 4T1 murine mammary carcinoma cells (12). In this study, we used the recombinant adenovirus RCAS1 (Ad-RCAS1) and GST-RCAS1 fusion protein, to investigate the apoptosis of T lymphocytes and immune cells derived from leukemia cell lines induced by RCAS1. We detected the expression of the RCAS1 receptor (RCAS1R) in the cell lines, and investigated the mechanism behind the apoptosis induced by RCAS1. We used immunohistochemical analysis to examine the expression of this molecule in human breast tissues.

Materials and methods

Reagents. Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA, USA). GST monoclonal antibody (MoAb) was purchased from Kangchen Biotech Co. (Shanghai, China). The B-PER GST Fusion Purification kit was purchased from Pierce Biotechnology (Rockford, IL, USA). Mouse GAPDH MoAb, donkey anti-rabbit IgG (H+L) horseradish peroxidase (HRP)-, donkey anti-goat IgG (H+L) HRP- and goat anti-mouse IgG (H+L) HRP-conjugated antibodies were all from Cell Signaling Technology, Inc. (Danvers, MA, USA). Goat polyclonal antibodies (PoAbs) to RCAS1 (N18 and C20; sc-23396 and sc-23394, respectively; Santa Cruz

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Biotechnology, Inc., Santa Cruz, CA, USA), rabbit PoAbs to glycogen synthase kinase 3β (GSK3β) and phospho-Ser9-GSK3β (BioVision Technologies, Exton, PA, USA) were commercially purchased. RCAS1 primer was synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). This study was carried out in accordance with the Declaration of Helsinki. All the data presented and materials used in this manuscript were approved by the Ethics Committee of the Children's Hospital of Zhejiang University School of Medicine, Hangzhou, China.

Cell culture and treatment. A293 (human embryonic kidney), HeLa (adenocarcinoma), K562 (chronic myelogenous leukemia), Jurkat (acute T cell leukemia), THP1 (acute monocytic leukemia) and E003 (acute B cell leukemia) cells were from the American Type Culture Collection (Manassas, VA, USA). All the cells were grown in RPMI-1640 supplemented with 10% (v/v) fetal calf serum, 4.5 g/l D-glucose, non-essential amino acids (100 μM each), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine at 37°C in a 5% CO_2 atmosphere. HeLa cells were transfected with the Ad-RCAS1 and Ad-LacZ vectors, using Lipofectamine 2000 according to the manufacturer's instructions. Following transfection, RT-PCR and western blot analysis were carried out to determine the expression of RCAS1, β-actin or GAPDH, and the effectiveness of the assay was determined by the negative control. K562, Jurkat, THP1 and E003 cells were cultured in 6-well flat bottom plates ($5x10^5$ cells/well), and then Ad-RCAS1 [$5x10^7$ plaque forming units (PFU), multiplicity of infection (MOI) 100] and Ad-LacZ ($5x10^7$ PFU, MOI 100) were added to the medium. After 24 h of culture, the cells were harvested for apoptosis assay.

Amplification of recombinant adenovirus Ad-RCAS1 and Ad-LacZ. Recombinant adenovirus Ad-RCAS1 and Ad-LacZ were gifts from Professor Cao (Second Military Medical University, Shanghai, China). For recombinant adenovirus multiplication, A293 cells with 80% fusion were replenished with fresh medium and subsequently infected with 100 μl of Ad-RCAS1 or Ad-LacZ. After the cytopathic effect was achieved (approximately 24 to 72 h), the cells were collected and washed 3 times with PBS, then metered in a total volume of 200 μl. After 6 freeze-thaw cycles, the cells were centrifuged at 5,000 rpm at 4°C for 15 min, and the virus was released into the supernatants (approximately 200 μl). Following amplification, all viruses were mixed and filtered through a Gelman syringe filter (0.2 μm) for the experiments. Virus titer (PFU/ml) was determined by a 50% tissue culture infective dose (TCID50) assay (13).

Isolation and culture of T cells and CD4+ T cells. Peripheral blood was obtained from volunteers (Blood Center, Zhejiang, China). Peripheral blood mononuclear cells (PBMCs) were obtained by Percoll density gradient centrifugation using lymphocyte separation medium (Henxin Bio Co., Shanghai, China) from human peripheral blood. T cells ($1x10^8$) were purified using MACS Columns (Miltenyi Biotec). CD4+ T cells and CD4- T cells were acquired. The PBMCs and CD4+ T cells were then cultured in RPMI-1640 in 6-well flat bottom plates (Orange Scientific), supplemented with 200 U/ml interleukin (IL)-2. The old medium was replaced with fresh medium every 2 days. On day 7, the cells were treated with 1 μg/ml phytohemagglutinin (PHA), then cultured for 24 h. On day 8, the cells were randomly divided into 3 groups: controls, Ad-RCAS1 and Ad-LacZ; after 24 h of culture, the cells were harvested for apoptosis assay (propidium iodide and Annexin V staining).

Apoptosis assay. The cells were washed, resuspended in staining buffer, and stained with propidium iodide and Annexin V, according to the manufacturer's instructions. Stained cells were analyzed using a FACSCalibur (Becton-Dickinson). The Annexin V-FITC Apoptosis Detection kit was purchased from BioVision Technologies.

