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Upregulation of Retinoblastoma Protein Phosphorylation in Gingiva after Cyclosporine A Treatment: An *in vivo* and *in vitro* Study

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RUNNING TITLE: CsA upregulates pRb phosphorylation in gingiva

KEY WORDS: gingiva, cyclosporine-A, retinoblastoma protein, phosphorylation

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ABSTRACT

Objective: To *in vivo* and *in vitro* examine the expression of genes and proteins associated with gingival cell proliferation after cyclosporine A (CsA) treatment.

Background: CsA can induce gingival cell proliferation; however, the precise molecular regulation of the proliferation is uncertain.

Materials and Methods: Forty Sprague Dawley rats with right maxillary posterior edentulous gingivae were assigned to a CsA group (30 mg/kg daily, administered orally) or a control group (mineral oil only). The animals were killed four weeks after treatment. The edentulous gingivae were dissected out and analyzed for the expression of proliferating cell nuclear antigen (PCNA), cyclin D1, cyclin-dependent kinase 4 (CDK4), and retinoblastoma protein (Rb1) mRNA and/or protein and phosphorylated Rb1 (pRb1) by real-time RT–PCR or immunohistochemistry. In human gingival fibroblast (HGF) cultures, the expression of PCNA, CDK4, cyclin D1, and Rb1 proteins and Rb1 phosphorylation were determined by western blotting after CsA treatment (0–10^4 ng/mL concentrations).

Results: Significantly greater expression of *Pcna* and cyclin D1 (Ccnd1) mRNAs was observed in the gingivae of CsA-treated animals than in those of the controls. More gingival cells were immunohistochemically positive for cyclin D1, CDK4, and pRb1 in the CsA group than in the control group. Increased expression of cyclin D1, CDK4, and PCNA proteins was observed in HGFs after CsA treatment. The phosphorylation of Rb1 was enhanced in HGFs after CsA treatment at concentrations of 10^2–10^3 ng/mL.

Conclusion: The increases in cyclin D1, PCNA, and CDK4, together with the enhanced phosphorylation of Rb1, suggest that CsA promotes cell cycle progression through the G_1/S transition in the gingiva.

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INTRODUCTION

Systemic cyclosporine A (CsA) administration induces gingival overgrowth, which is characterized by the overproduction of extracellular matrix components, a large number of fibroblasts, and epithelial thickening (1). The direct and indirect effects of the drug on gingival fibroblasts and their metabolism have been examined (2-4). CsA treatment can induce the proliferation in gingival fibroblasts (1, 3, 5-8), but the precise molecular regulation of CsA-stimulated gingival cell proliferation is not yet completely clear.

The progression of the cell cycle is governed by a family of cyclin-dependent kinases (CDKs) and their regulatory subunits, the cyclins (9-11). A key role of the CDKs is to inactivate, by phosphorylation, the negative regulators of progression, notably retinoblastoma protein (Rb1), to permit cell exit from G_1 and entry into S phase (12-13). Proliferating cell nuclear antigen (PCNA) is a protein that functions as an auxiliary protein for DNA polymerase d (14-15). PCNA is expressed at the beginning of late G_1 stage. Its expression increases 2–3-fold in S phase, and decreases at the S/G_2 transition and during G_2/M.

Studies have shown a precise correlation between the proliferative state of the cell and the cell cycle: increasing through G_1 phase, peaking at the G_1/S phase transition, decreasing through G_2 phase, and reaching low levels at the G_2/M transition and in M phase (16-17). Checkpoints are now known to exist at every single point in the cell cycle. The first checkpoint, called the “restriction point”, is located at the end of the G_1 phase of the cell cycle, just before entry into S phase. The restriction point is predominantly controlled by the action of the CDK inhibitor p16. This protein inhibits CDK4/6 and ensures that they can no longer interact with cyclin D1 to cause cell cycle progression. Once the CDK4/6–cyclin D complex is activated, it phosphorylates Rb1, **which results in its dissociation from E2F.** ~~which abolishes the inhibition of the~~

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~~transcription factor E2F.~~ E2F is then able to induce the expression of cyclin E, which interacts with CDK2 to allow the G_1/S phase transition(18). These cyclin–CDK complexes induce the phosphorylation of Rb1, and phosphorylated Rb1 (pRb1) promotes cell cycle progression through the G_1/S transition (19-20). Because the precise molecular regulation of CsA-stimulated gingival cell proliferation remains unclear, the expression of genes or proteins associated with its proliferation, such as PCNA, cyclin D1, CDK4, and Rb1, and the phosphorylation of Rb1, were investigated *in vivo* in a rat model and *in vitro* in primary cultured HGFs after their treatment with CsA.

