Downregulation of ERK1/2 activity by CaMKII modulates $p21^{Cip1}$ levels and survival of immortalized lymphocytes from Alzheimer's disease patients.

[1]Noemí Esteras, [1,6]Carolina Alquézar, [2,5]Félix Bermejo-Pareja, [3]Emilia Bialopiotrowicz, [3,4]Urszula Wojda, and [1,6]Ángeles Martín- Requero.

[1]Department of Cellular and Molecular Medicine. Centro de Investigaciones Biológicas (CSIC) Madrid, Spain [2]Hospital Doce de Octubre, Madrid, Spain, [3]Laboratory of Neurodegeneration, International Institute of Molecular and Cell Biology in Warsaw [4]Laboratory of Preclinical Testing of Higher Standard, Nencki Institute of Experimental Biology, Warsaw, Poland,[5]Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), [6]Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER) Spain.

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Address for correspondence:

Dr. Ángeles Martín-Requero
Centro de Investigaciones Biológicas (CSIC)
Ramiro de Maeztu 9
28040 Madrid, SPAIN
Phone: 34-91-837-3112
Fax: 34-91-536-0432
E-mail: amrequero@cib.csic.es

Abstract

Previously, we reported a Ca^{2+}/calmodulin (CaM)-dependent impairment of apoptosis induced by serum deprivation in Alzheimer's disease (AD) lymphoblasts. These cell lines showed downregulation of ERK1/2 activity and elevated content of p21 compared to control cells. The aim of this study was to delineate the molecular mechanism underlying the distinct regulation of p21 content in AD cells. Quantitative RT-PCR analysis demonstrated increased p21 mRNA levels in AD cells. The ERK1/2 inhibitor, PD98059, prevented death of control cells and enhanced p21 mRNA and protein levels. The CaM antagonist, CMZ, and the CaMKII inhibitor, KN-62, normalized the survival pattern of AD lymphoblasts by augmenting ERK1/2 activation and reducing p21 mRNA and protein levels. Upregulation of p21 transcription in AD cells appears to be the consequence of increased activity of FOXO3a as the result of diminished ERK1/2-mediated phosphorylation of this transcription factor, which in turn facilitates its nuclear accumulation. MDM2 protein levels were decreased in AD cells relative to control lymphoblasts, suggesting an impairment of FOXO3a degradation.

163 words

1. Introduction

Alzheimer's disease (AD) is a late-onset human neurodegenerative disorder marked by a progressive dementia and a spectrum of behavioral alterations. Whereas the hallmarks of AD, neurofibrillary tangles and amyloid-β containing plaques are well established, the cause of neuronal loss remains largely elusive. Cumulative evidence has associated the aberrant re-expression of some cell cycle regulatory proteins with neuron vulnerability and neurodegeneration in AD (McShea, et al., 2007, Webber, et al., 2005). Cell cycle re-entry appears to represent an early and critical event in AD, leading to the development of AD-related pathology such as hyperphosphorylation of tau and amyloid-β deposition and ultimately inducing neuronal cell death (Bonda, et al., 2010, Lee, et al., 2009). It is believed that certain neurons are able to reactivate cell-cycle activity in response to different triggers of neuronal apoptosis, including the withdrawal of growth factors (Park, et al., 1998) and other detrimental factors (Kruman, et al., 2002, Verdaguer, et al., 2002, Zhu, et al., 2007). Abnormal cell cycle re-entry leads to neuronal dead, however, in AD, the activation of neuronal cell cycle may result in apoptosis avoidance (Jellinger, 2006, Raina, et al., 2000), allowing the cells to arrest in G2, accumulating oxidative damage which in turn would induce its death according to the two-hit hypothesis (Moh, et al., 2011, Zhu, et al., 2004).

Cell cycle dysregulation is not restricted to neurons, since peripheral cells from AD patients such as lymphocytes or fibroblasts have been shown to display cell cycle-related alterations (Bialopiotrowicz, et al., 2011, de las Cuevas, et al., 2003, Nagy, et al., 2002, Stieler, et al., 2012, Tatebayashi, et al., 1995, Zhang, et al., 2003). It appears therefore that cell cycle disturbances represent a systemic aspect of the disease. While very limited data exist on the contribution of aberrant cell cycle in lymphocytes to the

clinical phenotype of AD, there is no doubt that peripheral cells from patients provide convenient material to study cell cycle-related events associated with neurodegeneration. Previous work from this laboratory indicated that immortalized lymphocytes from AD subjects show enhanced proliferative activity (de las Cuevas, et al., 2003, Muñoz, et al., 2008) and more resistance to serum withdrawal-induced apoptosis in a Ca^{2+}/CaM-dependent manner (Bartolome, et al., 2007, de las Cuevas, et al., 2005). It was also shown that both CaM content and activity were enhanced in AD lymphoblasts (Esteras, et al., 2012). Two cell cycle regulatory proteins, the CDK inhibitors $p27^{Kip1}$ and $p21^{Cip1}$, from now on p27 and p21, are ultimately responsible for the enhanced proliferation and increased resistance to cell death, respectively. Whereas downregulation of p27 induces the enhanced proliferative response of immortalized lymphocytes from AD patients (Muñoz, et al., 2008), upregulation of p21 seems to help AD cells to escape from serum deprivation-induced apoptosis (Bartolome, et al., 2010).

A number of recent studies pointed out that in addition to being an inhibitor of cell proliferation, p21 may protect cells from apoptosis (Gartel, 2009). For example, it has been reported that upregulation of p21 blocked the oxidative stress-induced death of human myeloma U266 cells (Kim, et al., 2001) and rendered resistance to chemotherapy drugs in other types of cancer cells (Gareau, et al., 2011). Thus the increase in p21 cellular content in AD lymphoblasts may confer these cells a survival advantage.

This work was undertaken to unravel the molecular mechanisms underlying the Ca^{2+}/CaM-mediated resistance of AD lymphoblasts to cell death induced by serum deprivation and the role of Ca^{2+}/CaM in the regulation of p21 cellular levels. We tested the hypothesis that enhanced Ca^{2+}/CaM signaling would protect AD cells from apoptosis via upregulation of p21. Our data suggest that CaMKII indirectly regulates the

FOXO3a-mediated activation of p21 transcription by preventing its phosphorylation mediated by ERK1/2 and subsequent translocation and degradation of this transcription factor via an MDM2-mediated ubiquitin-proteasome pathway.

2. Methods

2.1. Materials.

All components for cell culture were obtained from Invitrogen (Carlsbad, CA). Calmidazolium (CMZ), KN-62, pifithrin-α (PFT-α) and serum replacement were obtained from Sigma Aldrich (Alcobendas, Spain). LY294002 and PD98059 were obtained from Calbiochem (Darmstadt, Germany). PVDF (polyvinylidene difluoride) membranes for Western blots were purchased from Bio-Rad (Richmond, CA). Rabbit anti-human polyclonal antibodies, such as FOXO3a, ERK1/2, CaMKII, p53 and phospho-p53 (Ser 15) and rabbit anti-human monoclonal antibodies such as p21 and phospho-ERK1/2 were from Cell Signaling. Rabbit anti-human phospho-CaMKII (Thr286) (sc-12886-R), and mouse anti-human α-tubulin (sc-23948) were from Santa Cruz Biotechnologies (Santa Cruz, CA). Rabbit anti-human β-actin antibody was from Sigma. Rabbit anti-human MDM2 was from Millipore (Darmstadt, Germany). Mouse anti-human Lamin B was from Calbiochem. The enhanced chemiluminiscence (ECL) system was from Amersham (Uppsala, Sweden). All other reagents were of molecular grade.

2.2. Cell lines

A total of 34 patients diagnosed in the department of Neurology of the University Hospital Doce de Octubre (Madrid, Spain) of probable Alzheimer according to NINCDS-ADRDA (National Institute of Neurological and Communicative Diseases and Stroke-Alzheimer's Disease and Related Disorders Association) criteria were used in this study. Of the 34 patients, 20 had mild AD (DSM-III-R, Mini Mental State Examination (MMSE) score between 18-24), 7 had moderate AD (MMSE: 10-18), and 7 had severe AD (MMSE: <10). A group of 23 non-demented age-matched individuals was used as control. In all cases peripheral blood samples were obtained after written

informed consent of the patients or their relatives. A summary of demographic characteristics of all subjects enrolled in this study is reported in Table 1.

Establishment of lymphoblastic cell lines was performed in our laboratory as previously described by infecting peripheral blood lymphocytes with the Epstein-Barr virus (Koistinen, 1987). Cells were grown in suspension in T flasks in an upright position, in approximately 8 mL of RPMI-1640 (Gibco, BRL) medium that contained 2 mM L-glutamine, 100 μg/mL penicillin/streptomycin and, unless otherwise stated, 10 % (v/v) fetal bovine serum (FBS) and maintained in a humidified 5% CO_2 incubator at 37 ºC. Fluid was routinely changed every two days by removing the medium above the settled cells and replacing it with an equal volume of fresh medium.

