

Analysis of cell cycle arrest and apoptosis induced by RCAS1

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Abstract. A tumor-associated antigen RCAS1 (receptor binding cancer antigen expressed on SiSo cells) induces cell cycle arrest and apoptosis to a putative RCAS1 receptor (RCAS1-R) expressing cells such as T, B, and natural killer cells. Its expression is related with clinical poor prognosis of some malignant tumors. It is suggested that the expression of RCAS1 in tumor cells plays an important role in evasion from host immune system resulting tumor progression, invasion and metastasis. However, the mechanism of RCAS1 induced cell cycle arrest and apoptosis has not been clarified. In this study, we established a mouse L cell line transformed with tetracycline-induced *rcas1* gene expression system and analyzed the RCAS1 functions. We showed that RCAS1 induced cytochrome c release and activation of caspase-3 for apoptosis. Moreover, we investigated cell cycle associated proteins and revealed that cyclin D3 decreased significantly and no change was seen in the expression levels of the other proteins. These results suggest that cyclin D3 is one of the key target molecules in the RCAS1-RCAS1-R signaling pathway.

Introduction

Monoclonal antibody (mAb) 22-1-1 was raised against SiSo (human uterine carcinoma cell lines) (1). Based on studies investigating the binding of mAb to SiSo cells, a novel tumor-associated antigen was cloned, and designated a 'receptor-binding cancer antigen expressed on SiSo cells' (RCAS1) (2). RCAS1 is a type-II membrane protein forming homo-oligomers through C-terminal coiled-coil structures. It also exists in soluble form, probably through alternative splicing (2). Immunohistochemical studies show that RCAS1 is expressed in various malignant tumors, and its expression is one of the important clinicopathological parameters correlating with patient survival (3-13). Soluble RCAS1 has also been

detected in blood serum by enzyme-linked immunosorbent assay (ELISA), and is probably released from the membrane as a result of ectodomain shedding (14,15). The clinical significance of serum RCAS1 levels as a novel tumor marker has been studied for screening of several carcinomas (15-22). RCAS1 is the ligand for a putative receptor expressed by various human cell types such as erythroid leukemia, K562 cells, and normal peripheral blood lymphocytes (T, B, and NK cells), especially after activation by the immune system (2). RCAS1 inhibits cell growth and induces apoptosis in cells expressing the RCAS1 receptor (RCAS1-R) (2). Knock-down of RCAS1 expression by RNA interference restores T cell growth and proliferation by inhibiting apoptosis, and partially reversing T cell IFN- γ secretion (23). Therefore, it has been suggested that RCAS1 might play an important role in tumor progression, invasion, and metastasis through evasion of host immune surveillance (2,4,6,22,24). Recent studies show that RCAS1 induces apoptosis through the loss of mitochondrial transmembrane potential and the activation of caspases (2,3). However, it is still unclear how RCAS1 induces cell growth inhibition and apoptosis in RCAS1-R-expressing cells. To investigate the mechanism of apoptosis induced by RCAS1, we used mouse L cells expressing RCAS1-R to establish a cell line transformed with a tetracycline induced *rcas1* expression system (tet-on system). This cell line (L/ind RCAS1) underwent apoptosis after the induction of RCAS1 expression. Here, we describe the time-dependent expression of cell cycle and apoptosis-related molecules after induction of *rcas1* expression.

Materials and methods

Establishment of the doxycycline inducible rcas1 expression cell line L/ind RCAS1. The tet-on system was used to obtain a stable cell line in which doxycycline (Dox) could be used to induce *rcas1* expression. An *EcoRI-XbaI* cDNA fragment containing the full coding region of *rcas1* was inserted into the *EcoRI-XbaI* site of the pUHD 10-3 plasmid in a sense orientation, and an *EcoRV* fragment containing a hygromycin-resistant gene, controlled by the PGK promoter, and a polyadenylation site was ligated at the *Pvu II* site of the plasmid (pUHD 10-3/RCAS1/Hyg). The linearized pUHD 10-3/RCAS1/Hyg and pUHD 172-1neo plasmids were co-transfected into L cells using the FuGENE HD Transfection Reagent (Roche Applied Sciences, Germany). Stable transformants were obtained by neomycin and hygromycin selection. Sub-clones were cultured with 0.5 μ g/ml of Dox