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MATERIAL AND METHODS

In vivo experiment:

Forty male five-week-old Sprague Dawley rats, weighing 120–150 g, were randomly assigned to a CsA group or a control group after a three-week healing period after the extraction of all their right maxillary molars (24). Animals in the CsA group received CsA (30 mg/kg body weight in mineral oil; Sandimmun, Sandoz, Basel, Switzerland) daily by gastric feeding for four weeks, whereas the control group rats received mineral oil only. At the end of the study, all animals were killed with carbon dioxide inhalation. Ten edentulous gingival specimens from each group were immediately frozen in liquid nitrogen and stored at –70 °C for the real-time reverse transcription–polymerase chain reactions assay (real-time RT–PCR) of genes related to cell proliferation, including *Pcna* and *Ccnd1* paraffin embedding, serial tissue sections were cut buccopalatally at a thickness of 4 μm, and analyzed using immunohistochemistry (IHC) to evaluate the expression of cyclin D1, CDK4, and pRb1 proteins (Santa Cruz Biotechnology, Inc., Santa Cruz,CA, USA).

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Real-time RT–PCR assay:

Total RNA from homogenized gingival tissue was extracted with TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA, USA), and quantified by spectrophotometry at 260 nm. On a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA), 5 μg of each total RNA was reverse transcribed with SuperScript III at 55 °C for 1 h into total cDNA, which was used as the template for the subsequent PCR reactions and analysis. Confirmation of *Pcna* and *Ccnd1* gene expression was confirmed using the ABI Real-time PCR System (25). In brief, the desired probes and primers for rat *Pcna*, *Ccnd1*, and β-actin (*Actb*) were selected from TaqMan

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Assay-on-Demand gene expression. TaqMan PCR was conducted in triplicate with 50 μL reaction volumes of 1 × PCR buffer A, 2.5 mM $MgCl_2$, 0.4 μM each primer, 200 μM each dNTP, 100 nM probe, and 0.025 U/μL Taq Gold. Each primer/probe set (5–10 μL) was then added and the PCR was conducted with the following cycling parameters: 95 °C for 12 min × 1 cycle, and (95 °C for 20 s, 60 °C for 1 min) × 40 cycles. The data were analyzed with sequence detection software that calculates the threshold cycle for each reaction(26).

Immunohistochemistry:

After deparaffinization and hydration, the tissue sections were boiled in Dako buffer (Dako Denmark, Produktionsvej 42, Glostrup, Denmark) for 15 min for antigen retrieval. The endogenous peroxidase activity was quenched by incubation for 5 min with 0.1% hydrogen peroxide in distilled water. The sections were then incubated for 2 h with unconjugated primary polyclonal antibodies directed against CDK4, cyclin D1, or pRb1. This was followed by further incubation with biotinylated secondary antibody, streptavidin-conjugated horseradish peroxidase complexes, and 3-amino-9-ethyl carbazole solution for a further 30 min, 30 min, and 10 min, respectively. The specimens were then counterstained with hematoxylin, dehydrated, and mounted. The cells positively stained for CDK4, cyclin D1, and pRb1 were examined under a microscope. In this study, five consecutive tissue sections were selected from each study animal to measure and compare the percentages of positively IHC-stained cells in the gingival stroma in the control and CsA-treated rats (25, 27).

In vitro experiment:

As in our previous study, the connective tissue fragments of gingiva were digested for 24 h in medium containing 10% FBS and 2 mg/mL collagenase (Sigma-Aldrich Inc.,

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St. Louis, MO, USA) (27). The fragments were then placed in culture flasks to allow the cells to migrate from the explants, and the fibroblast cultures were maintained in 10% FBS in Dulbecco's modified Eagle's medium (DMEM)/F-12. After stimulation with various concentrations of CsA (including 1, 10, 10^2, 10^3, and 10^4 ng/mL) in dimethyl sulfoxide (DMSO, the solvent) for 24 h, the cells were harvested to determine their proliferation by the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA) and to evaluate the expression of PCNA, CDK4, cyclin D1, and Rb1 proteins and the phosphorylation of Rb1, by western blotting.