2.3. Cell survival assay.

The cell suspension was mixed with a 0.4% (w/v) Trypan Blue solution (Sigma), and the number of live cells was determined using a hemocytometer. Cells failing to exclude the dye were considered nonviable. In addition, apoptosis was characterized by chromatin condensation/fragmentation, as determined by cell fixation followed by DAPI staining and fluorescence microscopy examination.

2.4 Determination of cell proliferation

Cell proliferation was assessed by the 5-bromo-2'-deoxyuridine (BrdU) incorporation method, using an enzyme-linked immunoassay kit procured from Roche (Madrid, Spain). Cells (100,000 cells/well) were seeded in 96-well microtiter plates. Four hours prior to the end of the interval of measurement, BrdU (10 μM) was added. The cells were fixed with precooled 70% ethanol for 30 min at -20 ºC and incubated with nucleases following manufacturer's recommendations. Cells were then treated for 30 min at 37 ºC with peroxidase-conjugated anti-BrdU antibody. The excess of antibody

was removed by washing the cells three times, followed by the addition of substrate solution. Absorbance was measured at 405 nm with a reference wavelength of 490 nm.

2.5. Preparation of whole-cell extracts and subcellular fractionation.

To prepare whole-cell extracts, cells were harvested, washed in PBS and then lysed in ice-cold buffer (50 mM Tris pH 7.4, 150 mM NaCl, 50 mM NaF, 1% Nonidet P-40), containing 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium pyrophosphate and protease inhibitor Complete Mini Mixture (Roche, Mannhein).

To separate the cytosolic and nuclear fractions, cells were harvested, washed in PBS and then lysed in ice-cold hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM ethylendiaminetetraacetic acid (EDTA), 0.1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM PMSF and protease inhibitor mixture. After extraction on ice for 15 min, 0.5% Nonidet P-40 was added and the lysed cells were centrifuged at 4000 rpm for 10 min. Supernatants containing cytosolic proteins were separated, and nuclei were washed twice with the hypotonic buffer, and then lysed in hypertonic buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM ethylendiaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM PMSF and protease inhibitor mixture. After extraction on ice for 30 min, the samples were centrifuged at 15000 rpm for 15 min at 4 ºC. Antibodies to α-tubulin and to lamin B were used to assess the purity of the fractions.

The protein content of the extracts was determined by the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA).

2.6. Western Blot Analyses

50-100 μg of protein from cell extracts were fractionated on a SDS polyacrylamide gel, and transferred to a PVDF membrane which was then blocked with 1-5% bovine serum

albumin (BSA) or 5% nonfat milk, and incubated overnight at 4ºC, with primary antibodies at the following dilutions: 1:1000 anti-p21, 1:1000 anti-FOXO3a, 1:2000 anti-phospho ERK1/2, 1:1000 anti-ERK1/2, 1:500 anti-pCaMKII, 1:1000 anti-CaMKII, 1:5000 anti-β-actin, 1:1000 anti-α-tubulin, 1:1000 anti-Lamin B, 1:1000 anti-phospho p53 (Ser 15), 1:1000 anti-p53 and 1:1000 anti-MDM2. Signals from the primary antibodies were amplified using species-specific antisera conjugated with horseradish peroxidase (Bio-Rad) and detected with a chemiluminiscent substrate detection system ECL (Amersham). The specificity of the antibodies used in this work was checked by omitting the primary antibodies in the incubation medium. Protein band densities were quantified using Image J software (NIH, Bethesda, Maryland, USA) after scanning the images with a GS-800 densitometer from BioRad.

2.7. Quantitative Real-Time PCR.

Total RNA was extracted from cell cultures using Trizol™ reagent (Invitrogen). RNA yields were quantified spectrophotometrically and RNA quality was checked by the A260/A280 ratio and on a 1.2% agarose gel to observe the integrity of 18S and 28S rRNA. RNA was then treated with DNase I Amplification Grade (Invitrogen). One microgram was reverse transcribed with the Superscript III Reverse Transcriptase kit (Invitrogen). Quantitative real-time PCR was performed in triplicates using TaqMan Universal PCR MasterMix No Amperase UNG (Applied Biosystems) reagent according to the manufacturer's protocol. Primers were designed using the Universal ProbeLibrary for Human (Roche Applied Science) and used at a final concentration of 20 μM. The sequences of the forward and reverse primers used are the following: for p21 5'-cgaagtcagttccttgtggag-3' and 5'-catgggttctgacggacat-3'; for β-actin 5'-ccaaccgcgagaagatga-3' and 5'-ccagaggcgtacagggatag-3'.

Real time quantitative PCR was performed in the BioRad iQ5 system using a thermal profile of an initial 5-min melting step at 95 ºC followed by 40 cycles at 95 ºC for 10 s and 60 ºC for 60 s.

Relative mRNA levels of the genes of interest were normalized to β-actin expression using the simplified comparative threshold cycle delta *CT* method [$2^{-(\Delta CT\,p21 - \Delta CT\,Actin}$].

2.8. Confocal laser scanning microscopy

Immortalized lymphocytes from control and AD individuals were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and incubated in serum-free RPMI medium for 24 hours. Cells were fixed for 15 minutes in 4% paraformaldehyde in PBS and blocked and permeabilized with 0.5% Triton X-100 in PBS-0.5% BSA for 20 minutes at room temperature. Then, cells were incubated with rabbit anti-p21 monoclonal antibody (Cell Signaling). After washing with PBS, cells were incubated with Alexa Fluor 488-conjugated anti-rabbit antibody. For nuclear staining, samples were incubated with DAPI (Sigma) at 4 mg/mL in PBS for 15 minutes at room temperature. The preparations were mounted on FluorSave™ Reagent (Calbiochem, Madrid, Spain) and visualized with the Leica TCS-SP2-AOBS confocal microscope system (Heidelberg, Germany).

2.9. Statistics

Statistical analyses were performed on GraphPad Prism 5 for Macintosh (La Jolla, CA, USA). All the statistical data are presented as mean ± standard error of the mean (SEM). Statistical significance was calculated using an unpaired, two-tailed Student's *t*-test or, when appropriated, by analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* testing. A value of $p<0.05$ was considered significant.

3. Results

3.1. CaM and ERK1/2 control of cell survival and p21 content.

Data in Fig. 1 confirm and extend our previous findings that lymphoblasts from AD patients show increased resistance to cell death induced by serum deprivation in a Ca^{2+}/CaM and ERK1/2 activity dependent manner (Bartolome, et al., 2007, Bartolome, et al., 2010). The cell response to serum deprivation was not dependent on disease's severity, the average percentage of cell survival after 72 h of serum deprivation was 98±2, 97±3 and 95±4 in lymphoblasts from mild (DSM-III-R, Mini Mental State Examination (MMSE) score between 18-24), moderate (MMSE: 10-18) or severe (MMSE: <10) AD patients, respectively. Further experiments were carried out with cell lines randomly selected among control individuals and the three groups of AD patients. The CaM antagonist, calmidazolium (CMZ) as well as the CaMKII inhibitor KN-62, rescued the normal cell response to serum deprivation, inducing apoptosis in AD cells, suggesting the involvement of CaMKII in the regulation of cell survival. The fact that these drugs didn't affect the survival of control cells suggests a threshold for CaM content/activity as the survival signal. On the other hand, the inhibitor of ERK1/2, PD98059, prevented the serum deprivation-induced apoptosis in control cells without significantly changing survival of AD lymphoblasts (Fig. 1B). To examine the apoptotic status of control and AD lymphoblasts after serum deprivation, cells were observed in a fluorescence microscopy following DAPI staining, which specifically binds to DNA. As shown in Fig. 1, the addition of CMZ or KN-62 to AD lymphoblasts induced chromatin condensation in some nuclei while PD98059 prevented the serum deprivation-induced apoptosis in control cells (Fig. 1C). Taken together, these observations suggest a crosstalk between CaMKII and ERK1/2 in regulating the cellular response to serum deprivation. An inverse relationship was found between CaMKII and ERK1/2 activities,

as well as between ERK1/2 phosphorylation status and p21 levels in human lymphoblasts. As shown in Fig. 1D, AD lymphoblasts displayed higher activity of CaMKII, as monitored by the enhanced autophosphorylation, together with decreased ERK1/2 activity, when compared with control cells. In parallel, levels of p21 increased in agreement with previous reports (Bartolome, et al., 2010). Moreover, as it was the case for changes in other cell cycle-related events reported in AD lymphoblasts (Bartolome, et al., 2009), we did not observe significant differences in p21 content among mild, moderate or severe AD patients (results not shown). Inhibition of CaMKII with CMZ treatment in AD cells restored the activity of ERK1/2 and diminished the p21 cellular content up to the levels observed in control cells. Under these conditions, AD cells underwent apoptosis as control cells did. Thus, it appears that CaM/CAMKII/ERK1/2 signaling pathway controls cell survival under trophic factor deprivation by regulating p21 levels.

