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Transcriptional upregulation of p19INK4d upon diverse genotoxic stress is critical for optimal DNA damage response

Julieta M. Ceruti [1], María E. Scassa [1], Mariela C. Marazita, Abel C. Carcagno, Pablo F. Sirkin, Eduardo T. Cánepa *

Laboratorio de Biología Molecular, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria Pabellón II Piso 4, 1428 Buenos Aires, Argentina

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ABSTRACT

p19INK4d promotes survival of several cell lines after UV irradiation due to enhanced DNA repair, independently of CDK4 inhibition. To further understand the action of p19INK4d in the cellular response to DNA damage, we aimed to elucidate whether this novel regulator plays a role only in mechanisms triggered by UV or participates in diverse mechanisms initiated by different genotoxics. We found that p19INK4d is induced in cells injured with cisplatin or β-amyloid peptide as robustly as with UV. The mentioned genotoxics transcriptionally activate p19INK4d expression as demonstrated by run-on assay without influencing its mRNA stability and with partial requirement of protein synthesis. It is not currently known whether DNA damage-inducible genes are turned on by the DNA damage itself or by the consequences of that damage. Experiments carried out in cells transfected with distinct damaged DNA structures revealed that the damage itself is not responsible for the observed up-regulation. It is also not known whether the increased expression of DNA-damage-inducible genes is related to immediate protective responses such as DNA repair or to more delayed responses such as cell cycle arrest or apoptosis. We found that ectopic expression of p19INK4d improves DNA repair ability and protects neuroblastoma cells from apoptosis caused by cisplatin or β-amyloid peptide. Using clonal cell lines where p19INK4d levels can be modified at will, we show that p19INK4d expression correlates with increased survival and clonogenicity. The results presented here, prompted us to suggest that p19INK4d displays an important role in an early stage of cellular DNA damage response.

1. Introduction

The outcome of DNA damage is diverse and generally adverse. Cancer development requires accumulation of numerous genetic changes, which are believed to initiate through the presence of unrepaired lesions in the genome. In the absence of proficient repair, genotoxic agents can lead to crucial mutations of vital cellular genes, via replication of damaged DNA, contributing to oncogenesis (Helleday et al., 2008; Zindy et al., 1997). Furthermore, evidence suggests that DNA damage may be a central event in neurodegeneration (Swarbrick et al., 2000; Rolig and McKinnon, 2000).

In view of the diversity of lesions, cells have developed a remarkable repertoire of responses to different stresses, particularly those causing structural damage to the genome. Upon exposure to DNA damaging agents, mammalian cells initiate a series of complex biochemical reactions designed to ensure the integrity of the genetic material. Mechanisms, which have evolved to detect structural alterations of the genome and induce cell cycle checkpoints, function in parallel with the biochemical machinery that repairs the DNA damage (Matsuoka et al., 2007; Rouse and Jackson, 2002).

Multicellular organisms have the ability of eliminating the damaged cells by triggering their death. Therefore, a cell will first try to repair any DNA damage and survive; conversely, when DNA damage overwhelms repair capacity the cell will switch to apoptosis. The signals that govern the decision whether a cell initiates DNA repair, enters cell cycle arrest, or undergoes apoptosis and the means by which the cell integrates information from these pathways are not yet understood (Zhivotovsky and Kroemer, 2004). Progression through each phase of the cell cycle is governed by cyclin-dependent kinases (CDKs). Cyclin-CDK complexes are negatively regulated by two classes of small peptides, the INK4 and Cip/Kip families of CDK-inhibitors (CKI). Members of INK4 family, comprising p16INK4a, p15INK4b, p18INK4c and p19INK4d, block the progression of the cell cycle by binding to either CDK4 or CDK6

* Corresponding author. Tel.: +54 11 45763342; fax: +54 11 45763342.
E-mail addresses: julce@qb.fcen.uba.ar (J.M. Ceruti), mscassa@qb.fcen.uba.ar (M.E. Scassa), marazita@qb.fcen.uba.ar (M.C. Marazita), abelc@qb.fcen.uba.ar (A.C. Carcagno), psirkin@qb.fcen.uba.ar (P.F. Sirkin), ecanepa@qb.fcen.uba.ar (E.T. Cánepa).
[1] These authors contributed equally to this work.

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and inhibiting the action of cyclin D. The four proteins share a similar structure dominated by several ankyrin repeats. Although they appear to be structurally redundant and equally potent as inhibitors, the INK4 family members are differentially expressed during development (Ortega et al., 2002). Actually, the reported evidence about different cellular processes in which they have been shown to participate, is growing (Scassa et al., 2007).

Several recent works have reported that p16INK4a and p19INK4d proteins could be involved in the cellular response to genotoxic agents (Al-Mohanna et al., 2007; Ceruti et al., 2005). In particular, there are strong evidences that, upon UV irradiation, p19INK4d plays a crucial role in regulating genomic stability and cell viability (Scassa et al., 2007). Notably, improvement of DNA repair in cells that overexpress p19INK4d inversely correlates with apoptosis triggered by DNA damage. These results, suggest that, in addition to its role in improving DNA repair, p19INK4d would negatively modulate DNA damage-induced apoptosis in mammalian cells. On this matter, recent studies performed in p19INK4d null mice, demonstrate that absence of p19INK4d rendered cells sensitive to apoptotic and autophagic cell death and suggest a possible role of p19INK4d induction in chemoprevention (Tavera-Mendoza et al., 2006a,b). In addition, p19INK4d expression is induced by UV light in various cell lines, but the regulatory mechanism involved is unknown (Ceruti et al., 2005).

Considering its function as cell cycle regulator, these observations suggest that p19INK4d may itself play a role in cellular DNA damage response (DDR) and would belong to a protein network that would integrate DNA repair, apoptosis and checkpoint mechanisms in order to maintain genomic integrity.