RT-PCR analysis. Total RNA was extracted from the cultured cells using TRIzol reagent (Dingguo Biotech Co., Beijing, China). Total RNA (1 μg) from each sample was reverse transcribed using a Reverse Transcription System kit (MBI Fermentas, Vilnius, Lithuania) in a total volume of 20 μl. cDNA synthesized from mRNA was quantified by PCR using specific primers as follows: RCAS1 sense, 5'-GCGAATTCTT ATGAAAGTTTCACACC-3' and antisense, 5'-GTGGATCC ATGGCCATCACCCAGTTT-3', with a predicted PCR product of 642 bp; β-actin sense, 5'-CCTAGAAGCATTT GCGGTGG-3' and antisense, 5'-GAGCTACGAGCTT GACG-3', with an expected PCR product of 402 bp. Cycling conditions were 30 sec of denaturation at 94°C, 30 sec of annealing at 56-60°C and 60 sec of extension at 72°C. The relative amount of mRNA was determined using Quantity One software (Bio-Rad, Hercules, CA, USA).

Western blot analysis. Cells were lysed in lysis buffer (Cell Signaling Technology, Inc.) for 30 min on ice. A BCA kit (Cat. no. 23225; Pierce Biotechnology) was then used to determine the concentration of the lysates. Cell extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The blot was probed with RCAS1 (C20 and N18), GSK3β and phosphorylated GSK3β (phGSK3β) PoAbs and GAPDH MoAb. Signals were developed using an enhanced chemiluminescence detection reagent. The relative protein level was determined with band intensities compared with GAPDH using the Quantity One software (Bio-Rad).

Receptor detection. The binding of GST-RCAS1 to RCAS1 receptor (RCAS1R)-expressing cells was determined by fluorescence-activated cell sorting (FACS) analysis. Briefly, after 3 washes with PBS, the K562, Jurkat or PHA-treated Jurkat cells ($3x10^5$) were incubated in 1 ml PBS, with the addition of 10 μl GST-RCAS1 (10 μg) fusion protein, GST protein (10 μg) and PBS, respectively, and incubated for 30 min at 4°C. After 3 washes, GST MoAb was added, followed by incubation for 30 min at room temperature. After 3 washes, the cells were labeled with anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) and incubated for 10 min at room temperature in the dark. After another 3 washes, the cells were resuspended in a final volume of 1 ml of PBS and acquired on a FACSCalibur (Becton-Dickinson). The experiments were repeated on three separate occasions and the percentage and fluorescence intensity of FITC-positive cells were detected by FACS.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. K562 and Jurkat cells were cultured in 96-well plates at a density of $1x10^4$ cells/well in RMPI-1640 supplemented with 5% fetal calf serum (FCS) in the absence or presence of Ad-RCAS1 or Ad-LacZ ($0.625x10^5$-$40x10^5$ PFU, MOI 12.5-400). After 48 h of incubation, 20 μl of MTT (5 mg/ml) (Sigma) were added to each well. The mixture was incubated for an additional 4 h and then centrifuged at 3,000 rpm at 4˚C for 10 min, and the supernatant was discarded. The precipitate was dissolved in 150 μl of DMSO and the absorbance values were read at 570 nm with a microplate reader (Molecular Devices, Palo Alto, CA, USA).

Co-immunoprecipitation. Cell lysates of K562 cells transfected with Ad-RCAS1 (300 μg/tube) were mixed with RCAS1 PoAb, control or GSK3β PoAb, and incubated for 6 h. Then a 3X reaction volume of Protein G Plus Agarose (60 μl) was added, followed by incubation for another 6 h. The cells were then washed 3 times in PBS, followed by the addition of the buffer (120 μl), and subjected to western blot analysis.

Histological examination of breast tissue. Different breast tissues for histological examination were collected from the Women's Hospital School of Medicine, Zhejiang University. Immunohistochemical analysis was performed by Chaoying Bio Co. (Shanxi, China). Slides (5-μm-thick) were deparaffinized, rehydrated and rinsed in distilled water. Endogenous peroxidase activity was blocked by 10 min of incubation in 3% H_2O_2 at room temperature. The slides were then rinsed and immersed in boiling citrate buffer (pH 6.0) in a microwave oven with 3 changes of buffer every 5 min. For the immunolocalization of RCAS1, the slides were treated with the goat PoAbs to RCAS1 (N18 and C20; dilution 1:100, 1:50) in a moist chamber overnight. The slides were subsequently rinsed in TBS buffer (pH 7.6) and incubated with secondary antibody [donkey anti-goat IgG (H+L) HRP] (dilution 1:500, 1:250) for 45 min at 37˚C. Visualization was performed using 3,3'-diaminobenzidine (DAB) as a chromogen for 10 min at room temperature. The sections were counterstained with hematoxylin and mounted in Glycergel. We assumed that positive reactivity for RCAS1 occurred when we observed a brown staining pattern.