To rule out that differences in p21 content could induce changes in the proliferative activity that could mask the distinct survival response of AD lymphoblasts, we carried out a comparative analysis of BrdU incorporation into DNA in control and AD cells after the treatment with the CaMKII or ERK1/2 inhibitors. As shown in Table 2, no differences were found in cell proliferation between control and AD lymphoblasts in the absence or in the presence of these inhibitors. Thus, under serum deprivation, the increased p21 content has a predominant anti-apoptotic role.

3.2. Regulation of p21 levels

To elucidate the molecular basis for the upregulation of p21 in AD lymphoblasts, we tested whether this effect was dependent on altered protein degradation. To this end, we evaluated the stability of the p21 protein after serum-deprivation, by treating control and AD cells with 20 μg/mL of cycloheximide to inhibit *de novo* protein synthesis. At the

indicated time, steady-state levels of p21 protein were determined by immunoblotting (Fig. 2). As in other cell types, p21 is a short-lived protein in human lymphoblasts. Inhibition of protein synthesis with cycloheximide led to a rapid decrease in p21 levels, with less than 50% remaining after 2 hours. It is shown that p21 half-life is identical in both control and AD lymphoblasts. We next determined, by quantitative RT-PCR, p21 mRNA levels. As shown in Fig. 3, lymphoblasts from AD patients show an increase in expression of p21 mRNA as compared with control cells, in consonance with the different content of this protein in control and AD lymphoblasts. Inhibition of CaMKII by CMZ or KN-62 abrogated the increased transcription of p21 in AD cells, while inhibiting the PI3K/Akt pathway by Ly294002 had no consequences in p21 mRNA levels (Fig. 3). On the contrary, the ERK1/2 inhibitor PD98059 was able to increase p21 mRNA levels in control cells without affecting the transcription of the gene in AD lymphoblasts. Under these conditions, and in agreement with previous reports (Bartolome, et al., 2010), control cells were found to be resistant to cell death induced by serum deprivation (Fig. 1C). Taken together, these results suggest that CaMKII regulates p21 mRNA expression levels by downregulating the activity of ERK1/2.

3.3. Regulation of p21 transcription

Transcriptional activation of p21 gene is regulated though p53-dependent and -independent mechanisms. We first tested whether the transcriptional activation of the p21 gene was dependent on p53 activity. For this purpose, we analyzed by Western Blot phospho-p53 at Ser 15 together with total p53 and p21 levels in the absence and in the presence of the p53 inhibitor pifithrin-α (PFT-α) (Zhu, et al., 2002b), and analyzed the influence of this inhibitor on cell survival after 72 h of serum withdrawal. Neither total nor phosphorylated (active form (Siliciano, et al., 1997)) p53 levels changed in AD lymphoblasts when compared with control cells despite the increased content of p21 in

AD cells (Fig. 4A). Treatment with PFT-α effectively decreased the levels of phospho-p53 and total p53, but had little effect on p21 levels (Fig. 4A) and didn't overcome the resistance of AD cells to death induced by serum withdrawal (Fig. 4B).

We next focused on p53-independent mechanisms of p21 transcriptional activation. The transcription factor FOXO3a has been shown to mediate the transcriptional transactivation of p21 gene in some type of cells (Hauck, et al., 2007), and it has also been reported that sustained ERK activation results in FOXO3a downregulation (Yang, et al., 2008). Levels of FOXO3a were first determined in control and AD lymphoblasts. As observed in Fig. 5A, AD cells show a higher content of FOXO3a than control cells. FOXO3a is known to be phosphorylated at Ser 294, Ser 344 and Ser 425 by ERK1/2 (Yang, et al., 2008). Phosphorylation of the molecule is required for the export of the transcription factor from the nucleus to be degraded (Obsil and Obsilova, 2008). We could not find a suitable phospho-specific antibody to recognize phosphorylated FOXO3a at these residues, to assess possible differences in phosphorylation status of FOXO3a between control and AD lymphoblasts as the result of their intrinsic differences in ERK1/2 activity (see Fig.1D). Thus, to address this issue, we performed subcellular fractionation experiments to determine whether there is a distinct distribution of FOXO3a in AD cells. We found a preferentially nuclear localization of FOXO3a in the nucleus, which is significantly enhanced in AD lymphoblasts (Fig. 5B). We did not detect significant changes in the cytosolic content between control and AD cells (Fig. 5), most likely due to possible differences in the rate of cytosolic degradation of this transcription factor in control or AD lymphoblasts. We then aimed at elucidating whether inhibiting ERK1/2 activation by PD98059 or alternatively increasing ERK1/2 phosphorylation with the CaM antagonist CMZ or the CaMKII inhibitor KN-62 impacts the nuclear accumulation of FOXO3a. First of all, Fig. 6A shows the efficacy of these

drugs modulating the activity of ERK1/2. Then, subcellular fractionation was performed to assess the nuclear content of FOXO3a under the different experimental conditions. Fig. 6B shows that PD98059 treatment effectively increased the nuclear levels of FOXO3a in control cells, while increasing the activation of ERK1/2 by inhibiting CaMKII by CMZ or KN-62 abrogated the enhanced nuclear levels of AD cells to levels similar to those of control cells (Fig. 6C).

After being exported from the nucleus, phosphorylated FOXO3a is then degraded in the cytosol via an MDM2-mediated ubiquitin-proteasome pathway (Yang, et al., 2008). Moreover, ERK1/2 has been shown to upregulate MDM2 protein expression levels (Phelps, et al., 2005, Ries, et al., 2000). On these grounds, we were interested at elucidating whether control and AD lymphoblasts display distinct MDM2 levels. As shown in Fig. 7, we observed higher levels of MDM2 and ERK1/2 activation in control lymphoblasts when compared with lymphoblasts from AD patients. On the other hand, the inhibition of ERK1/2 activity decreased significantly the cellular content of MDM2 protein (Fig. 7). Thus, it seems that MDM2 may function as the ubiquitin ligase for FOXO3a proteasome degradation in human lymphoblasts. Taken together the results suggest that downregulation of ERK1/2-mediated phosphorylation of FOXO3a in AD lymphoblasts may compromise its transport from the nucleus and its interaction with MDM2, to reduce the degradation of the molecule. The non-phosphorylated FOXO3a accumulated in more extent in the nucleus of AD lymphoblasts, thereby promoting the enhanced transcription of p21.

3.4. Intracellular localization of p21.

The p21 protein has been considered as a paradigm of protein showing completely different nuclear and cytosolic functions (Agell, et al., 2006, Coqueret, 2003). Therefore, we decided to examine whether there are differences in the subcellular localization of

p21 in control and AD lymphoblasts. For this purpose, we performed nuclear and cytoplasmic fractionation and analyzed the protein extracts by Western Blot. As shown in Fig. 8, p21 is accumulated in the cytoplasm rather than in nucleus, both in control and AD cells. Nuclear and cytoplasmic content of p21 are higher in AD lymphoblasts in consonance with the increased mRNA p21 levels. Similar results were obtained when cells were immunostained and processed for confocal laser imaging, as shown in Fig. 8.

4. Discusion

In AD lymphoblasts, the constitutive activation of Ca^{2+}/CaM-dependent signaling pathways enhances cell proliferation and survival. Among them, the PI3K/Akt and ERK1/2 seem to play important roles in controlling cell fate depending on growth factor availability (de las Cuevas, et al., 2010). These tumor-like features of lymphoblasts from AD patients were considered as systemic manifestations of the proposed relationship between cellular stress and unscheduled cell cycle entry observed in susceptible neurons in AD (Zhu, et al., 2004). While the CaM-dependent overactivation of PI3K/Akt regulates cell proliferation by enhancing the rate of p27 degradation (Muñoz, et al., 2008), here we report that ERK1/2 activation following serum deprivation is negatively regulated by CaMKII in AD cells, leading to an increase in FOXO3a activity, thereby enhancing the transcription of p21 and the survival of lymphoblasts from AD patients. Several lines of evidence support this assumption; first, CaMKII and ERK1/2 activation are inversely correlated in immortalized lymphocytes, CaMKII activity is increased in AD cells together with decreased ERK1/2 phosphorylation in the absence of serum; second, CaMKII inhibition restored ERK1/2 phosphorylation and decreased the levels of p21 in AD cells, thus sensitizing AD cells to apoptosis; and third, inhibition of ERK1/2 by PD98059 prevented the serum deprivation-induced death in control cells by increasing the cellular content of FOXO3a and the levels of p21.