In view of the plethora of types of lesions, no single repair process can cope with all kinds of damage. Instead, evolution has early selected an interwoven of DNA repair systems that as a whole cover most of the insults inflicted on the genetic information (Hoeijmakers, 2001). Taken into account that, independently of the type of lesion, the DDR is a common program that includes the execution of DNA repair, apoptosis and cell cycle arrest mechanisms, we hypothesize that proteins which are induced or activated in response to different genotoxics that cause distinct kind of lesions, are better qualified to be a member of such complex network.

Considering the preceding observation we decided to investigate the potential role of p19INK4d (hereafter referred as p19) in the DNA damage response triggered by different genotoxics that activate distinct DNA repair mechanisms. Furthermore, we analyzed the molecular mechanisms involved in the DNA damage-mediated p19 induction. In this study, we observed that p19 improves DNA repair, diminishes apoptosis and increases cellular survival independently of the genotoxic agent used to produce DNA damage. The present work demonstrates that genotoxics induce the expression of p19, providing strong evidences that this action is exerted at a transcriptional level, and suggests that would be independent of p53. Finally, cell transfection with damaged DNA molecules was not sufficient to induce p19 expression, suggesting that another event might be involved. The results presented here, prompted us to hypothesize that p19 displays an important role in an early stage of cellular DDR.

2. Materials and methods

2.1. Cell culture and transfections

BHK-21 (baby hamster kidney) fibroblasts cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, 100 mM non-essential aminoacids, and 2 mM glutamine at 37 °C in a humidified 5% CO_2 atmosphere. Culture of SH-SY5Y neuroblastoma cells was performed as previously described (Ceruti et al., 2005). For establishment of stable clones, the pMTCB6 vector, containing a NotI–BamHI fragment encoding the mouse p19 cDNA downstream the metallothionein promoter carrying a selectable neomycin-resistance gene (pMTp19S), was used (a generous gift from C. Sherr St. Jude Children's Hospital, Memphis). The pMTp19AS was constructed cloning the p19 cDNA in the reverse orientation. Transfections were performed using Lipofectamine™ 2000 Reagent (Invitrogen). Twenty-four hours after transfection cells were replated at low density to isolate single colonies. The clonal cell lines BHK-21empty, BHK-21p19S, BHK-21p19AS, from now on, derived from the transfectants were maintained in selective medium containing 300 μg/ml geneticin disulfate (G418, Calbiochem-Novabiochem). For metallothionein promoter induction stable transformants were treated with 50 μM $ZnSO_4$ for at least 4 h. Transfections of SH-SY5Y cells were performed using Lipofectamine™ 2000 Reagent (Invitrogen) following manufacturer's protocol. Before transfection 1×10^6 cells were seeded in 35 mm plates. pSG5p19 (2.5 μg) or pSG519AS (2.5 μg) and/or pBabePuro (0.5 μg) and Lipofectamine™ 2000 (10 μl) were used per plate. Twenty-four hours after transfection, selection was carried on with 2.5 μg/ml puromycin for 60 h.

2.2. UV irradiation

Exponentially growing cells were trypsinized and seeded at 50–60% confluence. Twenty-four hours after plating, cells were irradiated in open-dishes with the corresponding UV dose, 254 nm (range 240–280 nm) at room temperature from a Philips ultraviolet lamp (TUV15WG15T8) calibrated to deliver 0.25 mJ/cm^2 s. Following UV-irradiation, medium was replaced and cells were maintained at 37 °C in a 5% CO_2 humidified incubator along times indicated in each case. For each experiment, control cells were treated identically except for UV light exposure.

2.3. Oligonucleotides and plasmids

2.3.1. Single strand oligodeoxynucleotides (ODN)

CGATTTTGGATTGAAGCCAATATGATAACGAT (32 mer) and double strand ODN TTGGTTTTGTTTGCATCGATGACAGGGATTCTTTACTTGA (40 mer) were synthesized with phosphodiester linkage by Bio-Synthesis (Lewisville, TX) and diluted in H_2O to form a 500 μM stock. This stock was 64 mJ/cm^2 UV irradiated or not and added to culture dishes for use in experiments (200 nM). Cells were transfected with single or double strand ODN once and then harvested at the indicated time points. The pCMV-CAT vector (5 μg) encoding for the chloramphenicol acetyl transferase (CAT) reporter gene under the CMV promoter was treated or not with 64 mJ/cm^2 UV and transfected into BHK-21 cells using Lipofectamine™ 2000 Reagent.

The sequences of dumbbell-shaped decoy ODN are as follows: p53wt: 5′-GGGACATGTTCAAAGAACATGTCCCAACATGTTGAAACAA-CATGTT-3′; p53mut: 5′-GGGACATGTTCAAAGAATATATCCCAATATA-TTGAAACAACATGTT-3′. ODNs were annealed for 24 h with a steady temperature descent from 80 to 25 °C. T_4 DNA ligase (1 unit) was added to the mixture, followed by incubation for 24 h at 16 °C to generate covalently ligated dumbbell-shaped decoy ODN molecules. Cells were transfected with p53wt or p53mut decoy ODN (100 nM) as described above.

2.4. RNA extraction and Northern blot analysis

Total cellular RNA was isolated from cultures as described previously (Chomczynski and Sacchi, 1987). Ten micrograms of total RNA were denatured, electrophoresed in 1% glyoxal/agarose gels, and transferred to nylon membranes (GeneScreen Plus, PerkinElmer). The membranes were sequentially hybridized with

^{32}P-labeled probes to p19, p16^{INK4a}, p21^{WAF1} and β-tubulin. To detect p19 mRNA, a 24-mer ODN was synthesized complementary to bases +3 to +26 of human p19 mRNA (Ceruti et al., 2005). The ODN was 5′-end-labeled using [γ-^{32}P] ATP and T_4 polynucleotide kinase. Hybridization was carried out overnight at 68 °C in the same prehybridization solution by adding the ^{32}P-labeled ODN (3×10^5 cpm/cm^2) as previously described (Ceruti et al., 2005). To detect β-tubulin and other mRNAs, the corresponding cDNA, excised from the respective backbone vectors, was labeled by random priming using [α-^{32}P]dCTP and Klenow. Membranes were stripped, prehybridized, hybridized and washed in standard conditions as described before (Scassa et al., 2004). The membranes were scanned directly onto a Bio-Imaging Analyzer Fujifilm BAS-1800II.