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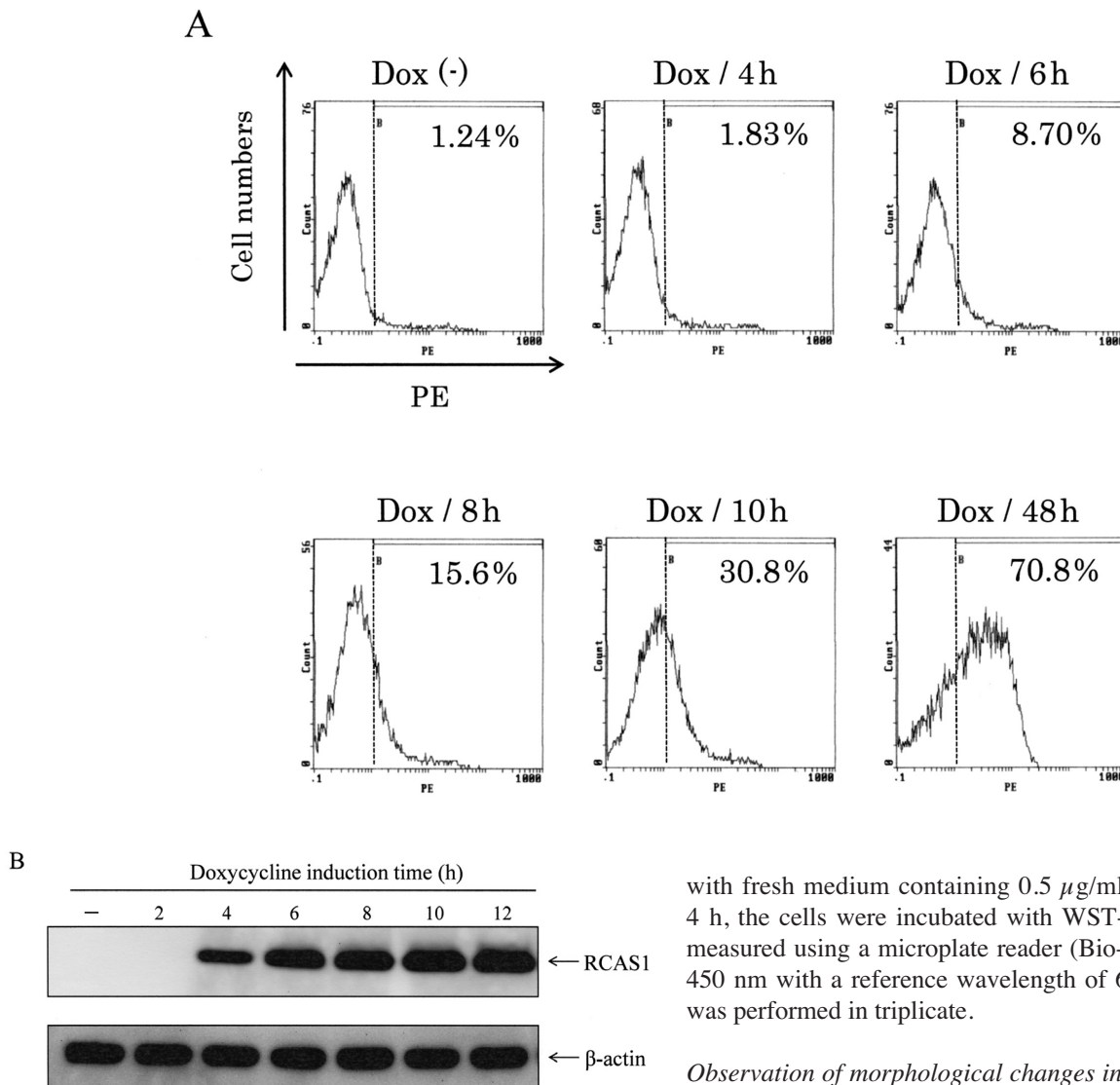


Figure 1. The expression of RCAS1 in L/ind RCAS1. RCAS1 was detected after 6 h of Dox induction and 70.8% of L/indRCAS1 cells expressed RCAS1 on the cell surface after 48 h (A). i.c.RCAS1 expression was seen 4 h after Dox induction and increased in a time-dependent manner until 12 h post-induction (B).

for 48 h and stained with mAb 22-1-1 to identify the doxycycline-inducible RCAS1 expressing clones (L/ind RCAS1).