FOXO3a is a member of the class O subgroup of the forkhead transcription factor family, which is expressed in many different tissues and involved in mediating the effects of growth factors on several cellular processes including cell cycle progression, glucose metabolism, and apoptosis (Accili and Arden, 2004, Greer and Brunet, 2008, Kops, et al., 2002). The activity of FOXO3a as a transcriptional regulator is primarily,

although not exclusively, controlled by its subcellular localization (Vogt, et al., 2005). In the absence of growth or neurotrophic factors, FOXO3a may localize to the cell nucleus, causing transcriptional induction of a variety of genes (Modur, et al., 2002). Our results indicate that the cellular content of FOXO3a is enhanced in serum-deprived AD lymphoblasts. Under these conditions, FOXO3a accumulated mainly in the nucleus, inducing the transcription of p21 and favoring the cell resistance to serum deprivation. FOXO3a levels appear to be controlled by CaM/CaMKII/ERK1/2-mediated phosphorylation, and subsequent degradation of the molecule in the cytosolic compartment by an MDM2-mediated ubiquitin-proteasome pathway as suggested by the observed decrease in the cellular content of MDM2 protein in AD lymphoblasts.

Experimental evidence supports the idea that important oncogenic kinases such as PI3K/AKT, IKK and ERK1/2 are involved in regulating FOXO3a nuclear localization and activity through phosphorylation at different sites (Hu, et al., 2004, Plas and Thompson, 2003, Yang, et al., 2008). However, in human lymphoblasts, inhibition of PI3K/Akt has no consequences in the transcription of p21 (this manuscript) or in p21 levels and cell survival (Bartolome, et al., 2010). Our data indicate that inhibiting ERK1/2 activity by PD98059 might prevent phosphorylation of FOXO3a, allowing the nuclear accumulation of FOXO3a, increasing its transcriptional activity and blocking the serum withdrawal-induced death in control lymphoblasts.

In addition to the increased transcriptional activation of p21 gene observed in AD lymphoblasts, we also found an increase in the cytosolic content of p21 of AD cells. The cytoplasmic p21 is thought to be a positive modulator of cell survival (Blagosklonny, 2002, Coqueret, 2003).

On the other hand, we did not observe changes in the rate of p21 degradation following serum deprivation. This observation is in contrast with the previously reported increase

in p21 degradation in AD lymphoblasts under proliferating conditions (Sala, et al., 2008). Thus, it seems that serum stimulation or withdrawal induced opposite changes in p21 cellular content by affecting protein stability or transcription respectively.

The increased levels of p21 in AD lymphoblasts associated with enhanced resistance to death induced by serum deprivation reported here, are in line with the observation that upregulation of p21 blocked the oxidative stress-induced death of human myeloma U266 cells (Kim, et al., 2001) and rendered resistance to chemotherapy drugs in other types of cancer cells (Gareau, et al., 2011). Thus the increase in p21 cellular content in AD lymphoblasts may confer these cells a survival advantage similar to that described for cancer cells (Weiss, 2003). Increased levels of p21 may protect cells from apoptosis through different mechanisms: p21 can interact and inhibit the activity of caspases (Roninson, 2002), it can also bind to E2F1 and MYC preventing their transcriptional induction of pro-apoptotic proteins (Abbas and Dutta, 2009). On the other hand, p21 can mediate the upregulation of anti-apoptotic proteins (Dotto, 2000). Interestingly, upregulation of p21 has also been associated with blockade of oxidative stress-induced apoptosis in fibroblasts from AD patients (Naderi, et al., 2006). In this sense, it is worth to note that a selective impairment of mechanisms involved in cell death has been previously reported in peripheral cells from AD patients (Eckert, et al., 2001, Morocz, et al., 2002, Naderi, et al., 2006, Uberti, et al., 2002) although there are conflicting results as to whether cells from AD patients are more resistant or more vulnerable to situations that promote cell death. Most likely these discrepancies result from the different cell types and stress-inducing conditions used.

AD cells treated with the CaM antagonist CMZ or the CaMKII inhibitor KN-62 undergo significant apoptosis in the absence of serum in the culture medium as they do control cells. The effects of these compounds are accompanied by activation of ERK1/2

together with decreased levels of FOXO3a and p21. Inactivation of CaMKII has no effects in control cells suggesting a threshold for CaM activation as the survival signal. Our results are in consonance with previous reports indicating that CaM antagonists specifically resulted in apoptosis in tumorigenic mammary carcinoma cells, but did not affect normal mammary gland-derived epithelial cells (Deb, et al., 2004).

CaM/CaMKII downregulates ERK1/2 activity in human lymphoblasts by a mechanism not yet completely defined. Several mechanisms for CaM/CaMKII modulation of ERK1/2 have been proposed. For example, it was shown in thyroid cancer cells that CaMKII associates and phosphorylates Raf-1, contributing to ERK activation (Illario, et al., 2003). On the other hand, it was reported that CaM negatively regulates the Ras/Raf/MEK/ERK pathway in fibroblasts (Villalonga, et al., 2001). CaMKII can activate in vitro synGAP (Oh, et al., 2004), a Ras inhibitory GTPase expressed in neurons (Kim, et al., 1998), which may inhibit ERK activation. Recently, it has been shown that CaM may prevent the phosphorylation of K-Ras by different kinases and that inactivation of CaM leads to K-Ras activation (Alvarez-Moya, et al., 2011). Apparently, both the C-terminal polylysine region and the farnesylation of K-Ras are important for its specific interaction with CaM (Wu, et al., 2011). Whether there are similar mechanisms operative in human lymphocytes and impaired in AD patients will need further investigation.

In addition to the indirect effect of CaM/CaMKII on cellular content of FOXO3a levels, by preventing its phosphorylation by ERK1/2, and favoring thereafter the transcription of p21, one must consider the possibility that these genes belong to the recently described gene pool, apparently important for adaptive processes, whose expression is highly sensitive to nuclear Ca^{2+}/CaM signaling (Zhang, et al., 2009).

Impaired CaMKII activation has also been detected in AD brain, associated with

increased phosphorylation of tau and neurofibrillary tangle formation (McKee, et al., 1990). Similarly, abnormal ERK1/2 activation has been found in degenerating neurons (Zhu, et al., 2002a). ERK1/2 pathway appears to be actively involved in the pathogenesis of AD, by altering hippocampal synaptic plasticity, and contributing to memory deficit (Derkinderen, et al., 1999). On the other hand, increased FOXO3a activity has been found in the brain of the Tg2576 mouse model of AD compared with wild-type mice (Qin, et al., 2008) correlating with increased Aβ1-40 and Aβ1-42 peptide contents. It is also worth mentioning that overexpressed p21 has also been detected in the frontal cortex of AD brains (Engidawork, et al., 2001). Thus, our results obtained in peripheral cells from AD patients demonstrate that dysfunction of CaMKII/ERK1/2/FOXO3a signaling is not restricted to the brain, but rather represents a systemic aspect of the disease. These findings also suggest that changes in B-lymphocytes may contribute to the AD-specific phenotype, in agreement with previous reports highlighting the role of alterations in cells of the immune system in AD pathogenesis (Prinz, et al., 2011). While most of the studies on AD lymphocytes have been focused in detecting disease-specific changes that may serve as biomarkers, it remains to be established the clinical consequences, if any, of the altered response of lymphocytes in AD patients.

In summary, we have provided evidence that in the absence of serum, the survival of AD lymphoblasts depends on the CaMKII-induced downregulation of ERK1/2 activity, leading to increased accumulation of FOXO3a in the nucleus, and therefore to enhanced transcription of p21. The proposed scenario is represented schematically in Fig. 9. Our data indicate that the direct inverse coupling of CaMKII and ERK1/2 and ERK and FOXO3a activity function as key switches in controlling cell fate survival or death depending on growth factors availability. Finally, increased resistance of cells to death

induced by serum deprivation and elevated p21 levels appear to be early features in AD pathogenesis, since similar changes were observed in mild, moderate or severe AD patients.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Table 1

Summary of study population

		Control n=23	AD n=34
Age		75±1	76±5
Age range		(60-83)	(59-89)
Gender	Male	8	15
	Female	15	19

Control, healthy control individuals, no sign of neurological disease; AD, patients with a diagnosis of probable AD.

Table 2

BrdU incorporation into DNA of human lymphoblasts

Treatment	Absorbance 405/490 nm	
	Control	AD
None	0.111 ± 0.03	0.115 ± 0.01
CMZ 1 μM	0.114 ± 0.03	0.110 ± 0.005
KN-62 1 μM	0.108 ± 0.04	0.102 ± 0.06
PD98059 20 μM	0.124 ± 0.03	0.124 ± 0.02

Immortalized lymphocytes from control and AD patients (10^5 cells/well) were seeded in 96-well plates in RPMI medium containing 10% Serum Replacement (SR). After 24 h of serum deprivation, cells were pulsed with 10 μM BrdU for 4 h. DNA synthesis was assessed by BrdU incorporation method according to manufacturer's instructions. Values shown are the mean ± SEM of three different cell lines in each condition performed in triplicate.