2.5. Nuclear run-on transcription assay

Exponentially growing BHK-21 or SH-SY5Y cells were plated in 10 cm dishes (8×10^6/dish). After 24 h, fibroblasts were irradiated with 8 mJ/cm^2 UV and neuroblastoma cells treated with 10 μM β-amyloid peptide and incubated at 37 °C for 12 h. Identical number of BHK-21 or SH-SY5Y nuclei was used for preparation of nascent transcripts. To perform the nuclear run-on transcription, isolated nuclei were incubated in 50 μl reaction buffer (10 mM Tris–HCl, pH 8.4, 1.5 mM $MgCl_2$, 140 mM NaCl, 1 mM each of ATP, GTP, CTP and 0.34 μM [α-^{32}P]UTP purchased from New England Nuclear, 10 mM phosphocreatine and 100 μg/ml phosphocreatine kinase) for 20 min at 30 °C and radiolabeled RNA was extracted from each sample. All samples had very close total radioactivity and were hybridized to nylon membranes (GeneScreen Plus, PerkinElmer) that were previously slot-blotted with several linearized plasmid DNAs (5 μg) including pSG5p19, pBabePurop21^{WAF1}, pBluescriptSK(+)CyclinD1; pBluescriptSK(+)CDK4, pCMVGADD45, pCMVBcl-X_L. pBluescriptSK(+)β-tubulin was also blotted as control. After washing, the membranes were scanned directly onto a Bio-Imaging Analyzer Fujifilm BAS-1800II and quantified. Each experiment was repeated twice with independently labeled RNA obtained from separate preparations of nuclei.

2.6. Unscheduled DNA synthesis

SH-SY5Y cells were seeded at 1×10^6 cells per 35-mm plate and kept undisturbed until 80–90% confluence. Cells were washed with PBS and regular media was substituted for arginine-free medium containing 1% FBS. After 24 h, the medium was changed to fresh arginine-free medium containing 1% serum for additional 24 h. We determined that under these conditions, DNA semiconservative synthesis was completely inhibited. Cells were treated with 20 μM cisplatin or 10 μM β-amyloid peptide and further cultured in fresh arginine-free medium containing 1% serum and 10 μCi/ml [^{3}H]thymidine. At the indicated times, cells were washed three times with cold PBS, harvested and pelleted at $3000 \times g$ for 5 min. Cells were lysed with 5% TCA for 30 min and centrifuged at $10{,}000 \times g$ for 10 min. Pellet was washed twice with cold PBS and resuspended in 1 M NaOH. The incorporated radioactivity was quantified by scintillation counting. Unscheduled DNA synthesis was estimated as dpm/μg of protein.

2.7. Caspase-3 activity

Cells were plated at 1×10^6 cells/well on six plates exposed to 20 μM cisplatin or 10 μM β-amyloid peptide. After 12 h of genotoxic treatment cells were harvested with lysis buffer (50 mM Tris–ClH, pH 7.4, 1 mM EDTA, 10 mM EGTA, 10 μM digitonine, 0.5 mM PMSF, 10 μg/ml pepstatin, and 10 μg/ml aprotinin) at the indicated times, incubated for 30 min at 37 °C and centrifuged at $12{,}000 \times g$ for 20 min. The activity of caspase-3 in 150 μl cell lysates was determined using 100 μM of the synthetic caspase-3 substrate Ac-DEVD-pNA (Sigma) in reaction buffer (100 mM HEPES pH 7.5, 0.5 mM EDTA, 5 mM dithiothreitol and 20% v/v) in a final volume of 300 μl and incubated at 37 °C during 4 h. Color development was measured at 405 nm. Caspase activity was estimated at A_{405}/μg protein.

2.8. MTT assay

Cell viability was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Exponentially growing BHK-21empty, p19S or p19AS clonal cell lines (2×10^4 cells/well) were seeded in 24-well plates. After incubation with 50 μM $ZnSO_4$ for 6 h cells were treated with 20 μM cisplatin or 10 μM β-amyloid peptide. Twenty-four hours later, medium containing $ZnSO_4$ was removed and cells incubated for additional 5 days. At the indicated times, the culture media was replaced by 1 ml of fresh medium containing MTT (Sigma) to a final concentration of 1 mg/ml and incubated at 37 °C. After 4 h of incubation the medium and MTT were removed and 500 μl of isopropanol was added. Plates were shaken for 15 min and the absorbance in the solvent was determined at 570 nm. The background was substracted by using a dual-wavelength setting of 570 and 650 nm. Control included untreated cells and medium alone.

2.9. Clonogenic assay

Survival after cisplatin and β-amyloid peptide treatment was determined by clonogenic assay in BHK-21empty, p19S or p19AS clonal cells. Cells were plated as single cell and treated with different doses of either genotoxic after metallothionein promoter induction with 50 μM $ZnSO_4$ for 4 h. Twenty-four hours later, the medium containing $ZnSO_4$ removed. Cells were incubated for 6 days, rinsed in PBS and incubated with crystal violet stain (10% formaldehyde, crystal violet 5 mg/ml in ethanol). Plates were thoroughly rinsed with H_2O and allowed to dry. Colonies were counted, the sextuplicates were averaged and long term survival was determined as a percent of control. Colonies containing >50 cells were rated as deriving from viable, clonogenically capable cells.

3. Results and discussion

3.1. Cisplatin and β-amyloid peptide induce p19 in neuroblastoma cells

p19, improves DNA repair activity (Ceruti et al., 2005), promotes cell survival and decreases chromosomal aberrations in UV-damaged mammalian cells (Scassa et al., 2007), independently of CDK4 inhibition. Moreover, p19 expression is markedly augmented in response to UV damage in several cell lines. In the present study, we irradiated BHK-21 fibroblasts with UV and observed that p19 RNA increased in a time and dose dependent manner following treatment according to previous results (Fig. 1A). p21 mRNA was also induced upon UV damage, as expected (Al-Mohanna et al., 2007).