Flow cytometry. After Dox induction, 1×10^5 cells were harvested and washed three times with washing buffer (3% FCS, 2 mM EDTA, 0.1% NaN_3 in PBS). Cells were then incubated with mAb 22-1-1 on ice for 30 min. After washing, a phycoerythrin (PE)-conjugated goat anti-mouse IgM (μ -chain) antibody (Beckman-Coulter, Tokyo, Japan) was used as a secondary antibody. The stained cells were analyzed using an EPICS XL flow cytometer (Beckman-Coulter).

WST-1 assay. The viability of the L/ind RCAS1 cells was measured using the WST-1 assay (Takara Bio, Tokyo, Japan). Briefly, 3×10^3 cells were seeded in a 96-well plate and incubated overnight. The culture medium was then replaced

with fresh medium containing $0.5 \mu\text{g/ml}$ Dox. For the final 4 h, the cells were incubated with WST-1. Absorbance was measured using a microplate reader (Bio-Rad, model 550) at 450 nm with a reference wavelength of 630 nm. Each assay was performed in triplicate.

Observation of morphological changes in L/ind RCAS1 cells. Cells (3×10^4) were seeded in a glass-bottom dish (Matsunami Glass Ind., Ltd., Tokyo, Japan) and incubated overnight. Cells were then cultured with $0.5 \mu\text{g/ml}$ Dox. After Dox induction, the cells were observed using a fluorescence microscope BZ-8000 (Keyence, Osaka, Japan).

DAPI staining of apoptotic L/ind RCAS1 cells. Cells (3×10^4) were seeded in a glass-bottom dish and incubated overnight. The culture medium was exchanged for fresh medium containing $0.5 \mu\text{g/ml}$ Dox, and incubated for 48 h. After washing with PBS, the cells were fixed with 70% ethanol. Fixed cells were washed with PBS and stained with $1 \mu\text{g/ml}$ 4,6-diamidino-2-phenyl-indole solution (DAPI, Sigma-Aldrich, Tokyo, Japan). After washing, the cells were visualized using a fluorescence microscope BZ-8000 (Keyence).

Western blot analysis. After Dox induction, L/ind RCAS1 cells were lysed with RIPA buffer (0.5% NP-40, 0.25% sodium deoxycholate, 0.05% SDS, 150 mM NaCl, 50 mM HEPES, pH 7.4) containing a protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan). Lysates were centrifuged and supernatants collected. The protein concentration of each sample was measured using the BCA protein assay kit (Thermo Scientific, Rockford). The proteins were electrophoresed on 12% SDS-PAGE gels and transferred onto PVDF membranes.