Legends to the Figures

Figure 1

Survival of control and AD lymphoblasts in serum-deprived medium. Influence of CaMKII and ERK1/2 on cell survival and p21 content.

A. Scatter plot comparing cell survival following serum deprivation between lymphoblasts derived from control or Alzheimer's disease (AD) patients. Immortalized lymphocytes from control (open symbols) and AD individuals (filled symbols) were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and incubated in serum-free RPMI medium for 72 hours. Cell viability was determined by trypan blue exclusion under inverted phase-contrast microscopy. Statistical significance was determined by the Student's *t*-test. B. Percentage of surviving cells, measured as above, after treatment with 1 μM calmidazolium (CMZ), 1 μM KN-62 or 20 μM PD98059. Experimental conditions are identical to those described in A. Data shown are the mean ± SEM of six different experiments carried out with different cell lines, and expressed as percentage of the initial number of cells at day 0. *$p<0.05$ significantly different from untreated control cells. +$p<0.05$ significantly different from untreated AD lymphoblasts. C. Representative photomicrograph of DAPI-stained lymphoblasts following 72 hours of serum deprivation in the absence or in the presence of 1 μM CMZ, 1 μM KN-62 or 20 μM PD98059. Arrows show the presence of chromatin condensation/fragmentation indicating apoptosis. No signs of apoptosis were detected in cells treated with PD98059. Treatment of AD cells with CMZ or KN-62, sensitized AD lymphoblasts to death induced by serum deprivation. D. Effects of CMZ on CaMKII and ERK1/2 activities and p21 content in control and AD lymphoblasts. Representative immunoblots are shown. Densitometric analyses of phosphorylated CaMKII and p21 content represent

the mean ± SEM of four independent observations carried out with different cell lines. *p<0.05 significantly different from control cells. +p<0.05 significantly different from untreated AD lymphoblasts.

Figure 2

Half-life of p21 in control and AD lymphoblasts.

Lymphoblasts from control and AD subjects were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and incubated in the presence of cycloheximide (20 μg/mL) in serum-free RPMI medium. Cells were harvested 1, 3, and 6 hours thereafter and p21 was detected by immunoblotting. Blots from a representative experiment are shown. The decay of the p21 signal was graphed as a function of time post-cycloheximide addition, using data from different experiments carried out with of cell lines derived from six control and six AD subjects.

Figure 3

Relative p21 mRNA abundance in control and AD lymphoblasts.

Immortalized lymphoblasts from control and AD individuals were seeded at an initial density of 1 x 10^6 x mL^{-1} and cultured for 24 hours in serum-free RPMI medium as described in legend to Fig. 1 with 1 μM CMZ, 1 μM KN-62, 20μM PD98059 or 1μM Ly294002. Cells were then collected and subjected to quantitative RT-PCR. Relative mRNA levels of the p21 gene in the different experimental conditions were normalized to β-actin expression, and values for control cells were set as one. Values shown are the mean ± SEM for seven different control or AD cell lines. *p<0.05, **p<0.01 significantly different from control cells; +p<0.05, ++p<0.01 significantly different from untreated AD cells

Figure 4

Effects of pifithrin-α (PFT-α) on p53 and p21 content and in survival of control and AD lymphoblasts.

Lymphoblasts from control and AD subjects were incubated in serum-free RPMI medium for 72 hours in the absence or in the presence of 30 μM PFT-α. A. To assess the effects of the drug on p21 and p53 content and in p53 phosphorylation in Ser 15, cell extracts for Western Blot were prepared at 24 hours. A representative immunoblot is shown. Densitometric analyses of p21, p-p53 (Ser 15) and total p53 content represent the mean ± SEM from different observations carried out with four different cell lines. *$p<0.05$ significantly different from untreated control cells, +$p<0.05$ significantly different from untreated AD cells. B. Cell viability was determined by trypan blue exclusion under inverted phase-contrast microscopy. Data shown represent the mean ± SEM from different observations carried out with five different cell lines.*$p<0.05$, **$p<0.01$ significantly different from untreated control cells.

Figure 5

Relative FOXO3a abundance and subcellular localization in control and AD cells

A. Lymphoblasts from control and AD cells were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and serum-starved for 24 hours. At that time, cell extracts for Western Blotting were prepared. A representative immunoblot is shown. Densitometric analyses of FOXO3a content represent the mean ± SEM of different experiments performed with ten different control or AD subjets. *$p<0.05$ significantly different from control cells. B. Experiments were performed under the same conditions described in A. In this case, cells were fractionated into cytosolic and nuclear fractions and subjected to Western

Blot. Lamin B and α-tubulin were used as control of purity and loading of nuclear and cytoplasmic protein extracts, respectively. Data represent the mean ± SEM of different experiments carried out with eight different cell lines derived from control or AD patients. *p<0.05, significantly different from control nucleus.

Figure 6

Influence of CaMKII and ERK1/2 on FOXO3a nuclear localization

A. Cells were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and incubated in serum-free RPMI medium in the absence or in the presence of 20 μM PD98059, 1 μM CMZ or 1 μM KN-62 for 24 hours. Part of these cells was used to prepare whole-cell extracts, which were subjected to Western blot to check the effects of these drugs on ERK phosphorylation, as shown in a representative immunoblot. B. The rest of the cells were fractionated to get the nuclear fraction. Nuclear FOXO3a abundance as determined by Western Blot in the absence or in the presence of PD98059 was calculated with data derived from experiments using eight different cell lines. *p<0.05, significantly different from untreated control. C. Under the same conditions as B, nuclear FOXO3a abundance as determined by Western Blot was calculated in the absence or in the presence of CMZ and KN62. Representative immunoblots are shown. Four different cell lines derived from control or AD subjects were used to perform the experiments. *p<0.01, significantly different from untreated control; +p<0.05, significantly different from untreated AD; +p<0.01, significantly different from untreated AD. Densitometric analyses represent in all cases the mean ± SEM.

Figure 7

Effects of ERK1/2 on MDM2 content in control and AD lymphoblasts.

Cells were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and incubated in serum-free RPMI medium in the absence or in the presence of 20 μM PD98059 for 24 hours, when they were collected to prepare whole-cell extracts for Western Blot. A representative immunoblot is presented. Densitometric analyses of MDM2 content represent the mean ± SEM of different experiments carried out with six different cell lines from control or AD patients. *$p<0.05$ significantly different from untreated control.

Figure 8

Subcellular localization of p21 in control and AD lymphoblasts.

A. Lymphoblasts from control and AD patients were seeded at an initial density of 1 x 10^6 cells x mL^{-1}, incubated in the absence of serum for 24 hours, and then fractionated to determine by immunoblot the subcellular localization of p21. Antibodies to α-tubulin and to lamin B were used as control of purity and loading of cytoplasmic and nuclear protein extracts, respectively. A representative immunoblot is shown, whereas the densitometric analysis is presented below. Data represent mean ± SEM of eight experiments. *$p<0.01$ significantly different from cytosol of control cells. B. p21 localization was also studied by confocal microscopy. Under the same conditions than A, cells were stained with anti-p21 antibody followed by secondary antibody labeled with Alexa Fluor 488. DAPI was used for nuclear staining. Merged images depict a predominant cytosolic localization for p21, more evident in AD cells. Magnification: 63x.

Figure 9

Diagram summarizing the role of CaMKII/ERK1/2/FOXO3a pathway in regulating p21 content and survival of AD lymphoblasts.

In control cells, serum withdrawal promotes ERK1/2 activation, which then presumably induces phosphorylation of FOXO3a and its posterior degradation in the cytosolic compartment in an MDM2-mediated manner. In AD cells, ERK1/2 activation is downregulated by the enhanced CaMKII activity. Reduced ERK1/2 activity partially prevents phosphorylation and degradation of FOXO3a increasing thereby the transcriptional activation of p21, which in turn protects cells from apoptosis.