Statistical analysis. All experiments were repeated 2 or 3 times. Statistical analysis was carried out using the Student's t-test. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Identification of recombinant adenovirus Ad-RCAS1. To determine whether RCAS1 expression affects cellular apoptosis *in vitro*, we amplified recombinant adenovirus Ad-RCAS1. Ad-LacZ was amplified as the control. In this study, we amplified 5 ml Ad-RCAS1 and Ad-LacZ, respectively. TCID50 assay showed that Ad-RCAS1 and Ad-LacZ titers were $4.8x10^9$ and $4.2x10^9$ PFU/ml, respectively, then adjusted to a final titer of $4x10^9$ PFU/ml. HeLa cells were then transfected with Ad-RCAS1 or Ad-LacZ. The expression of RCAS1 was identified by RT-PCR and western blot analysis (Fig. 1A-C). Semi-quantitative RT-PCR analysis showed that the mRNA
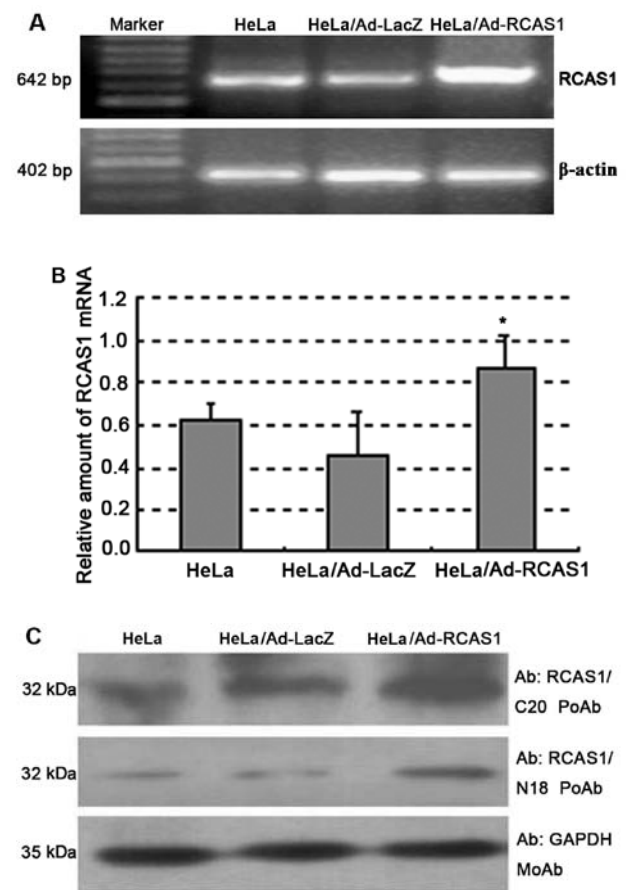

Figure 1. Identification of Ad-RCAS1 by RT-PCR and western blot analysis in HeLa cells transfected with Ad-RCAS1. *P<0.05. HeLa cells were transfected with Ad-RCAS1, or Ad-lacZ as the mock control using Lipofectamine 2000. The expression of RCAS1 in the Ad-RCAS1-transfected HeLa cells (HeLa/Ad-RCAS1) was detected by (A and B) RT-PCR and (C) western blot analysis with anti-N18 and anti-C20 antibody.The experiment was carried out 3 times and 1 representative result is shown.

expression of RCAS1 in the HeLa cells transfected with Ad-RCAS1 was higher than that in the HeLa cells transfected with Ad-LacZ or in the parental HeLa cells. Simultaneously, the density of the 32 kDa bands detected by western blot analysis using PoAb to RCAS1 (C20 and N18) was relatively higher in the cell lysates of the HeLa cells transfected with Ad-RCAS1 than that in the cell lysates of the HeLa cells transfected with Ad-LacZ or in the parental HeLa cells.