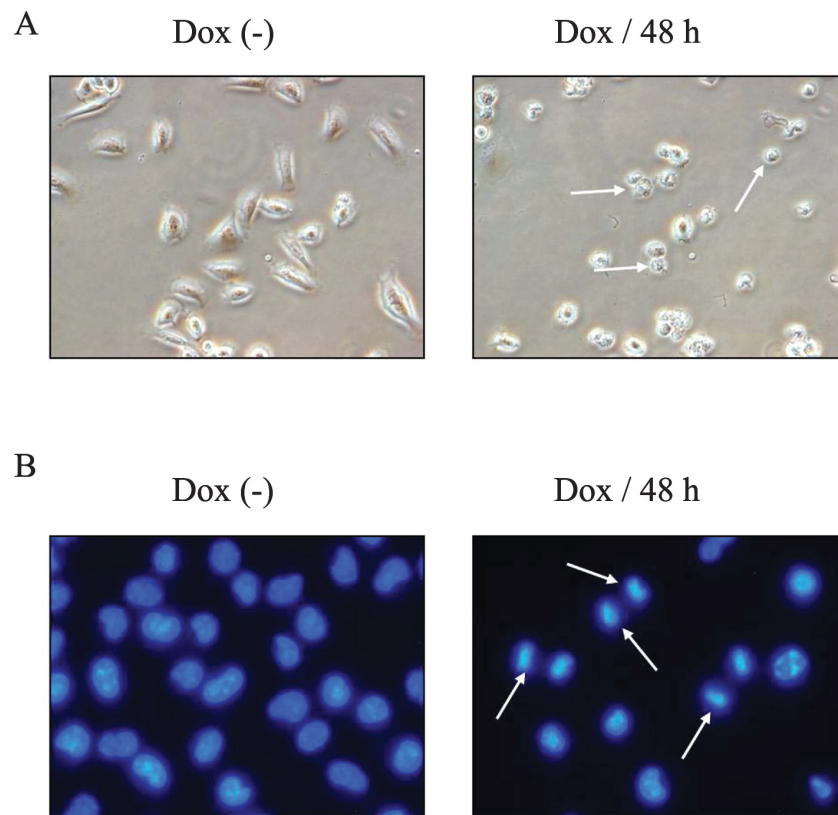


Figure 2. Cell morphological change of L/ind RCAS1. After 48 h of Dox induction, apoptotic cell morphological changes were observed. The white arrows indicate cell volume shrinkage and membrane blebbing (A) and chromatin condensation with DAPI staining (B) (x400).

The membranes were incubated with Blocking One buffer (Nacalai Tesque), followed by incubation with the following primary antibodies: mouse anti-RCAS1 (Oncogene Research Products, San Diego, CA, USA), rabbit anti-caspase-3 (Cell Signaling Technology, Beverly, MA, USA), rabbit anti-caspase-8 (AnaSpec, San Jose, CA, USA), mouse anti-cyclin D1, mouse anti-cyclin D2, mouse anti-cyclin E (Thermo Scientific), mouse anti-cyclin D3, mouse anti-cdk 2, mouse anti-cdk 4, mouse anti-p19^{INK4}, mouse anti-p27^{KIP1} (BD Transduction Laboratories, Tokyo, Japan). After washing, the membranes were incubated with peroxidase-conjugated goat anti-mouse IgG (γ -chain specific) or anti-rabbit IgG secondary antibodies. Immunoreactive proteins were visualized using an ECL Advance Western Blotting Detection kit (GE Healthcare Bio-Sciences, Tokyo, Japan). Protein content was normalized by reprobing the same membrane using a mouse anti- β -actin antibody as the primary antibody.

Detection of released cytochrome c. Cells treated with Dox were lysed with cytosolic fraction extract buffer (0.03% saponin, 250 mM sucrose, 20 mM HEPES, 5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4 containing protease inhibitor cocktails). After incubation on ice for 30 min, the cell lysates were centrifuged at 15000 rpm for 20 min at 4°C. Supernatants (cytosolic fraction) were collected and analyzed by Western blotting. For detection of cytochrome c, mouse anti-cytochrome c (BioLegend, San Diego, CA, USA) was used as the primary antibody, and peroxidase-conjugated goat anti-mouse IgG (γ -chain specific)

was used as the secondary antibody. β -actin and mitofilin were detected as cytosolic fraction and mitochondrial fraction markers, respectively.

Statistical analysis. All experiments were performed in triplicate and the results are represented as the mean \pm standard deviation (SD). Statistical significance was evaluated using a Student's t-test. A p-value of <0.05 was considered to indicate statistical significance.

Results

Expression of cell surface RCAS1 (22-1-1 antigen) and intracellular RCAS1 (i.c.RCAS1) by L/indRCAS1 cells. We examined cell surface RCAS1 (22-1-1 antigen) expression using flow cytometry. RCAS1 was detected after 6 h of Dox induction and 70.8% of L/indRCAS1 cells expressed RCAS1 on the cell surface after 48 h (Fig. 1A). Also, we analyzed the expression of intracellular RCAS1 (i.c.RCAS1) by Western blotting with a mouse anti-RCAS1 antibody, which recognizes the *rcas1* gene product, but not RCAS1 expressed on the cell surface. i.c.RCAS1 expression was seen 4 h after Dox induction and increased in a time-dependent manner until 12 h post-induction (Fig. 1B).

Cell growth inhibition and apoptosis induced by RCAS1 expression. We observed morphological changes in the L/ind RCAS1 cells consistent with apoptosis. Cell volume shrinkage and membrane blebbing were observed in the cells (Fig. 2A).