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Proliferation assay:

HGF cells were cultured in DMEM supplemented with 2 mmol/L L-glutamine and 10% fetal bovine serum (FBS). Cell viability was analyzed by MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI). In brief, HGF cells (3,000–10,000) were plated in each well of a 96-well tissue culture plate with 100 μL of growth medium. The cells reached 40–50% confluence 24 h after plating. The medium was then replaced with 100 μL of fresh medium containing different concentrations of CsA (10, 10^2, 10^3, or 10^4 ng/mL) or the control solvent-containing medium, and the cells were grown for 24 h. At the end of the treatment time, 20 μL of MTS solution was added to each well, the cells were incubated at 37 °C for 1–2 h, and the absorbance was read at 490 nm (28).

Western blotting:

Homogenates of primary cultured HGFs, lysed in lysis buffer, were centrifuged at 13,000 × *g* at 4 °C for 15 min, and boiled at 100 °C for 10 min. The protein concentrations were determined with a protein microassay using the BCA™ Protein

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Assay Reagent Kit (Pierce, Rockford, IL), and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 15% polyacrylamide gels and then electroblotted onto polyvinylidene difluoride membrane. Nonspecific binding was blocked by incubating the blots for 1 h in 5% FCS. After six washes in PBS-T (PBS containing 0.005% Tween 20), PCNA, CDK4, cyclin D1, Rb1, pRb1, and the internal control, α-tubulin, were detected by incubating them overnight with the appropriate primary antibody (29-30) at 4 °C and then with the secondary horseradish-peroxidase-conjugated goat anti-mouse or anti-rabbit IgG antibody, diluted 1:5,000, for 1 h. The antibody-reactive proteins were detected with enhanced chemiluminescence. The expression of Rb1, and pRb1 in the CsA-treated cells is presented as proportional increases or reductions relative to the levels observed in the control cells, calculated with densitometric analysis.

Statistical analysis:

Student's *t* test was used to evaluate the differences between the control group and the CsA group in the expression of the mRNAs (relative densities) for *Pcna* and *Ccnd1*, assessed by real-time RT–PCR, and the percentages of IHC-positive cells. One-way analysis of variance was used to evaluate the effect of CsA on cell proliferation in HGF cultures. $P < 0.05$ was selected as the level of significance.

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RESULTS

With real-time PCR, the relative intensities of the *Pcna* and *Ccnd1* mRNAs appeared significantly greater in the gingival specimens from CsA-treated animals than in those from the control rats (Figure 1). On average, the relative expression of the *Pcna* and *Ccnd1* mRNAs was 2.89-fold and 2.0-fold higher, respectively, in the CsA group than in the control group. IHC showed significantly more gingival cells positively stained for cyclin D1 and pRb1 in the CsA-treated group than in the control group (Figure 2). The mean number of CDK4-positive cells was higher in the CsA-treated group than in the control, but the difference was not statistically significant.

In the *in vitro* MTS assay, the optical density (OD) values were significantly higher for the cultures treated with 10^3 ng/mL CsA, but significantly lower for the cultures treated with 10^4 ng/mL CsA relative to the value for the DMSO-treated control (Figure 3). A dose-dependent increase in PCNA protein expression was observed with western blotting after the CsA treatments, and the expression of CDK4 and cyclin D1 was increased after CsA treatments in the dose range of $10–10^3$ ng/mL (Figure 4). Moreover, the phosphorylation of Rb1 was enhanced after treatment with CsA at doses in the range of $10^2–10^3$ ng/mL, but the levels of total Rb1 protein were similar after treatment with all doses of CsA (Figure 4).

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DISCUSSION

The effects of CsA on parameters related to the cell cycle and to the levels of proteins involved in the control and progression of the cell cycle were recently evaluated in hepatocyte cultures (23). An increase in the percentage of cells involved in the S phase of the cycle was observed, which correlated with increases in the levels of cyclins D1 and E, and PCNA, but with no modification to p27 expression (an inhibitory protein of CDKs) in cultured hepatocytes after exposure to CsA. In the present study, not only CDK4, PCNA, and cyclin D1, but also Rb1 and its phosphorylation (Ser780 and Ser807/811), which promotes the G_1/S transition in the gingival, were evaluated *in vivo* and *in vitro* after CsA treatment. Our *in vivo* data demonstrate that cyclin D1, CDK4, and pRb1 increased in the CsA group relative to their expression in the control group (Figure 2), whereas our *in vitro* results show increased expression for CDK4 and cyclin D1 and enhanced phosphorylation of Rb1 after CsA treatment (Figures 4). Moreover, significantly greater numbers of gingival fibroblasts were observed in the cultures treated with 10^3 ng/mL CsA compared with those in the cultures treated with DMSO (Figure 3). However, in this study, certain results were still inconsistent. For instance, CDK4, cyclin D1, and pRb1 increased in fibroblast cultures treated with 10^2 ng/mL CsA, but the expression of PCNA was greatest at a CsA concentration of 10^4 ng/mL. This may be attributable to the method used in the study to examine protein expression. For instance, CDK4, cyclin D1, and Rb1 regulate cell proliferation in the early stage of the cell cycle, but PCNA is present only in the late stage of cell proliferation, whereas the MTS assay measures the number of vital cell after cell proliferation is complete. Furthermore, the increased PCNA expression after treatment with 10^4 ng/mL CsA may be partly attributable to the accumulation of total protein present. To establish the actual explanations of these phenomena will require further detailed investigations.