Detection of apoptosis in T lymphocytes and immune cells derived from leukemia cell lines following treatment with RCAS1. To investigate whether RCAS1 can induced the apoptosis of T cells, we examined the percentage of apoptotic cells by propidium iodide and Annexin V staining. Twenty-four hours after the transfection of T cells isolated from peripheral blood with Ad-RCAS1, Ad-LacZ or the blank control, a small number of apoptotic cells was observed and no significant differences were detected in the T cells transfected with Ad-RCAS1 compared with those transfected with Ad-LacZ or the blank control (data not shown). After the T cells were stimulated with IL-2 (200 IU/ml) and PHA (1 μg/ml), we found that the apoptotic value of Ad-RCAS1 in the activated T cells was higher than that of the Ad-LacZ or the blank control
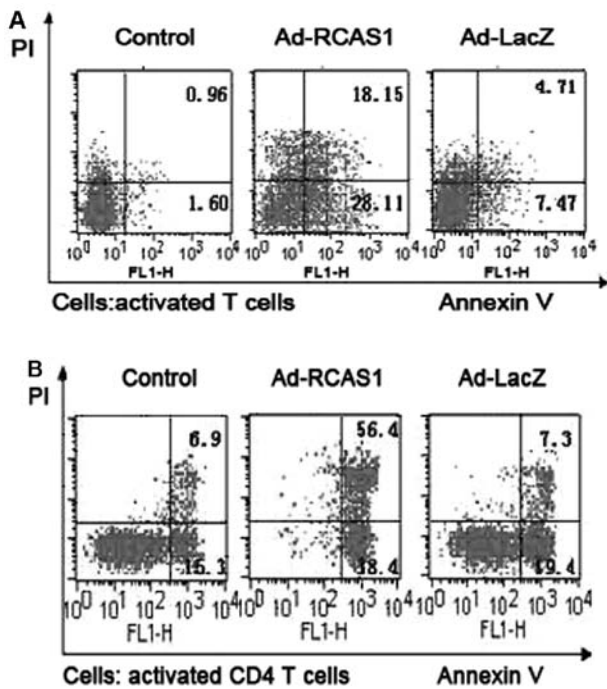
Figure 2. Effect of RCAS1 on activated T cell and CD4$^+$ T cell apoptosis *in vitro*. (A) Activated T cell and (B) CD4$^+$ T cell apoptosis detected by FACS with propidium iodide (PI) and Annexin V staining. Activated T cells and CD4$^+$ T cells were transfected with Ad-RCAS1, Ad-LacZ and medium, and harvested for apoptosis assay (PI and Annexin V staining). The experiment was carried out 3 times and 1 representative result is shown.
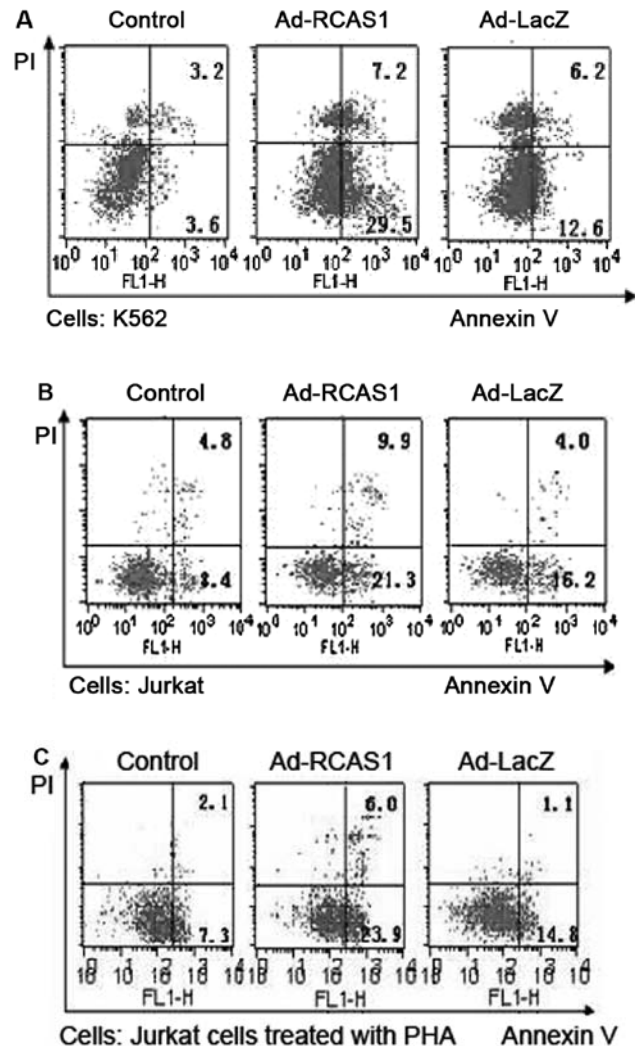

Figure 3. Apoptosis of Jurkat and K562 cells induced by RCAS1. The apoptosis of (A) K562, (B) Jurkat and (C) PHA-activated Jurkat cells was detected by FACS with propidium iodide (PI) and Annexin V staining. K562, Jurkat, PHA-activated Jurkat cells were transfected with Ad-RCAS1, Ad-LacZ and medium, and harvested for apoptosis assay (PI and Annexin V staining). The experiment was carried out 3 times and 1 representative result is shown.

group (Annexin V-positive cells 46.26, 12.18 and 2.56%, respectively) (Fig. 2A). We examined the effect of RCAS1 on activated CD4$^+$ T cell apoptosis. As shown in Fig. 2B, an increased number of apoptotic cells was observed in the activated CD4$^+$ T cells transfected with Ad-RCAS1 compared with those transfected with Ad-LacZ or the blank control (Annexin V-positive cells 94.8, 56.7 and 23.2%, respectively). These results suggest that RCAS1 overexpression induces the apoptosis of activated T cells and CD4$^+$ T cells.

Furthermore, using the same method, we examined whether RCAS1 affects the apoptosis of immune cells derived from leukemia cell lines, such as Jurkat, K562, THP1 and E003 cells. K562 cells exhibited higher levels of apoptosis following Ad-RCAS1 treatment, compared with the cells treated with Ad-LacZ or the blank control (Annexin V-positive cells 36.7, 18.8 and 6.8%, respectively) (Fig. 3A). However, RCAS1 had no effect on the apoptosis of THP1 and E003 cells (data not shown). For the Jurkat cells (acute T cell leukemia), the apoptotic value of Ad-RCAS1, Ad-LacZ and the blank control group was 31.2, 20.2 and 13.2%, respectively (Fig. 3B). The Ad-RCAS1 group had the highest rate of apoptosis, whereas the blank control group had the lowest rate. As shown in Fig. 3C, an increased number of apoptotic cells was observed in the Jurkat cells stimulated with PHA (1 μg/ml), and the difference in the number of apoptotic cells between the Ad-RCAS1 and Ad-LacZ or the blank control group was significant.