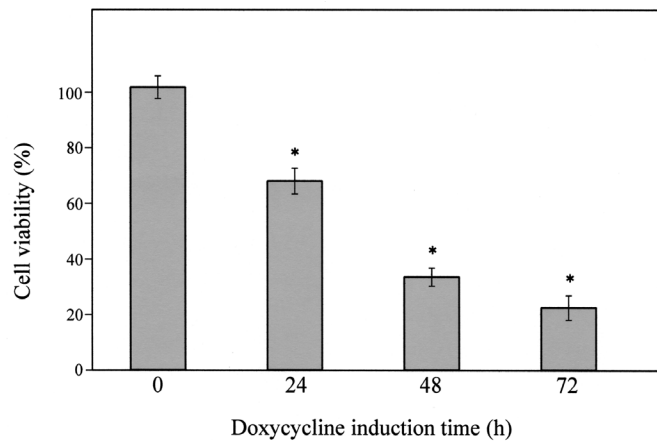


Figure 3. Cell viability. At each induction time with Dox, WST-1 assay was performed. A significant, time-dependent decrease in cell viability was detected ($66.80 \pm 3.92\%$, $32.97 \pm 0.55\%$ and $22.07 \pm 1.12\%$) at 24, 48 and 72 h post-induction, respectively. The results are presented as the mean \pm SD of three independent experiment. The asterisk indicates significant differences compared to the control ($p < 0.05$).

Moreover, chromatin condensation was detected by DAPI staining (Fig. 2B). A significant, time-dependent decrease in cell viability was also detected ($66.80 \pm 3.92\%$, $32.97 \pm 0.55\%$ and $22.07 \pm 1.12\%$) at 24, 48 and 72 h post-induction, respectively (Fig. 3).

Analysis of cytochrome c release and caspase-3 activation. In the mitochondrial apoptosis pathway, cytosolic cytochrome c is released from the mitochondria, and activation of caspases-3 and 9 is seen in apoptotic cells. Thus, we investigated cytochrome c release, and caspase-3 cleavage, in L/ind RCAS1 cells. Both cytochrome c release and caspase-3 cleavage were detected 12 h post-induction, and caspase-3 cleavage increased in a time-dependent manner (Fig. 4A and C). However, caspase-8 (associated with the death receptor pathway), was not activated (Fig. 4B).

Analysis of cell cycle-related proteins. RCAS1 induces cell cycle arrest in RCAS1-R-expressing cells. Thus, we analyzed the G1 phase-associated proteins, cyclin D1, D2, D3 and E, cdk 2, and 4, p19^{INK4} and p27^{KIP1} by Western blotting. Cyclin D3 expression decreased significantly 6 h post-induction, declining to about 40% of the control levels at 24 h (Fig. 5A). No change was seen in the expression levels of the other proteins (Fig. 5).

Discussion

Tumors are considered to be systemic diseases caused by over-proliferation of malignant cells driven by the interaction between the tumor tissue and the surrounding micro-environment. The immune system plays an important role in protecting an organism by detecting and eliminating tumor cells. It is known that IFN- γ receptor knockout mice have a high incidence of tumors (25). Moreover, the importance of antigen specific T cells in the eradication of tumors has been confirmed in animal models (26). These reports indicate the existence of immunological surveillance mechanisms operating

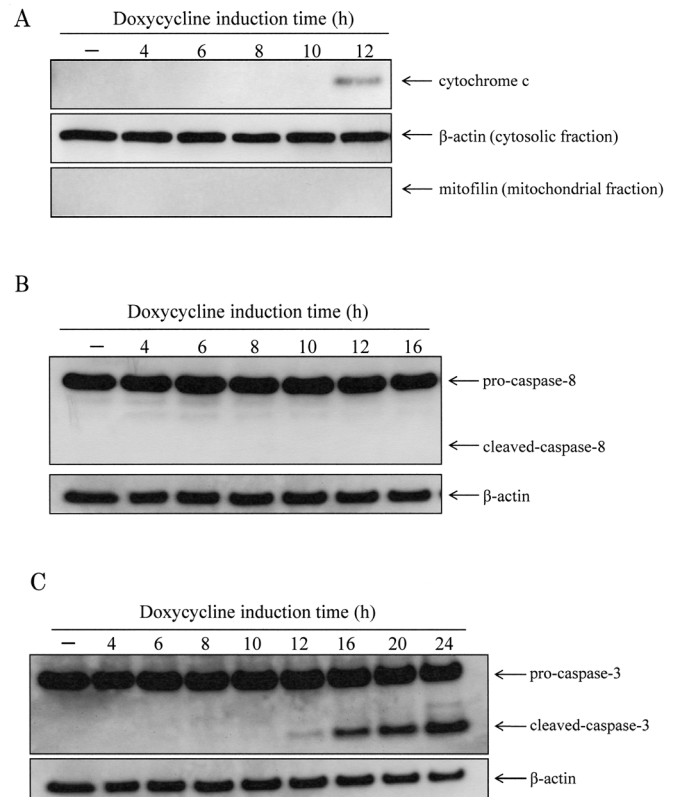


Figure 4. Analysis of apoptosis associated proteins by Western blotting. Cytosolic cytochrome c (associated with mitochondrial pathway) was detected at 12 h of Dox induction (A). Cleaved caspase-8 (associated with death receptor pathway) was not detected (B). Cleaved caspase-3 was detected at 12 h of Dox induction and increased time-dependently (C).

against tumors in the body. However, immune responses to tumors are not always complete, and tumor cells evade immune surveillance, survive and progress.