Figure 1

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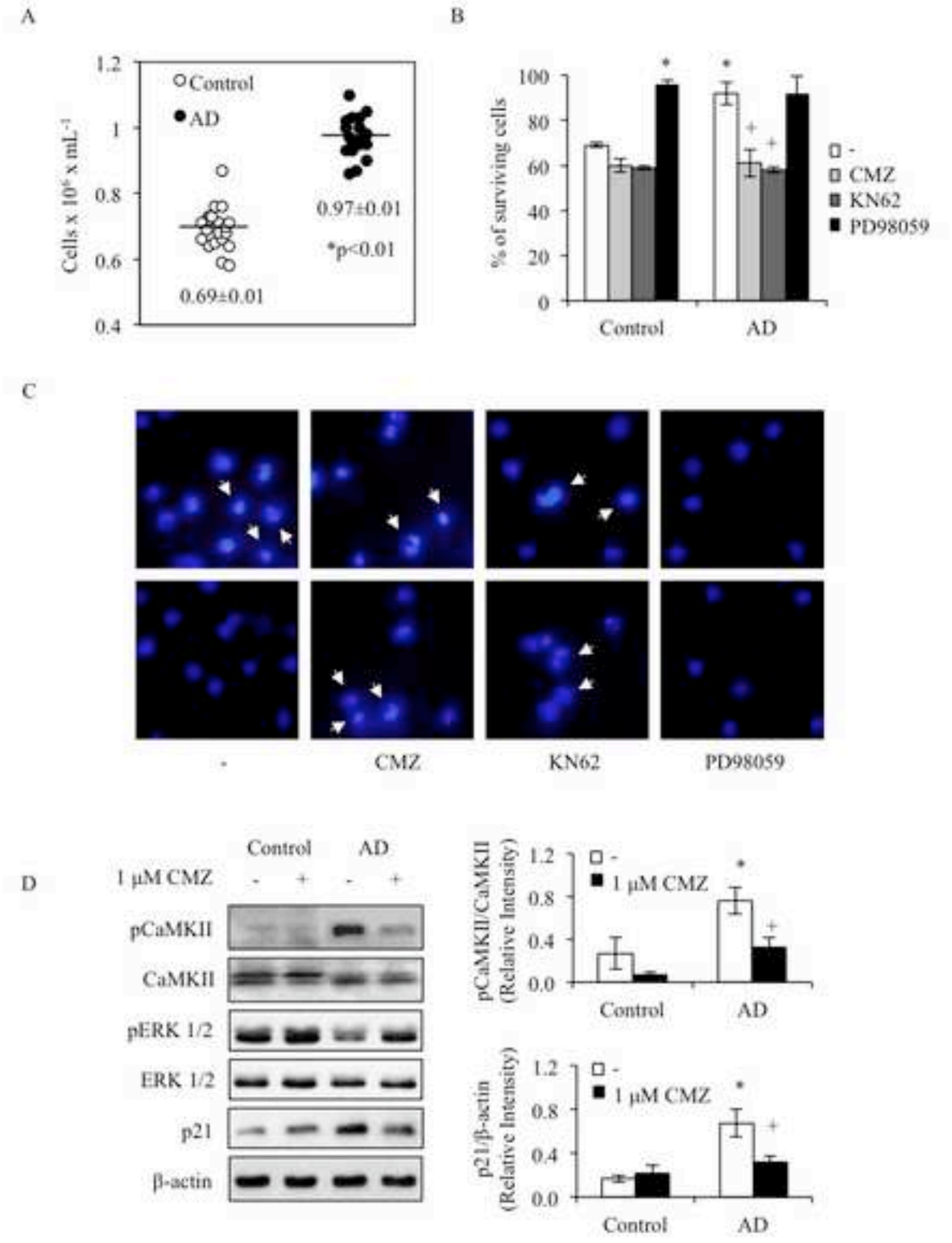

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Fig 1

Figure 2

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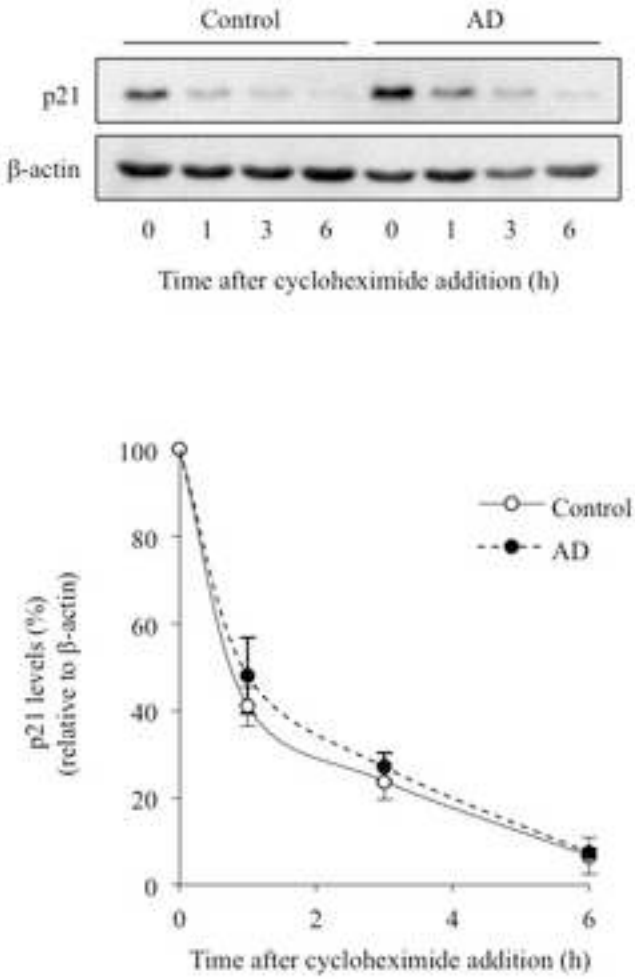

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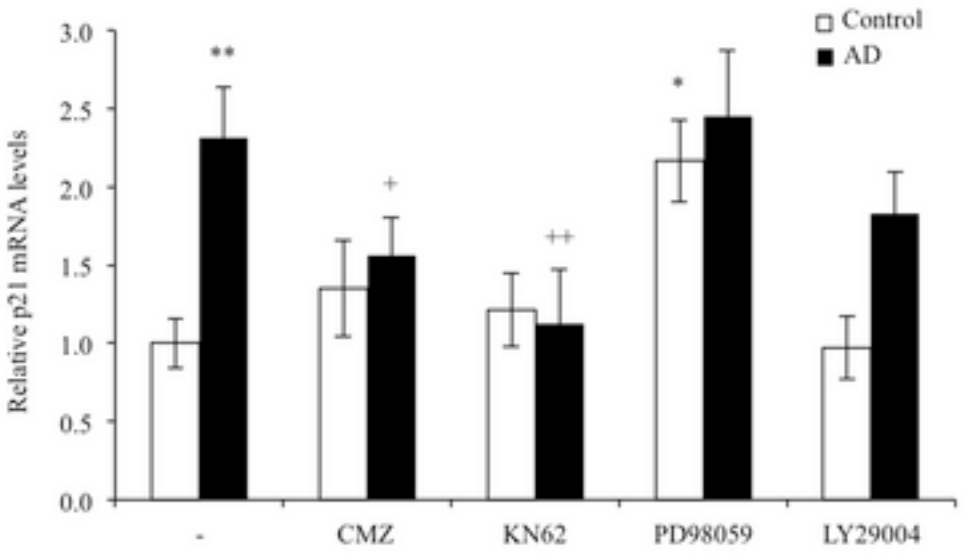

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Fig 3

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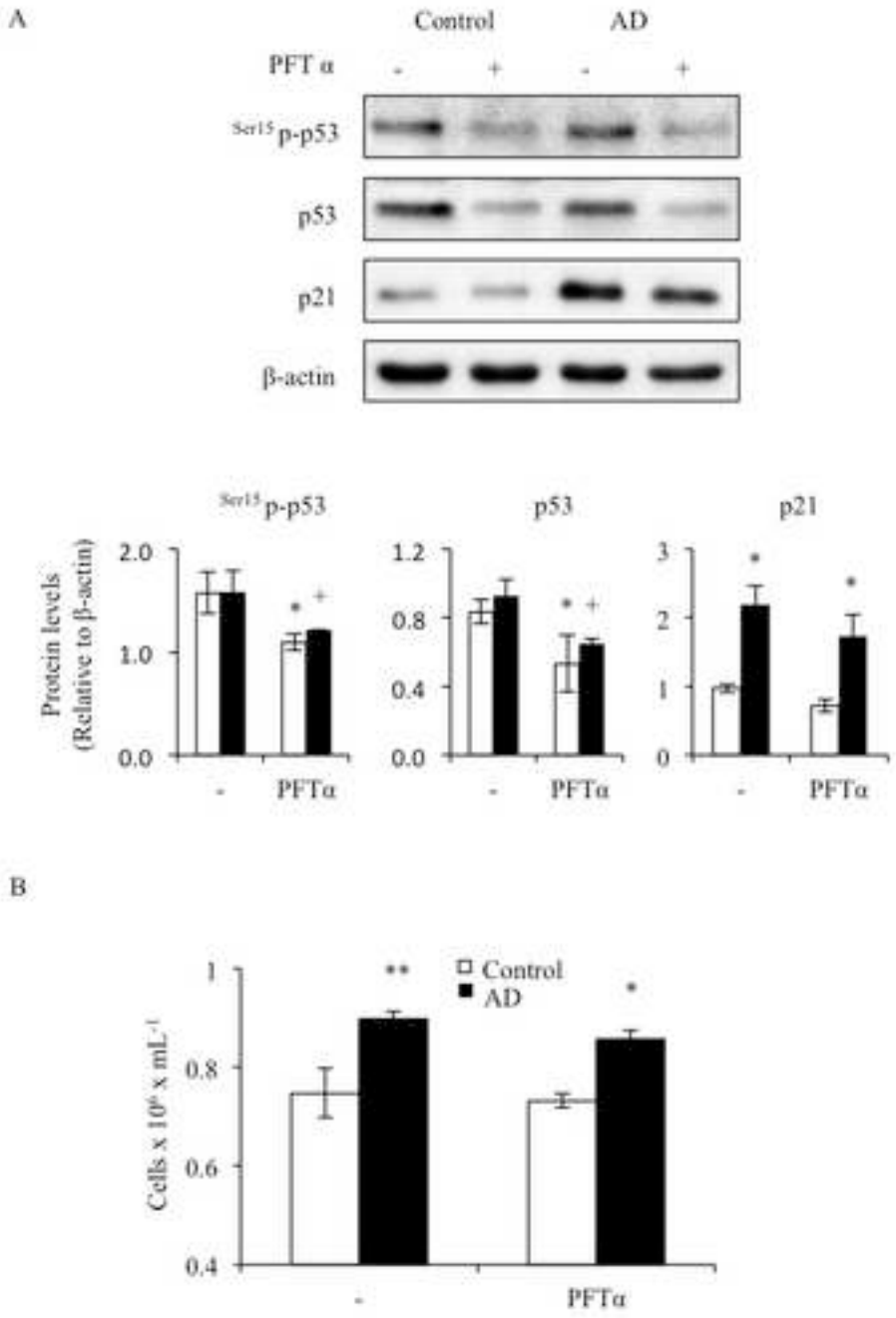