RCAS1 receptor detection. In our previous study, we induced the expression and purification of GST-RCAS1 fusion protein (11). In this study, using the GST-RCAS1 fusion protein, we examined whether RCAS1R is expressed on the cell membrane of Jurkat, K562, THP1 and E003 cells. In the K562 cells, RCAS1R expression was detected and there was a significant difference between the GST-RCAS1 group and the GST or control group; however, the percentage of FITC-positive cells was not very high (Fig. 4A). Of note, a low expression of RCAS1R was detected in the THP1, E003 (data not shown) and Jurkat cells (Fig. 4B). As shown in Fig. 4C, RCAS1 expression in the Jurkat cells co-cultured with GST-RCAS1 increased significantly following stimulation with PHA (1 μg/ml) compared with the cells co-cultured with GST or the blank control (FITC-positive cells 43.98, 1.59 and 4.07%, respectively). These results suggest that RCAS1R is not expressed in Jurkat cells; however, following stimulation with PHA, a moderate upregulation of its expression can be induced.

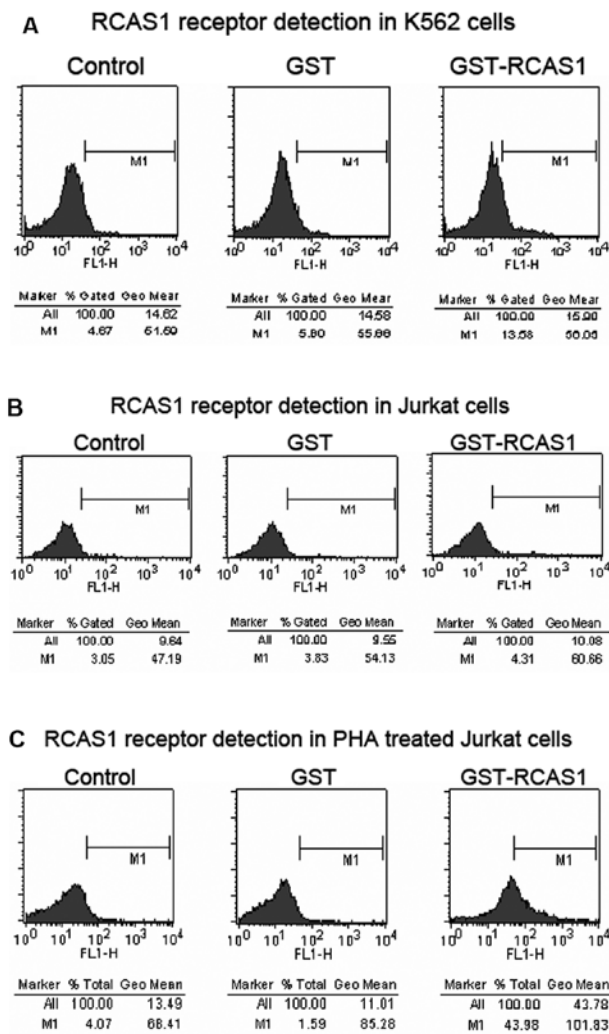*RCAS1 inhibits K562 and Jurkat cell proliferation.* We used MTT assay to examine whether RCAS1 affects the

Figure 4. Putative RCAS1 receptor detection by FACS using GST-RCAS1 fusion protein. Cell surface expression of RCAS1 in (A) K562, (B) Jurkat and (C) PHA-treated Jurkat cells determined by FACS using GST-RCAS1 fusion protein. GST protein or a blank were used as controls. The experiment was carried out 3 times and 1 representative result is shown.
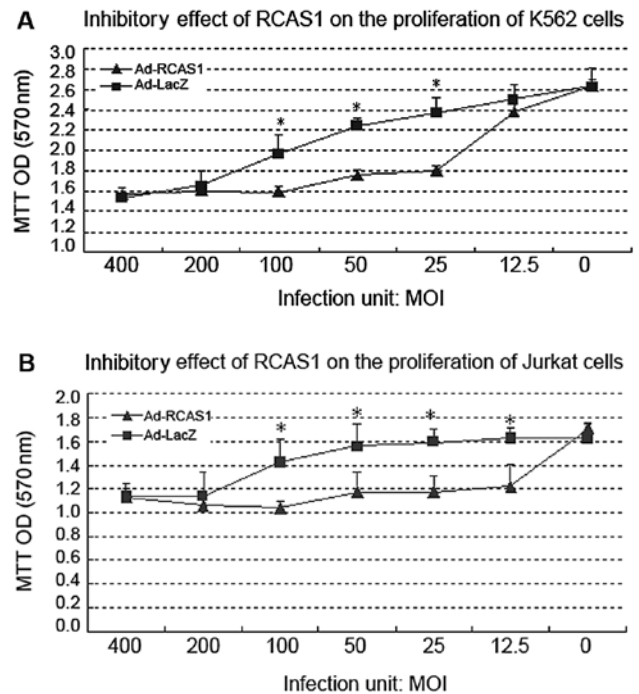

Figure 5. Effect of RCAS1 on (A) K562 and (B) Jurkat cell viability examined by MTT assay *in vitro*. After co-culture with Ad-RCAS1 (Ad-LacZ as the mock control) at different MOIs (0, 12.5, 25, 50, 100, 200 and 400), cell proliferation was evaluated. Data are presented as the means ± standard deviation. Similar results were obtained in 3 independent experiments. *$P<0.05$.