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In conclusion, the upregulation of Rb1 phosphorylation in the gingiva during CsA treatment was demonstrated *in vivo* and *in vitro*. Because the expression of CDK4, cyclin D1, and PCNA was also enhanced, we suggest that CsA, at the appropriate concentrations, can cause gingival cells to enter the G_1/S phase transition and thence to proceed to the DNA synthesis phase, leading to cell proliferation. These results support the growth-promoting effects of CsA in gingival cells.

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Figure 1: Expression of *Ccnd1* and *Pcna* mRNAs relative to that of *Actb* mRNA in the gingivae of rats in the control and CsA groups (five rats in each group) assessed by real-time RT–PCR (means and standard deviations; *significantly different from the control group at $P < 0.01$).

Figure 2: The histomicrographs show cells positively IHC-stained for CDK4, cyclin D1, and pRb1 in the gingivae of the control (left column) and CSA-treated animals (right column; the scale bar represents 50 μm). The lower graph shows a comparison of the positively stained cells in the control and CsA-treated animal groups (* $P < 0.05$, means and standard deviations of five rats in each group).

Figure 3: Effects of CsA on cell proliferation in HGF cultures. Four CsA concentrations (10, 10^2, 10^3, and 10^4 ng/mL) and the DMSO (solvent) control were tested. Cell proliferation was measured by MTS assay and the experiments were repeated three times (mean and standard error; *significantly different from the DMSO control at $P < 0.05$ by *t* test).

Figure 4: Effects of CsA on the levels of the proliferation-associated proteins CDK4, cyclin D1, and PCNA (A) and the retinoblastoma proteins Rb1 and pRb1 (B and C) in HGFs. The fibroblasts were collected after DMSO or CsA treatments for 24 h, and the expression of the proteins was examined by western blotting. The ratios of pRb1 to Rb1 expression are summarized in graph C after normalization to α-tubulin (means and standard deviations). The experiments were repeated three times.

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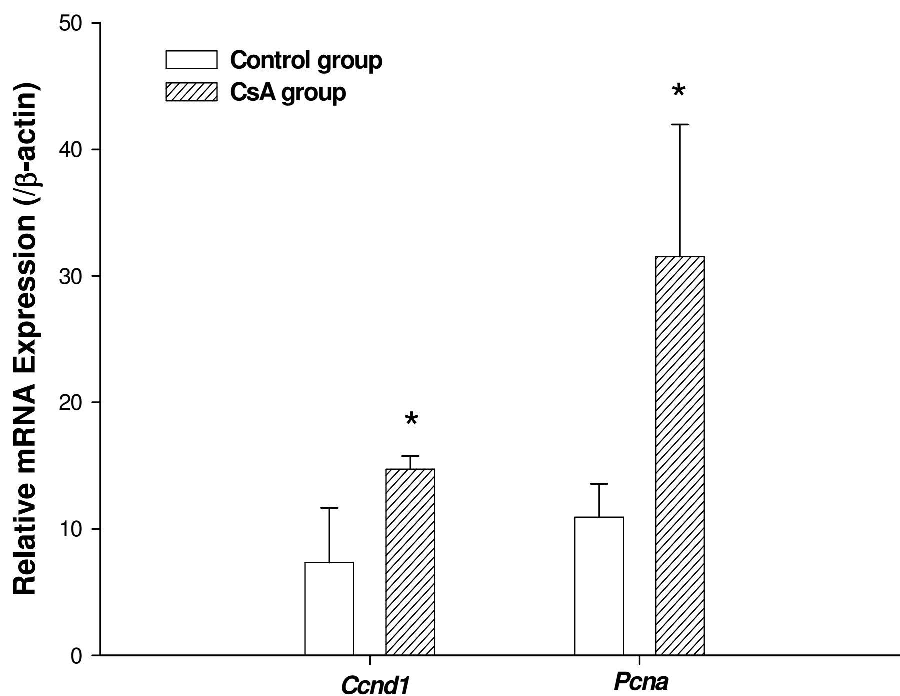