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Fig 4

Figure 5

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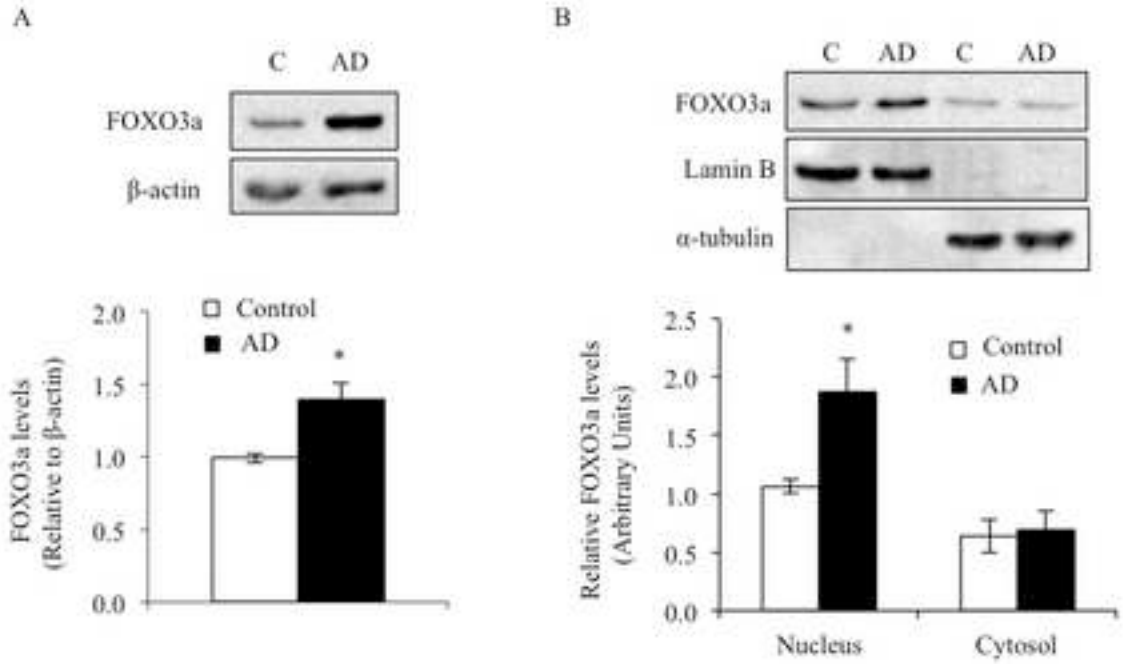

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Fig 5

Figure 6

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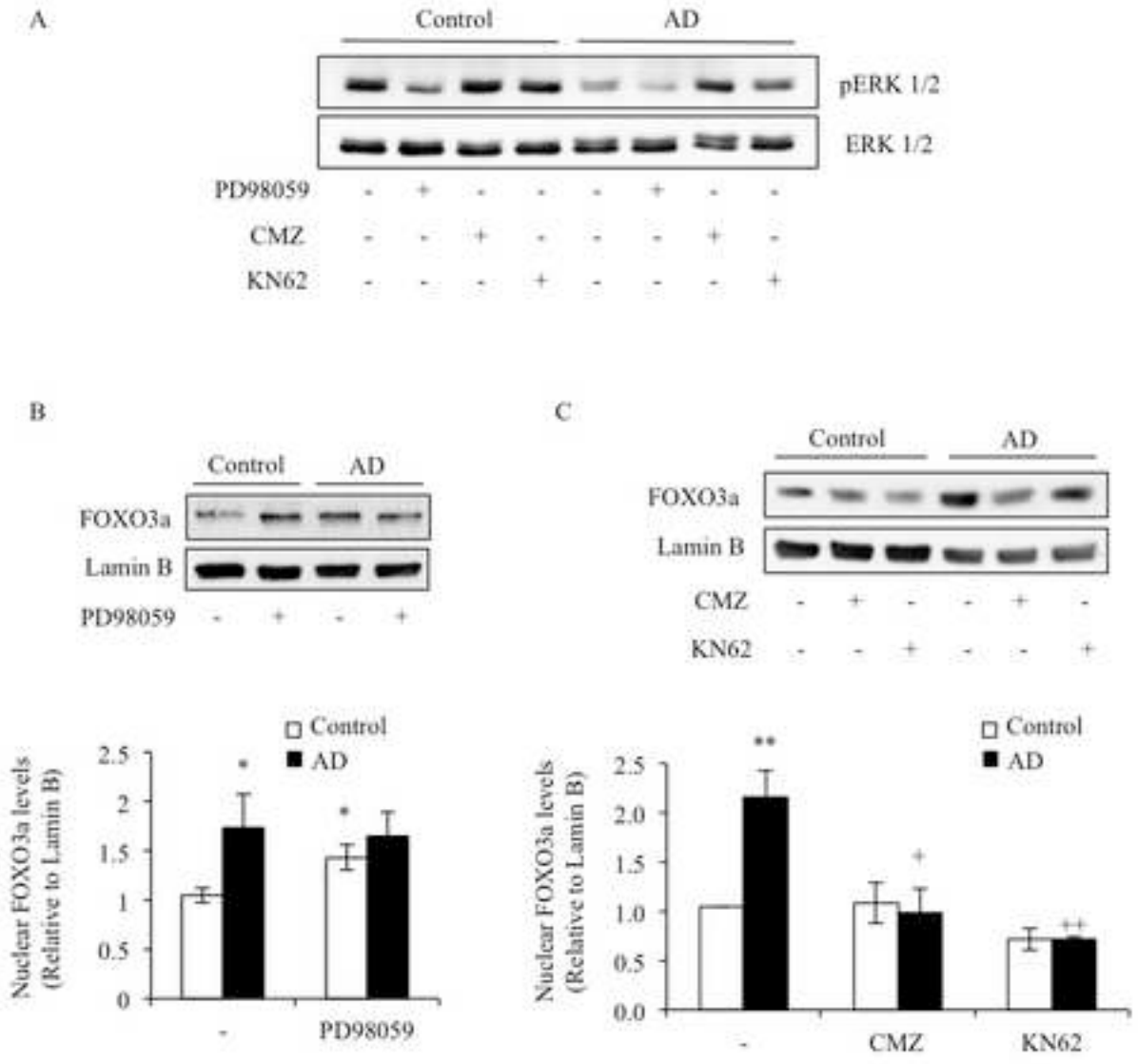