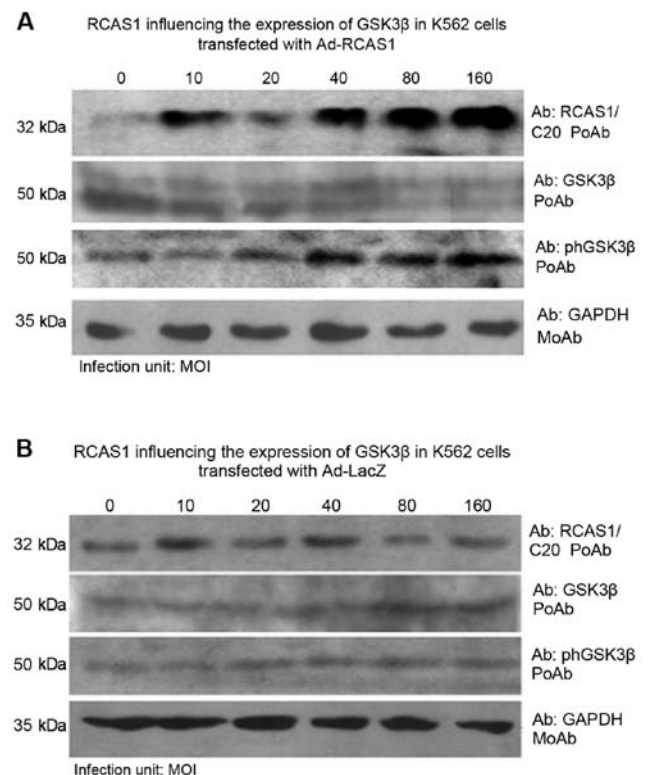Figure 6. Western blot analysis of the correlation between RCAS1 and GSK3β or phGSK3β. K562 cells were transfected with (A) Ad-RCAS1 or (B) Ad-LacZ at an MOI from 0 to 160, and the cell lysates were analyzed by western blot analysis using RCAS1, GSK3, or phGSK3β polyclonal antibody and GAPDH monoclonal antibody (MoAb) as the control. The experiment was carried out 3 times and 1 representative result is shown.

proliferation of K562 and Jurkat cells. Fig. 5A and B shows the inhibitory effect of RCAS1 on the proliferation of K562 and Jurkat cells at 48 h post-transfection at different MOIs of Ad-RCAS1 and Ad-LacZ. There was a significant difference in the cell viability between Ad-RCAS1 and Ad-LacZ in a MOI-dependent manner, indicating that RCAS1 inhibited K562 and Jurkat cell growth *in vitro*.

Correlation between overexpression of RCAS1 and GSK3β or phGSK3β. Using western blot analysis and co-immuno-precipitation, we examined whether GSK3β plays a role in the signaling pathway of cell apoptosis induced by RCAS1. When the MOI of Ad-RCAS1 was doubled in the K562 cells, the expression of RCAS1 in the K562 cells increased, but the expression of GSK3β decreased, and the expression of phGSK3β increased (Fig. 6A). No significant difference was observed in the K562 cells transfected with Ad-LacZ (Fig 6B), indicating that the overexpression of RCAS1 in the K562 cells resulted in the altered expression of GSK3β and phGSK3β. Our data suggest that the expression of RCAS1 negatively correlates
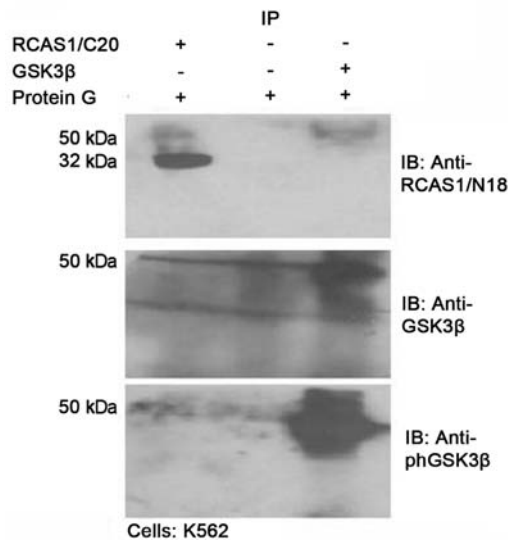
Figure 7. Results of the co-immunoprecipation assay. Lysates of K562 cells were immunoprecipitated with either RCAS1/C20 polyconal antibody (PoAb) or GSK3β PoAb and protein G as the control. The immunoprecipitated proteins were detected with RCAS1/N18 PoAb, GSK3β PoAb and phGSK3β PoAb. The experiment was carried out 3 times and 1 representative result is shown.

with the expression of GSK3β, but positively correlates with the expression of phGSK3β. Furthermore, we determined whether RCAS1 binds to GSK3β, leading to the phosphorylation of GSK3β by using co-immunoprecipitation. RCAS1 was not detected in the immunoprecipitates of K562 cells transfected with Ad-RCAS1, which were precipitated with anti-GKS3β and anti-phGKS3β antibody (Fig. 7). This result demonstrates that RCAS1 does not directly bind to GSK3β.