We then asked if the role of p19 in DNA repair ability is restricted to UV damage or if it participates in mechanisms triggered by other genotoxics. As brain is one of the tissues where p19 is predominantly expressed (Zindy et al., 1997) we choose neuroblastoma cells to address this issue. We first analyzed if p19 can also be induced by cisplatin or β-amyloid peptide. Cisplatin is a widely used chemotherapeutic agent that produces non-coding DNA adducts that interfere with replication and transcription (Damsma et al., 2007). β-Amyloid peptide is a component of the extracellular senile plaques which are present in patients with Alzheimer's disease,

and is believed to be neurotoxic. The mechanism of this toxicity is still unclear but it is known that oxidative stress and inflammation are implicated as mediators of it, and, in turn, these lesions, affect cellular components including proteins, membrane lipids and DNA (Suram et al., 2006). DNA-base excision repair (BER) is believed to be the major pathway for repairing deaminated bases and bases with oxidative damage generated by ROS (Hazra et al., 2007). Therefore, damage originated by cisplatin basically activates the Nucleotide Excision Repair (NER) mechanism, while lesions derived from accumulation of β-amyloid peptide cause the activation of BER and Homologous Recombination repair pathways.

To study the response of p19 against these genotoxic agents, we performed Northern blot analysis in neuroblastoma SH-SY5Y cells. Time course and dose response experiments revealed that p19 mRNA expression was significantly induced upon 10 μM cisplatin or 2 μM β-amyloid peptide (Fig. 1B and C). Importantly, a similar induction was observed with 5 Gy ionizing radiation or 1 μM camptothecin (data not shown). We then focused our attention on the effect of mentioned genotoxics in the expression of other CKIs. We observed that p16 mRNA level, another member of INK4 family, was neither altered by cisplatin nor by β-amyloid peptide, even at higher doses. This finding suggests that, as occur with UV, these two genotoxics would specifically modulate p19 gene expression. On the other hand, we found that p21 gene expression was also significantly increased with cisplatin, as previously described (Mitsuuchi et al., 2000). Notably, in SH-SY5Y cells this CKI was also upregulated under β-amyloid peptide treatment (Fig. 1B and C). So, diverse genotoxic agents that cause different types of DNA damage and that activate varied repair mechanisms, promote p19 gene induction.

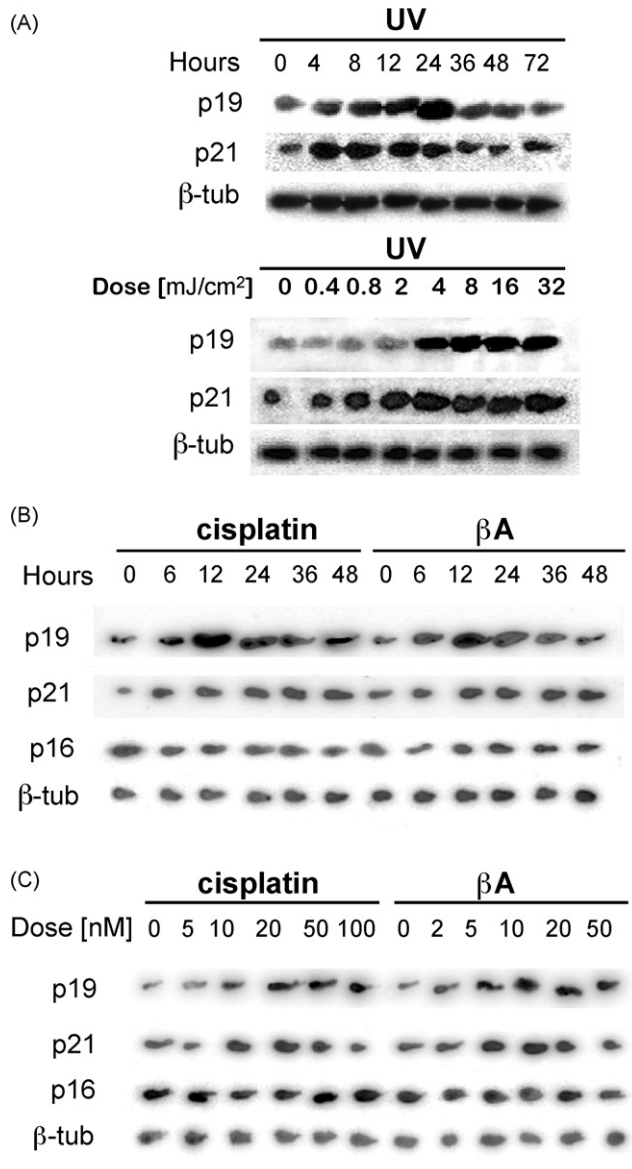

Fig. 1. Induction of p19INK4d mRNA levels by UV, cisplatin and β-amyloid peptide. (A) BHK-21 fibroblasts were irradiated with 8 mJ/cm^2 UV and harvested at the indicated time points following irradiation or inflicted with the indicated UV doses and harvested 8 h after irradiation. (B) SH-SY5Y neuroblastoma cells were treated with 20 μM cisplatin or 20 μM β-amyloid peptide (βA) and harvested at the indicated time points following treatment or (C) incubated with the indicated doses of cisplatin or βA and harvested 12 h after genotoxic insult. The expression of p19, p16 and p21 was assessed by Northern blotting, as described in Section 2. β-Tubulin (β-tub) was used as a loading control. Each figure shows a representative autoradiograph of two independent experiments.

3.2. Genotoxic agents accelerate p19 transcription initiation rate, and do not modify mRNA stability

The increase observed in p19 mRNA level after treatment with genotoxic agents could have different causes. One could be changes in the transcriptional rate level. In order to study the effect of genotoxics on the transcription initiation rate of p19, we performed run-on assays. Cells were irradiated with 8 mJ/cm^2 UVC or treated with 10 μM β-amyloid peptide and processed 24 or 12 h later, respectively. p19 transcription showed 3- and 2.5-fold increase, respectively, in damaged cells with respect to control cells (Fig. 2A and B). We also checked the expression of some genes known to be involved in cell cycle, DNA repair or apoptosis. Their transcription was induced or repressed in response to DNA damage, as previously reported. p21 and GADD45 were induced with UV and β-amyloid peptide 4.8- and 2.5-fold, respectively, while CDK4 and p16 expression remained unaltered (Al-Mohanna et al., 2007; Jabbur et al., 2000; Santiard-Baron et al., 1999). Cyclin D1 and Bcl-X_L presented a marked decrease in their initiation rates after UV irradiation or β-amyloid treatment, respectively (Hiyama and Reeves, 1999; Lutzen et al., 2004).