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Fig 6

Figure 7
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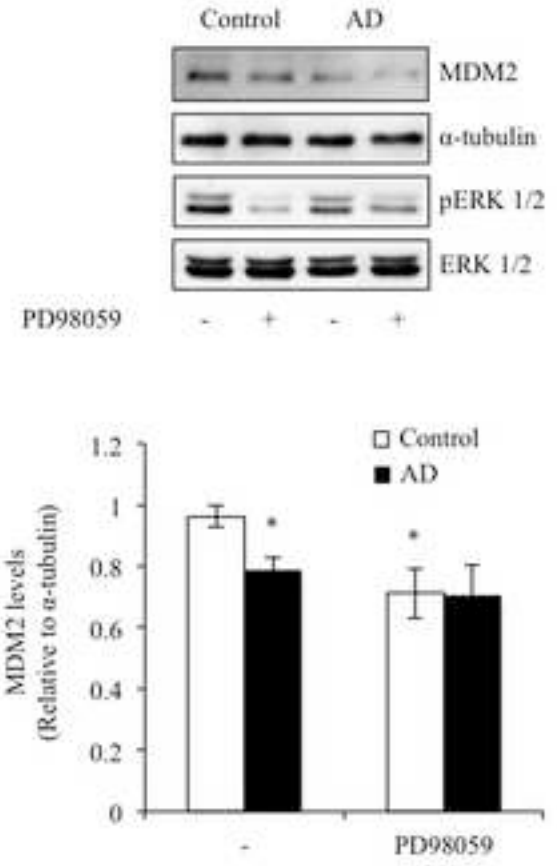

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Fig 7

Figure 8

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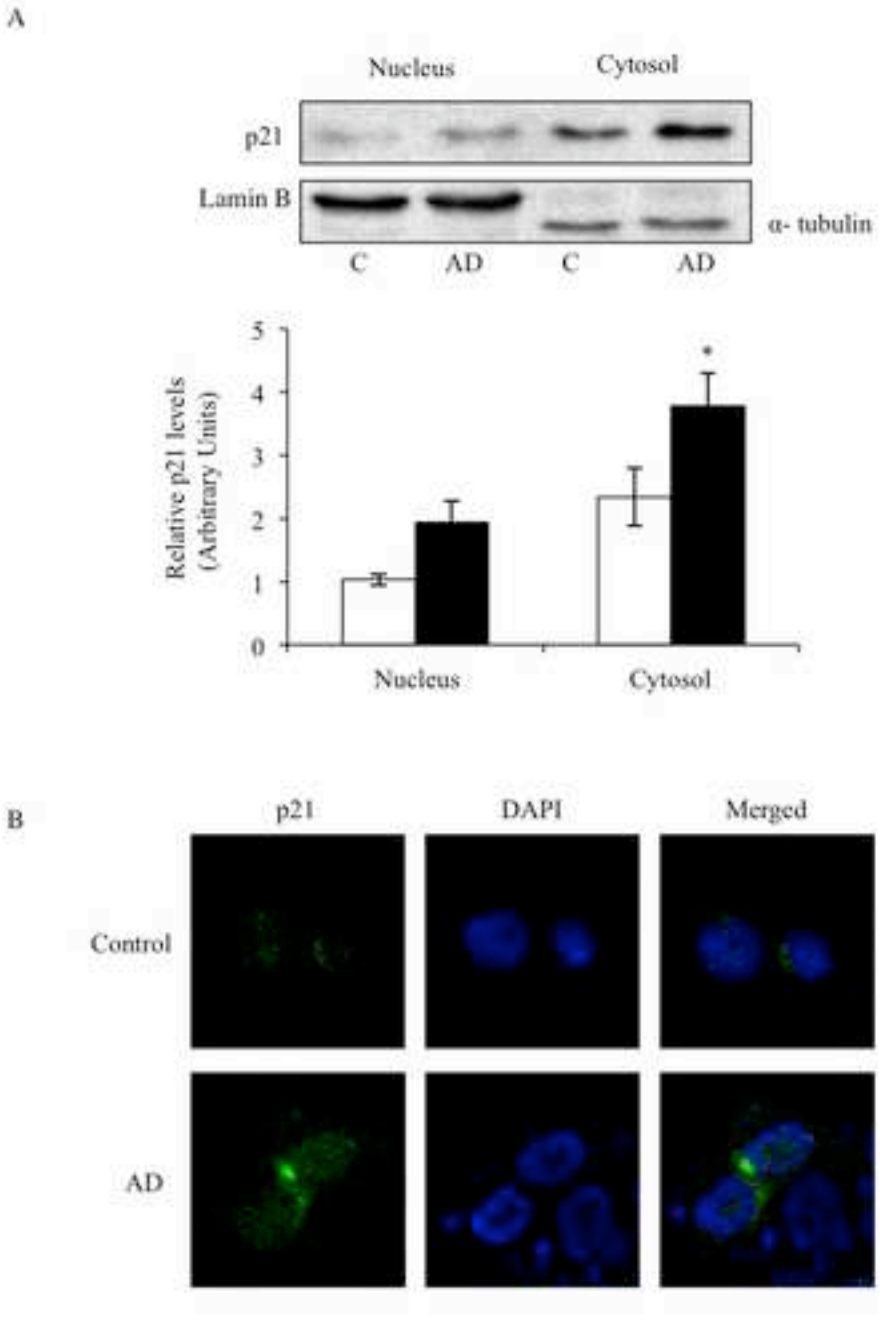

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Fig 8

Figure 9

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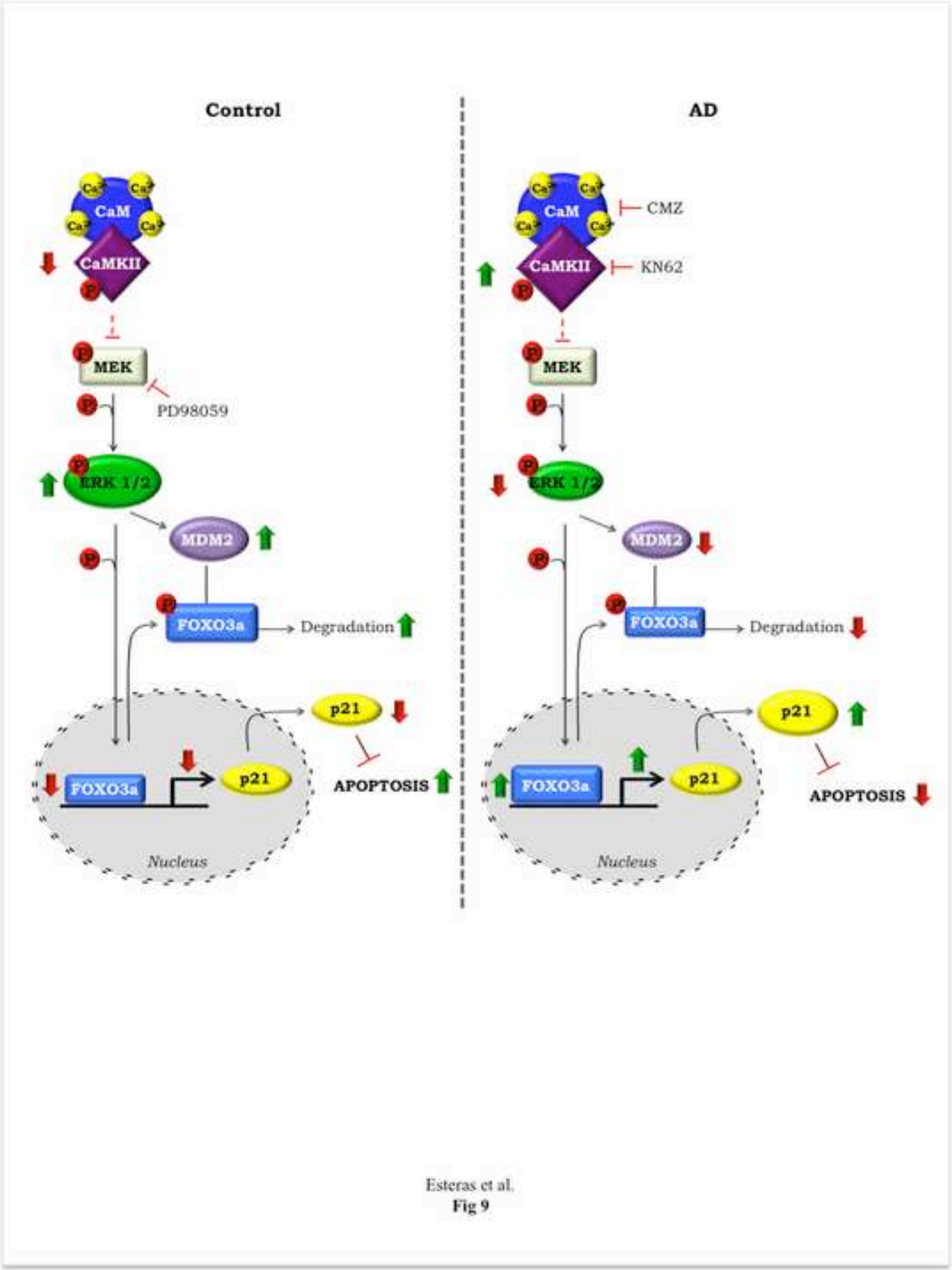

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Fig 9