RCAS1 expression identified as a brown staining pattern in breast cancer specimens. In our previous study, we reported that RCAS1 promoted tumor growth and metastasis in 4T1 murine mammary carcinoma cells (12). In this study, we collected different types of breast tissue from the Women's Hospital School of Medicine, Zhejiang University for histological examination. For the immunolocalization of RCAS1 the specimens were treated RCAS1 (N18 and C20) PoAb. Immunohistochemical analysis confirmed that the breast

cancer tissue expressed RCAS1; however, the normal breast tissue did not express RCAS1 (Fig. 8).

Discussion

RCAS1 is a type-II membrane protein, expressed in estrogen target organs, as well as in various malignancies. Nakashima *et al* (6) showed that RCAS1 was a transmembrane molecule, and that activated T cells expressed a putative RCAS1R, and apoptosis was induced after the binding between RCAS1 and RCAS1R. However, other studies have provided conflicting results without showing any significant correlation. Engelsberg *el al* (14) showed that RCAS1 was predominantly located on Golgi and that RCAS1 was not able to induce apoptosis, also confirmed by the study of Reimer *et al* (15). In our study, we constructed the GST-RCAS1 fusion protein and recombinant adenovirus Ad-RCAS1 to examine the biological function of RCAS1 *in vitro* in inducing the apoptosis of immune cells.

To determine whether RCAS1 expression affects cell apoptosis *in vitro*, we amplified the recombinant adenovirus Ad-RCAS1. Prior to its use, Ad-RCAS1 was analyzed by RT-PCR and western blot analysis. HeLa cells are a cervical adenocarcinoma cell line, which does not express RCAS1 (6). In our study, HeLa cells were transfected with Ad-RCAS1, thus inducing the expression of RCAS1 in the HeLa cells. Cells were also transfected with Ad-LacZ as the controls. Using RT-PCR, we found that RCAS1 was expressed in the HeLa cells transfected with Ad-RCAS1, the HeLa cells transfected with Ad-LacZ and in the parental HeLa cells; this results is contradictory to the results of other studies (6). Semi-quantitative RT-PCR analysis showed that the mRNA expression of RCAS1 in the HeLa cells transfected with Ad-RCAS1 was higher than that in the HeLa cells transfected with Ad-LacZ or in the parental HeLa cells; this difference was statistically significant. Simultaneously, 32 kDa bands were detected by western blot analysis using RCAS1 PoAb (C20 and N18) in the 3 groups of HeLa cells, but the density of the bands was upregulated in the HeLa cells transfected with Ad-RCAS1 compared to the HeLa cells transfected with Ad-LacZ or in the parental HeLa cells.

After amplifing recombinant adenovirus Ad-RCAS1 and Ad-LacZ, we determined and corrected the virus titer. Due to the biological effects of adenovirus on cells, a consistent concentration should be used for experiments. The virus titer
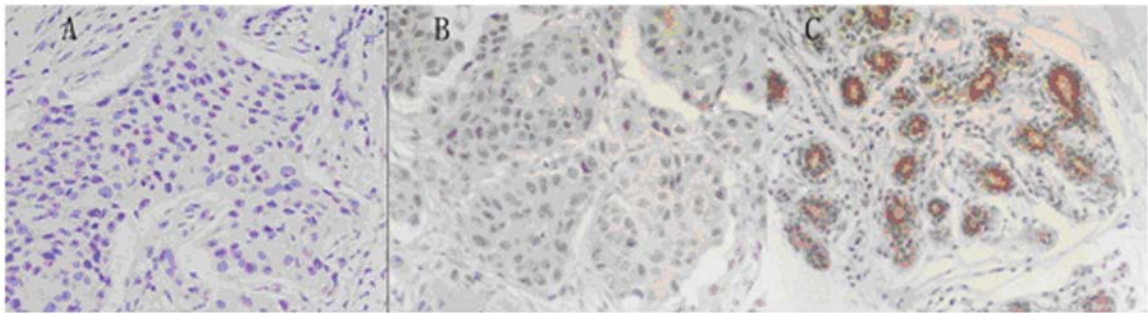

Figure 8. Detection of RCAS1 expression in different breast tissue specimens. (A) In the normal breast tissue, RCAS1 expression was not detected. Immnohistochemical detection of (B) RCAS1/C20 and (C) RCAS1/N18 in breast cancer tissue. Brown staining indicates RCAS1 expression. Original magnification, x200.

(PFU/ml) was determined by TCID50 assay. Ad-RCAS1 and Ad-LacZ titer were then adjusted to the same concentration.