While transcriptional regulation of gene expression is often associated with increased mRNA abundance, the rate of mRNA decay also plays an important role in the regulation of mRNA steady-state levels and therefore gene expression. To address this issue we studied the effect of UV and β-amyloid peptide on p19 mRNA stability. We irradiated BHK-21 cells or treated SH-SY5Y cells with β-amyloid peptide and inmediatelly added the inhibitor of transcription actinomycin D. Values of p19 half-life obtained in these experiments were 190 min in control cells, 185 min in UV irradiated cells (Fig. 2C) and 200 min in β-amyloid peptide treated cells (Fig. 2D).

The fact of adding actinomycin D simultaneously with the damaging agent implies a limitation. If UV light needs the early transcription of intermediary molecules to increase p19 mRNA stability, this requirement would be abrogated because all transcription would be inhibited. Therefore, we performed another assay adding actinomycin D 3 h after UV infliction. Similarly, in this case, there was no difference in the rate of p19 mRNA degradation after UV damage (data not shown). These results strongly suggest that variations in p19 mRNA stability do not contribute to genotoxic-mediated increase in p19 expression.

Damage induced by genotoxics at the molecular level initiates the activation of transcription factor pathways, which in turn regulate the expression of a number of genes termed the "DNA damage response genes". At present, the regulatory sequences of p19 responsible to confer UV responsiveness remain elusive. In silico analysis of 5′-regulatory region of p19 revealed the presence of high homologous binding sites for NF-κB, E2F, Sp1, NF-Y and

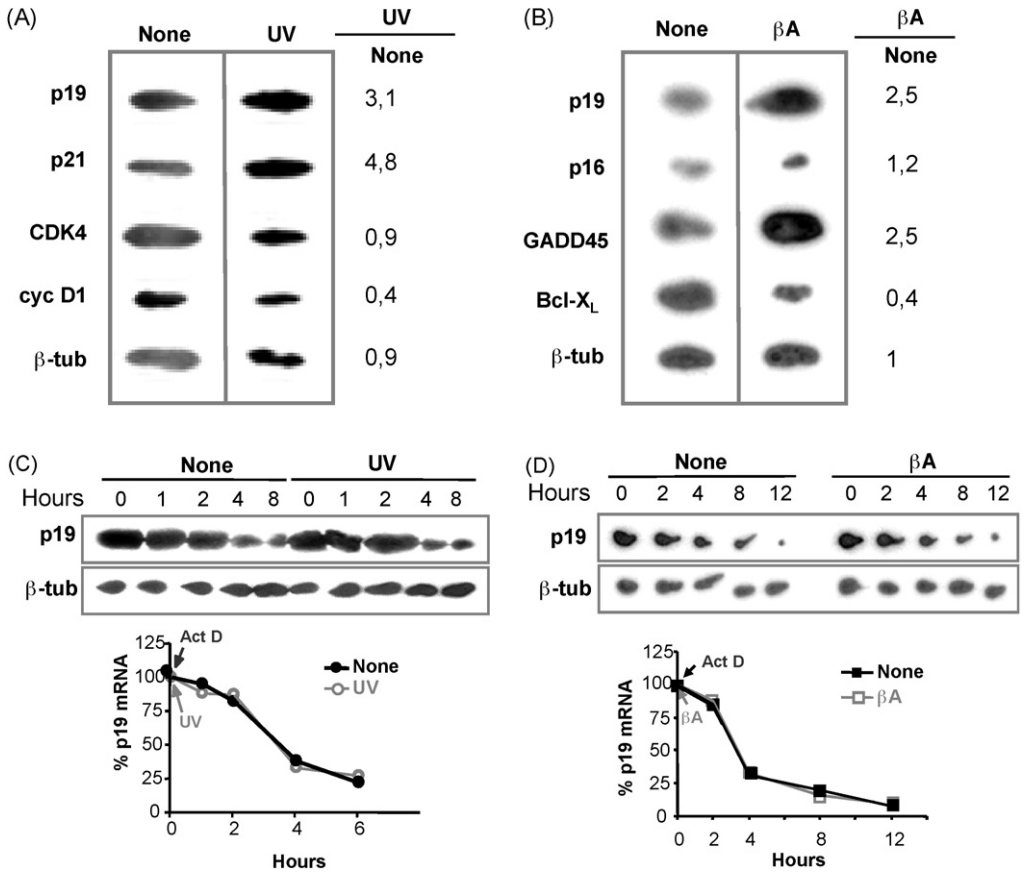

Fig. 2. Nuclear run-on shows that p19INK4d expression is transcriptionally regulated. (A) BHK-21 fibroblasts were irradiated with 8 mJ/cm^2 UV and harvested 24 h following irradiation. Denatured DNA fragments containing the coding sequences of p19, p21, CDK4 and cyclin D1 (cycD1) were slot-blotted onto a nylon membrane and hybridized with [^{32}P]UTP-labeled nascent RNA prepared from BHK-21 cells. (B) SH-SY5Y neuroblastoma cells were treated with 10 μM β-amyloid peptide (βA) and harvested 12 h following treatment. Denatured DNA fragments containing the coding sequences of p19, p16, GADD45 and Bcl-X_L were slot-blotted onto a nylon membrane and hybridized with [^{32}P]UTP-labeled nascent RNA prepared from SH-SY5Y cells. Transcription rates were normalized to β-tub signal. In (A) and (B), values indicate the transcription rate of treated versus untreated cells. (C) BHK-21 cells were irradiated or not with 8 mJ/cm^2 UV, immediately incubated with 1 μM actinomycin D (ActcD) and harvested at the indicated time points after irradiation. (D) SH-SY5Y neuroblastoma cells were simultaneously treated with 10 μM β-amyloid peptide and 1 μM actinomicyn D and harvested at the indicated time points after treatment. In (C) and (D), the expression of p19 was assessed by Northern blotting, as described in Section 2. β-tub was used as loading control. Each figure shows a representative autoradiograph of two independent experiments.