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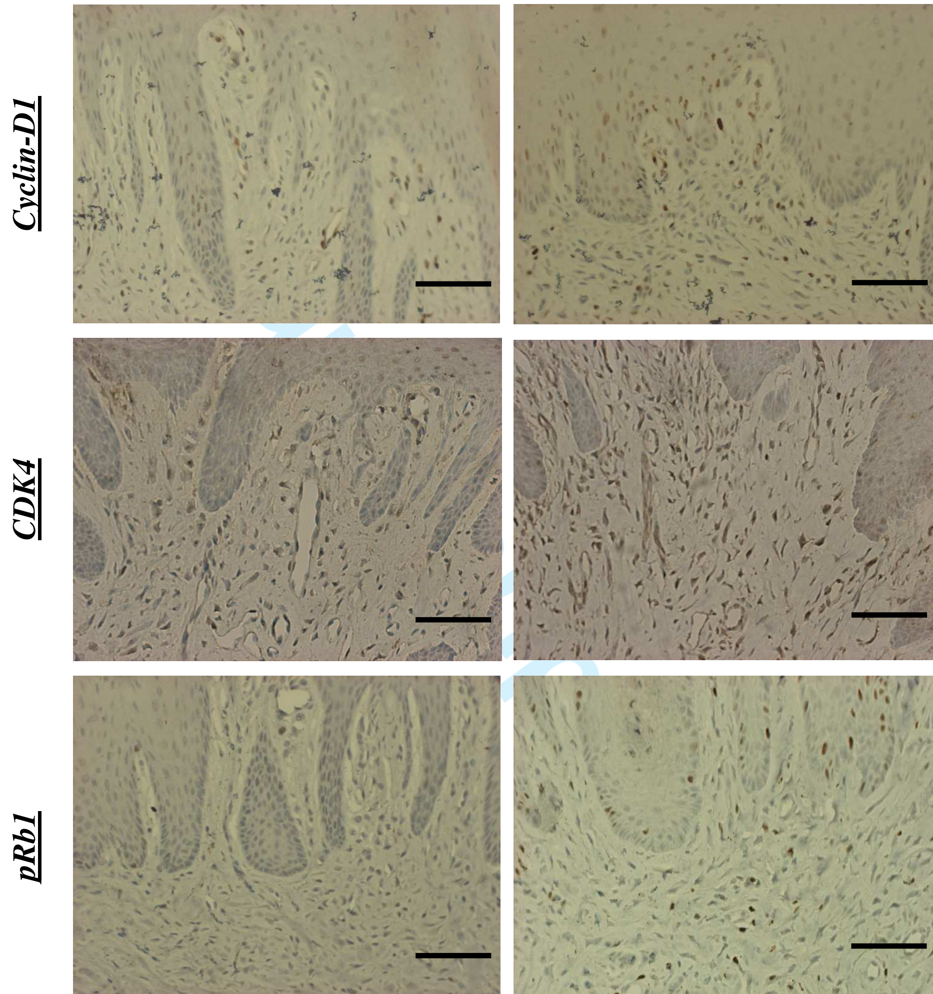