RCAS1 is a tumor-associated antigen expressed on the cell surface and in the cytoplasm of various cancer cells (3-13). RCAS1 acts as a ligand for a putative receptor (RCAS1-R), which is expressed on various human cell lines and induces cell growth-inhibition and apoptosis (2). Immune cells such as T, B and NK cells express RCAS1-R, especially when they are activated, and they induce cell growth-inhibition and apoptosis via RCAS1 (2). This phenomenon may be related to the mechanism of tumor evasion from the immunological surveillance, and it has been suggested that RCAS1 might play an important role in tumor progression, invasion and metastasis.

In this study, to investigate the mechanism of RCAS1-induced cell cycle arrest and apoptosis, we established a transformant in which *rcas1* expression was induced by doxycycline (L/ind RCAS1 cells). Here, we demonstrate that RCAS1 induced apoptosis is characterized by morphological changes, and chromatin condensation. In addition, we show that the viability of L/ind RCAS1 cells decreased significantly after *rcas1* induction. Various apoptotic stimuli are known to activate caspases, and signal transduction may occur by two distinct pathways; the death receptor pathway or the mitochondrial pathway. In the death receptor pathway, upon interaction with ligands such as FasL and tumor necrosis factor- α (TNF- α), death receptors recruit adaptor

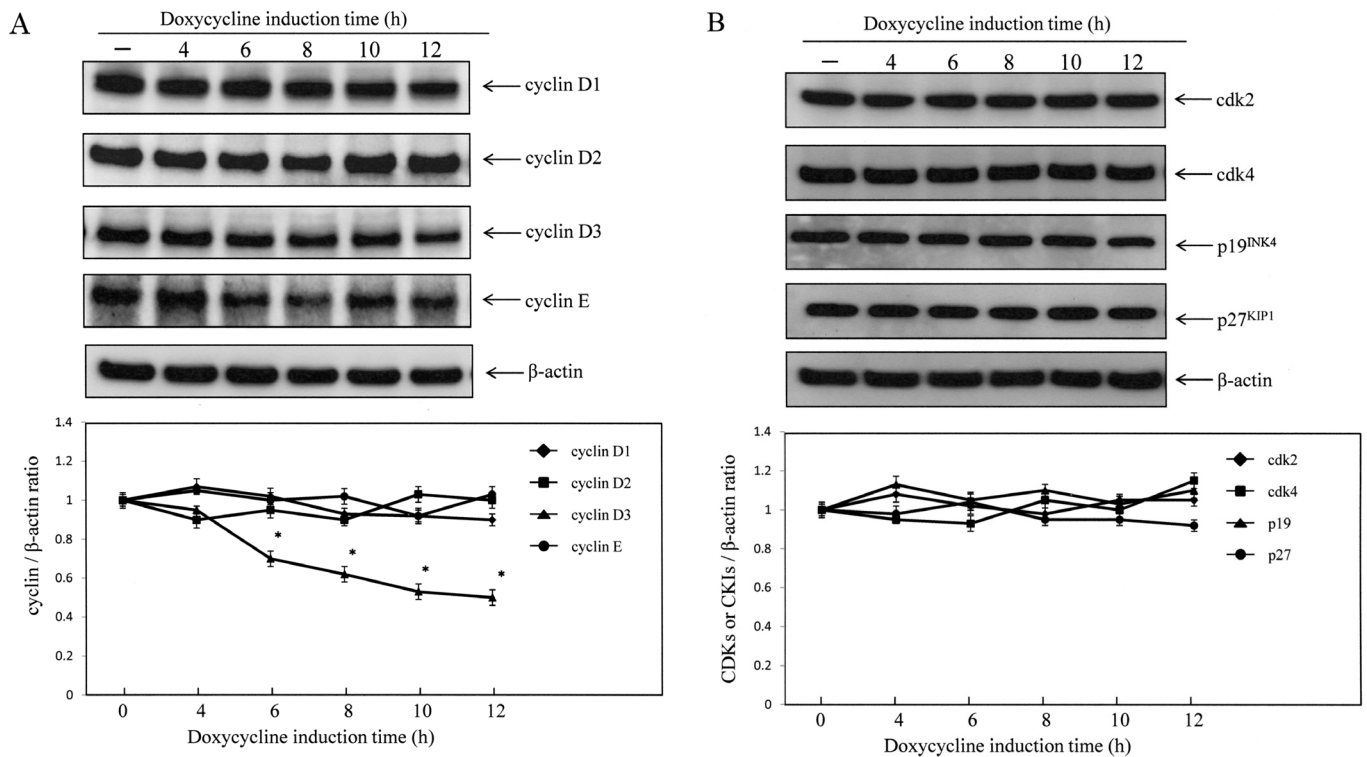


Figure 5. Analysis of cell cycle associated proteins by Western blotting. Cyclin D3 expression decreased significantly 6 h post-induction, falling to about 40% of the control levels at 24 h (A). No change was seen in the expression levels of the other proteins. The results are presented as the mean \pm SD of three independent experiment. The asterisk indicates significant differences compared to the control ($p < 0.05$).

proteins and activate caspase-8 (initiator caspase). Active caspase-8 then cleaves caspases-3, and 7 (effector caspases), which induce apoptosis. In the mitochondrial pathway, death signals stimulate mitochondria directly, resulting in the release of cytochrome c, which binds to an adaptor protein Apaf-1 and recruits initiator caspase-9. Active caspase-9 then activates caspases-3 and 7 to induce apoptosis. Therefore, distinct initiator caspases (caspase-8 or caspase-9) are activated via two different pathways, whereas the effector caspases are common to both pathways.