Egr-1 transcription factors. Several studies described participation of these factors in the cellular DNA-damage response (Ravi et al., 2008; Blattner et al., 1999; de Belle et al., 1999; Hu et al., 2000) and, moreover, some are known for exerting growth-suppressing activities (de Belle et al., 1999; Virolle et al., 2001). Moreover, the functionality of Sp1 and Egr-1 elements present in p19 promoter has been reported (Virolle et al., 2003; Yokota et al., 2004). Therefore, it stands to reason that, at least in some of the cell lines assayed, one or more of the aforementioned factors would be involved in p19 induction. Actually, in our lab several studies are under progress in order to uncover the molecular events implicated in genotoxic-mediated p19 regulation at gene and protein level.

3.3. Transcriptional induction of p19 after DNA damage needs de novo protein synthesis

Genotoxic agents regulate cellular processes as protein localization, posttranslational modifications and protein stability, some of them, concerning protein synthesis (Stokes et al., 2007; Gentile et al., 2003). We investigated if DNA damage-induced p19 gene expression does require de novo synthesis of some factor/s involved in the aforementioned cellular processes. To this end, we analyzed p19 mRNA expression along time in UV damaged cells, previously incubated with 10 μM cycloheximide for 3 h in order to inhibit protein synthesis. As shown in Fig. 3, this antibiotic partially impairs UV-mediated p19 up regulation. Cycloheximide does not affect basal transcription of p19, neither by its inhibitory effect on protein synthesis, nor as a potential DNA damaging agent. This result suggests, that protein synthesis is required, at least in part, to obtain maximal induction of p19 after damage. It is conceivable that cycloheximide is affecting the abundance of a molecule (inducible or labile) which would be acting directly or indirectly as a positive regulator.

3.4. The only presence of UV-damaged DNA molecules does not suffice to induce p19

Our current results indicate that p19 expression responds to many different DNA damaging agents. Taking this into account, we then asked: Which are the molecular actors that transduce stress signals to p19 promoter? Are UV signals triggered on the cell surface necessary to achieve the complete endpoint response? or Does the mere presence of DNA lesions suffice to produce the observed induction of this INK4?.

To answer these questions, first we transiently transfected cells with single strand or double strand ODN, harboring sequences with adjacent thymidines. We designed them in this way, to allow dimer formation after UV irradiation, a type of photolesion that is recognized by NER machinery. Moreover, damaged single strand

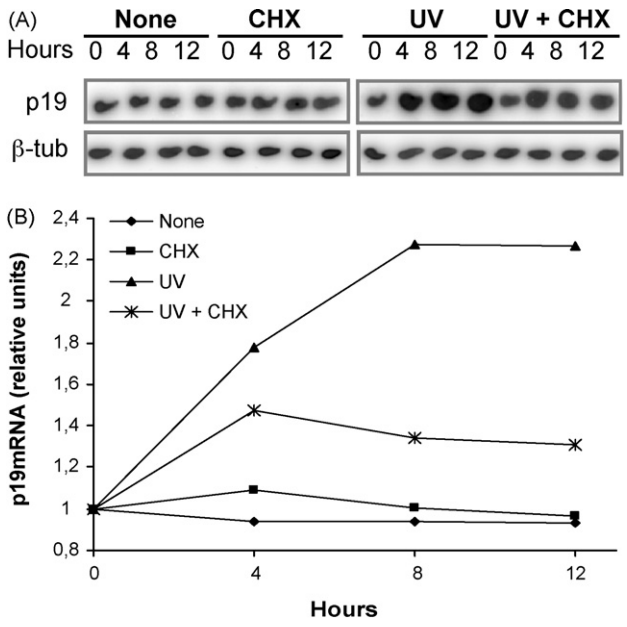

Fig. 3. DNA-damage induced p19INK4d upregulation requires active protein synthesis. (A) BHK-21 fibroblasts were irradiated with 8 mJ/cm^2 UV or not, after incubation with 10 μM cycloheximide (CHX) for 3 h, and harvested at the indicated time points following irradiation. The expression of p19 was assessed by Northern blotting as described in Section 2. β-Tubulin (β-tub) was used as loading control. The figure shows a representative autoradiograph of two independent experiments. (B) p19 mRNA level was quantified for one representative experiment with ImageJ software.

ODN were used to resemble the fragments of approximately 32 nucleotides excised by this repair mechanism. Then we examined expression of p19, p16 and p21. It was reported that thymidine dinucleotide (pTpT) stimulates melanogenesis in mammalian cells and in intact skin, mimicking the effects of UV irradiation. Moreover, pTpT enhanced DNA repair capacity, in part by upregulating a set of genes involved in DNA repair (ERCC3 and GADD45) and cell cycle inhibition (p21) (Eller et al., 1997). Notably, only p21 gene was induced when cells were transfected with the mentioned damaged DNA structures. The presence of UV treated DS oligonucleotides caused a robust induction of p21 as early as 4 h after treatment, while irradiated SS oligonucleotides provoked a slight increase (Fig. 4A).

UV-induced intrastrand DNA cross-link give rise to photoproducts that are potent inhibitors of transcription by RNA polymerase II (RNAP II). Bulky adducts present in the template strand of a transcribed gene very efficiently block elongating RNAP II (Lindsey-Boltz and Sancar, 2007). Thus, RNAP II stalled at damaged bases constitutes itself a signal for DNA repair, DNA damaged checkpoints and apoptosis. To deepen our understanding into the nature of the signals that mediate p19 induction we also transfected cells with an expression plasmid, encoding CAT gene, irradiated or not with 64 mJ/cm^2 UV and studied the role of RNAP II blockage in this event. The effectiveness of UV irradiation and transcription recovery was previously tested by host cell reactivation assays (Scassa et al., 2007). Only cells transfected with damaged plasmid exhibited a robust increase in p21 expression, similar to that elicited in UV irradiated cells. Importantly, with any of the DNA structures assayed, neither p19 nor p16 mRNA levels were affected (Fig. 4A). So, the presence of damaged DNA is not enough to induce p19 transcription.