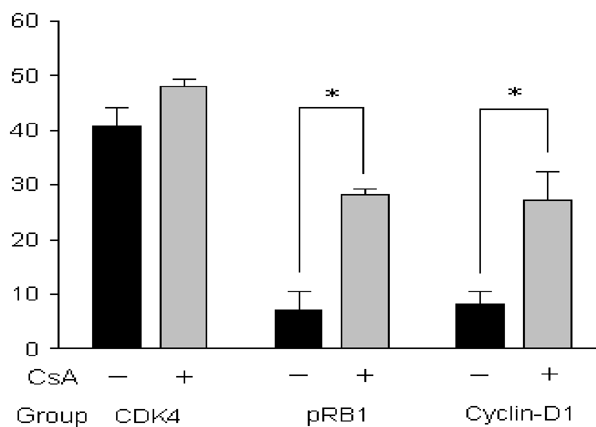

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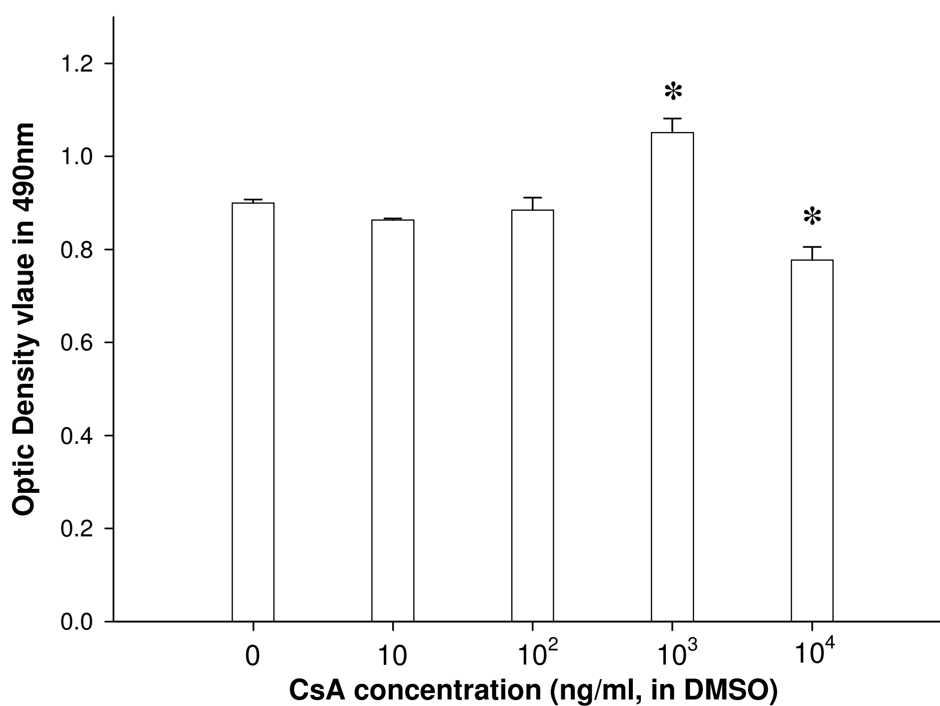

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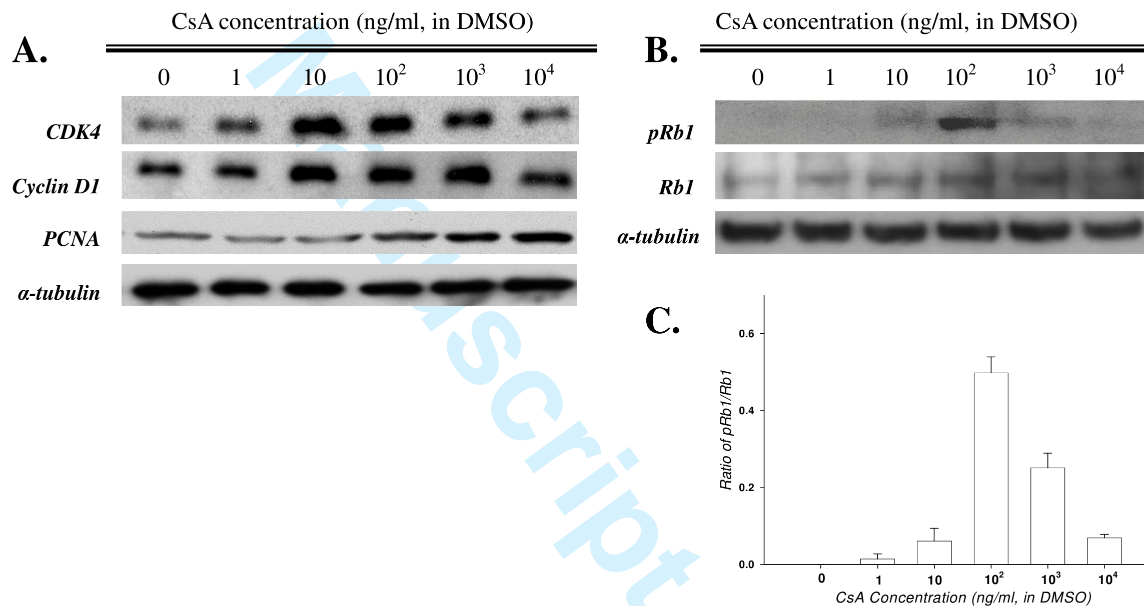

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