We performed further studies to elucidate the mechanism of apoptosis induced by RCAS1. Western blotting experiments showed that the release of cytochrome c from mitochondria into the cytosol and the subsequent activation of caspase-3 were detected 12 h after Dox induction. However, we found that caspase-8 was not activated by RCAS1 expression. It has been reported that RCAS1 induces loss of mitochondrial transmembrane potential in RCAS1-R-expressing cells (3), and that ZVAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl-ketone), a caspase inhibitor, strongly inhibits RCAS1-induced apoptosis (2). These results suggest that the apoptotic pathway activated by RCAS1 is likely to be through the mitochondrial pathway and not the death receptor pathway. RCAS1 also induces cell cycle arrest in RCAS1-R-expressing cells. Thus, to investigate the mechanism of cell cycle arrest induced by RCAS1, we assessed the expression levels of cell cycle related proteins. Cell cycle analysis revealed that cyclin D3 levels were decreased 6 h post-induction, declining to about 40% of the control levels after 24 h, while other cell cycle-related proteins such as cyclin D1, 2 and E, and CDKs and CKIs, did not decrease. Cyclin D3 is a member of the D-

type cyclin family, and plays a pivotal role in tightly controlling the progression from G1 to S phase of the cell cycle. After combining with CDKs 4 and 6, it phosphorylates the retinoblastoma (Rb) protein, eventually promoting the entry of the cell into S phase. Our study suggests that RCAS1-induced reductions in cyclin D3 levels in RCAS1-R-expressing cells, results in the inhibition of RB phosphorylation leading to cell cycle arrest. Also, we analyzed the cell cycle-associated proteins in another cell line, K562 (human chronic myelogenous leukemia), and confirmed that RCAS1 stimulation specifically reduces cyclin D3 levels (data not shown).

In conclusion, we show that RCAS1 inhibits cell proliferation by inducing G1 arrest via the specific down-regulation of cyclin D3, and subsequently induces apoptosis via the mitochondrial pathway. Dox-induced i.c.RCAS1 and RCAS1 expression was seen after 4 and 6 h, respectively, and a decrease in cyclin D3 levels was seen after 6 h. Thus, cyclin D3 levels decline immediately after induction of both i.c.RCAS1 and RCAS1 expression. These results suggest that cyclin D3 is one of the key molecules involved in the RCAS1-RCAS1-R signaling pathway. Future experiments will be conducted to identify the regulatory factors controlling cyclin D3 expression in the RCAS1-RCAS1-R signaling pathway at the mRNA level.

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