We next asked whether the NER pathway had to be fully activated in order to trigger p19 induction. To find the answer, we analyzed p19 mRNA expression in response to different genotoxic agents, in neuroblastoma cells, inhibiting or not NER mechanism. We incubated cells with the NER inhibitor F11782 (Barret et al., 2002), under the same experimental conditions previously used to evaluate DNA repair (Ceruti et al., 2005). F11782 blocks endonuclease activity of ERCC1/XPF and ERCC1/XPG complexes and is used as an anticancer drug. Then, SH-SY5Y cells were damaged with 8 mJ/cm^2 UV or incubated with 20 μM cisplatin or 10 μM β-amyloid peptide and samples were harvested 24 h later to analyze mRNA level. As NER machinery does not repair β-amyloid peptide induced lesions, we used β-amyloid peptide as control, to ensure that F11782 inhibitor is not affecting the activity of proteins involved in DNA damage responses initiated by genotoxics different from those that cause intrastrand cross-links. p19 expression augmented in all cases of damage independently of the presence of NER inhibitor (Fig. 4B). This result indicates that p19 is induced before endonucleases can release the damaged DNA fragment, supporting the concept that excised repair fragments are not sufficient to activate the response.

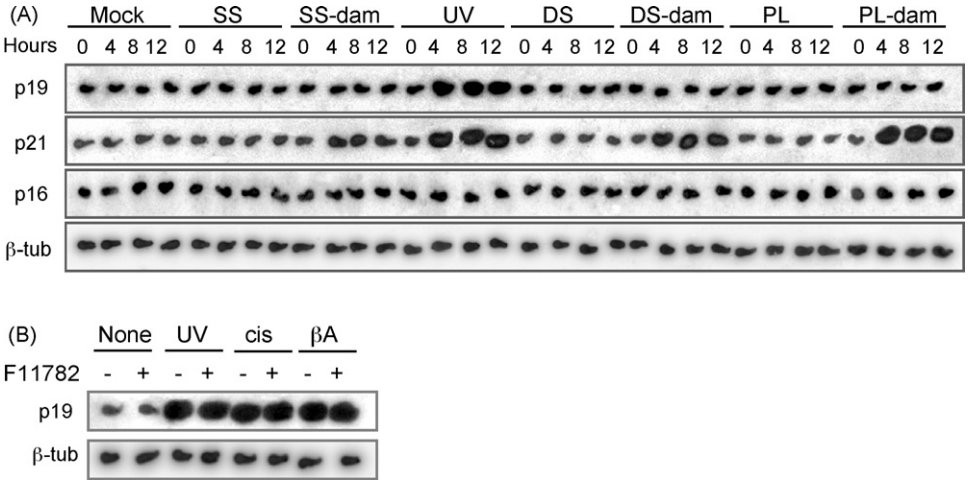

Fig. 4. (A) Damaged-DNA structures treatment does not suffice to transcriptionally upregulate p19. BHK-21 fibroblasts were mock treated (MOCK), transfected with undamaged (SS) or 64 mJ/cm^2 UV-damaged SS oligonucleotides (SS-dam) or irradiated with 8 mJ/cm^2 UV (UV), or transfected with undamaged (DS) or 64 mJ/cm^2 UV-damaged DS oligonucleotides (DS-dam) or transfected with pCMVcat (PL) or UV-irradiated plasmid (PL-dam) and harvested at the indicated time points following treatment. (B) p19 induction after DNA damage is insensible to NER inhibition. SH-SY5Y neuroblastoma cells were irradiated with 8 mJ/cm^2 UV or treated with 20 μM cisplatin (cis) or 10 μM β-amyloid peptide (βA) in the presence or absence of 50 μM F11782 and harvested 12 h following treatment. NER inhibitor was added 30 min prior to genotoxic insults. The expression of p19 and p21 was assessed by Northern blotting, as described in Section 2. β-Tubulin (β-tub) was used as loading control. Each figure shows a representative autoradiograph of two independent experiments.

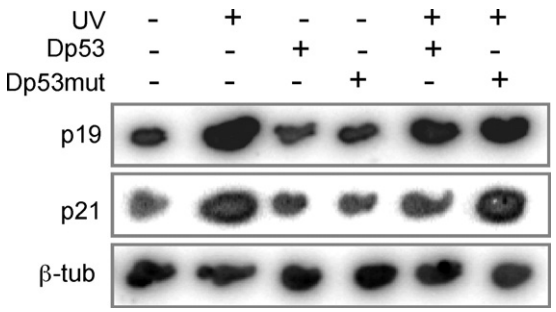

Fig. 5. p19 induction after UV irradiation is p53-independent. BHK-21 cells were irradiated with 8 mJ/cm^2 UV and treated with p53 wild type (Dp53) or mutated (Dp53mut) decoy ODN (100 nM), as indicated. The expression of p19, p21 and β-tubulin mRNA was assessed by Northern blotting, as described in Section 2. The figure shows a representative autoradiograph of two independent experiments.

As a whole, these results allow us to hypothesize that p19 induction after UV irradiation would need another event involved in DDR mounted by the cell, like extracellular signals, subsequent activation of secondary transduction cascades, or chromatin distortion generated by the lesions.

3.5. DNA damage mediated induction of p19 is independent of p53

ATM and ATR stimulate DNA repair and regulate cell cycle in response to DNA damage (Kuhne et al., 2004). Both kinases are required to activate p53 after ionizing irradiation however, only ATR is necessary to activate p53 after UV (Siliciano et al., 1997; Tibbetts et al., 1999). It is known that p21 is predominantly regulated by p53 under cellular stress, and induced at the transcriptional level after UV, ionizing irradiation or hypoxia (O'Reilly et al., 2005). Moreover, our current results, consistently with those reported by Goukassian et al. (1999) show that p21 mRNA is augmented in presence of oligonucleotides that contain thymidine dimers, meaning that the damaged DNA itself is a signal capable of inducing this gene. The observation that stalled RNAP II leads to p53 induction (Derheimer et al., 2007), would support the hypothesis that p21 induction observed after transfection with damaged plasmid could be mediated by this transcription factor.