In our study, we separated peripheral blood lymphocytes from human peripheral blood as the cellular model to investigate whether RCAS1 induces apoptosis. Unfortunately, we did not observe any cell apoptosis. These results were consistent with those of a previous study on RCAS1 and cell apoptosis (6); namely that RCAS1 does not induce the apoptosis of naïve T cells. Of note, we observed a significant difference in cell apoptosis between the Ad-RCAS1 group and the Ad-LacZ or control group after the cells were stimulated with IL-2 and PHA. We also observed an increased number of apoptotic cells in the activated CD4$^+$ T cells transfected with Ad-RCAS1 and the percentage of Annexin V-positive cells was >90%. These results suggested that activated T cells and CD4$^+$ T cells overexpressed RCAS1 following transfection with RCAS1, leading to the induction of apoptosis. In the activated CD4$^+$ T cells, the high number of apoptotic cells (>90%) may be due to MACS.

Furthermore, using the same method, we also detected the effects of RCAS1 on the apoptosis of immune cells derived from leukemia cell lines, such as Jurkat, K562, THP1 and E003 cells. We observed an increased apoptosis of K562 cells in the RCAS1 group compared with the other groups. However, RCAS1 had no effect on the apoptosis of THP1 and E003 cells, and a low number of apoptotic cells was observed in the Jurkat cells transfected with RCAS1. Following treatment with PHA, the overexpression of RCAS1 significantly increased the apoptosis of Jurkat cells. In order to elucidate the mechanism behind these phenomenon, we examined whether the receptor expression differed in these cells. In our previous study, we induced the expression and purification of GST-RCAS1 fusion protein (11); in this study, we used this GST-RCAS1 fusion protein to detect RCAS1R expression in these cells. Of note, RCAS1R expression was detected in the K562 cells, but low expression levels of RCAS1R were detected in the THP1, E003 and Jurkat cells. RCAS1 expression in the Jurkat cells increased significantly following stimulation with PHA. These results suggest that RCAS1 does not induce the apoptosis of THP1, E003 and Jurkat cells which do not express RCAS1R; however, RCAS1 induces the apoptosis of K562 and PHA-treated Jurkat cells which express RCAS1R. Thus, RCAS1 may be a possible mechanism involved in the induction of cell apoptosis by binding to RCAS1R. The other mechanisms involved remain to be elucidated.

We also used MTT assay to examine the inhibitory effect of RCAS1 on the proliferation of K562 and Jurkat cells. In this study, there was a significant difference in cell viability between the RCAS1 group and other groups in a MOI-dependent manner, indicating that RCAS1 inhibited K562 and Jurkat cell growth *in vitro*. This result were consistent with the result of cell apoptosis.

Consisting of 2 isoforms, GSK3α and GSK3β, GSK3 was originally considered to be inexorably linked to glycogen metabolism (16,17). Increasing knowledge has altered the status of GSK3β to that of a broadly influential enzyme that is a crucial regulator of a nubmer of cellular functions, including cellular structure, growth, motility and apoptosis (18). GSK3β activity was significantly reduced by the phosphorylation of the N-terminal serine, Ser9, in GSK3β, and Ser21 in GSK3α.

One of the isoforms, GSK3β, has been reported to be crucial to cell survival, including the attenuation of apoptosis and NF-κB activation (19). GSK3 regulates Toll-like receptor (TLR)-mediated inflammatory response by differentially affecting the nuclear amounts of the transcription factors, NF-κB subunit p65 and cAMP response element-binding protein (CREB), interacting with the co-activator CREB-binding protein (CBP) (20). In this study, we detected GSK3β activity in the K562 cells transfected with Ad-RCAS1 and Ad-LacZ. The upregulation of phGSK3β was detected in the K562 cells transfected with RCAS1, whereas total GSK3β downregulated, suggesting that the overexpression of RCAS1 results in the phosphorylation of GSK3β by a certain pathway. Co-immunoprecipitation assay showed that RCAS1 did not directly bind to GSK3β, indicating that RCAS1 possibly interacted with the protein located upstream of GSK3β. The inhibition of the GSK3β activity and thus the inhibiton of the NF-κB signaling pathway may be involved in the apoptosis of K562 cells induced by RCAS1, although the precise mechanism involved needs to be further elucidated.

A number of previous studies have linked the exposure to RCAS1 as an important factor associated with cancer (21,22). We used immunohistochemical analysis to evaluate RCAS1 expression in breast tissues. RCAS1 expression was observed in the breast cancer tissues, but not in normal breast tissue, suggesting that RCAS1 expression is an important event associated with cancer.

In conclusion, we demonstrate that RCAS1 induces the apoptosis of activated T cells, K562 cells and PHA-activated Jurkat cells. The possible mechanism involved may be that the expression of RCAS1R can be induced. RCAS1 inhibits the growth of Jurkat and K562 cells, and is expressed in breast cancer tissues. In addition, the downregulation of GSK3β may play a certain role in the cell growth inhibition by RCAS1. Our data provide insight into the mechanism through which tumor cells escape from immune surveillance and provide a strategy for the therapeutic intervention of cancer.

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