The relevance of p53 in the DNA damage response and the differences observed between p21 and p19 regulation after UV, prompted us to elucidate whether this tumor suppressor is involved in p19 modulation. To address this issue, we used an experimental approach to evaluate the effect of p53 displacement from its target promoters on UV mediated p19 upregulation. We supplied BHK-21 cells with dumbbell-shaped decoy ODN (100 nM) harboring the p53 consensus binding site or a mutant version. As shown in Fig. 5, p19 upregulation was similar with both type of decoy ODNs, indicating that induction is not sensitive to p53 displacement. Conversely, p21 upregulation was significantly affected only when cells were treated with p53 consensus version of decoy ODN. Taking into account our previous findings describing the enhanced ability displayed by p19-overexpressing Saos-2 cells (p53 deficient) to repair UV-induced DNA damage (Ceruti et al., 2005) we propose that p19 not only is induced in a p53 independent manner upon DNA damage but also contributes to DDR without requiring the presence of p53.

3.6. p19 improves DNA repair and diminishes apoptosis in neuroblastoma cells independently of the genotoxic agent

Overexpression of p19 increases the repair of UV-damaged DNA. Moreover, DNA repair synthesis was dramatically reduced in

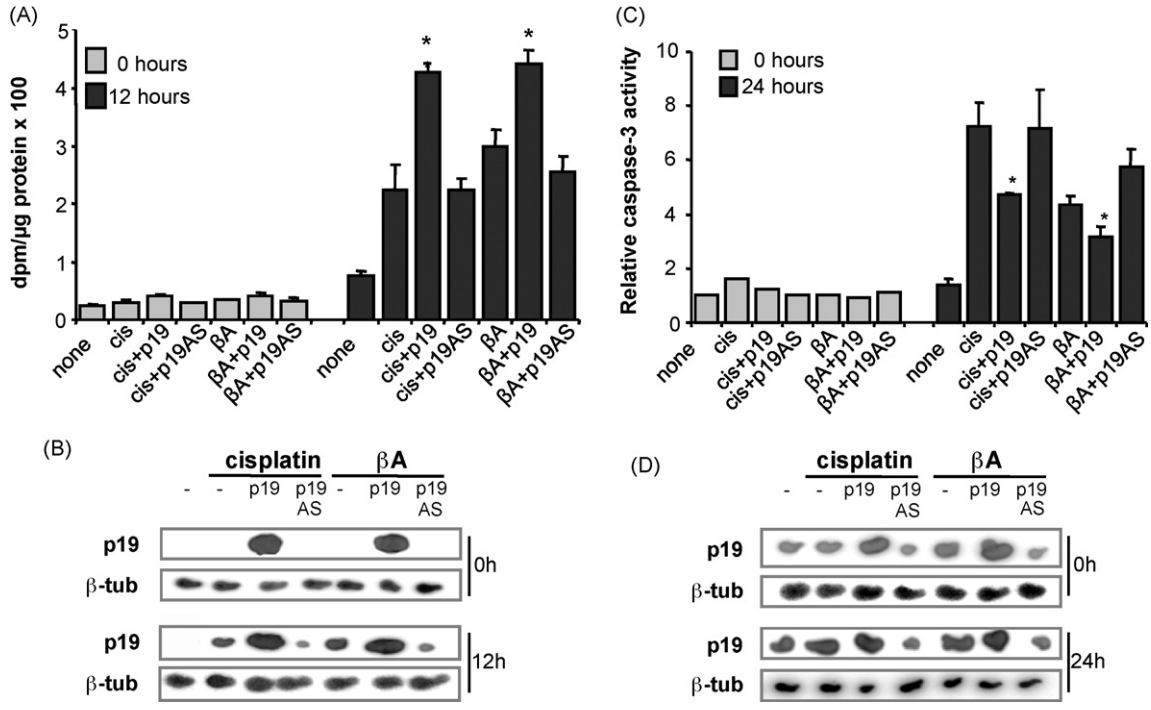

Fig. 6. p19 enhances the ability to repair cisplatin or β-amyloid peptide induced DNA lesions and diminishes apoptosis of neuroblastoma cells. SH-SY5Y cells were transfected with 2.5 μg of the vector encoding sense (p19) or antisense (p19AS) cDNA along with 0.5 μg of pBabePuro. Twenty-four hours after transfection regular medium was substituted for arginine-free medium containing 1% FBS and then cells were treated for additional 60 h in the presence of puromycin. Resistant cells were treated with 20 μM cisplatin (cis) or 10 μM β-amyloid peptide (βA) for 12 h. (A) After genotoxic insults, cells were incubated with 10 μCi [^{3}H]thymidine and 12 h later cell lysate were tested for UDS. (C) Twenty-four hours later cells were tested for caspase-3 activity. Results are expressed as percentage of caspase-3 activity with respect to basal activity of cell lysates derived from nontransfected and without genotoxic addition at time point 0 h, which was set to 100. In (A) and (C) bars represent the mean ± S.E. of three different experiments performed in duplicate. Student *t*-test was used to compare samples cotransfected and genotoxic treated with samples noncotransfected and cisplatin or β-amyloid treated (*$P < 0.05$). (B, D) Expression level of p19 mRNA from treated or untreated samples were assessed by Northern blotting, as described in Section 2. β-Tubulin (β-tub) was used as a loading control. The figure shows a representative autoradiograph of two independent experiments.

UV-irradiated p19-deficient cells. These data indicate that enhancement of DNA repair results specifically from overexpression of p19, but also that endogenous p19 is necessary to drive a complete response to UV damage (Ceruti et al., 2005; Scassa et al., 2007). To deepen in the study about the effect of p19 on DNA repair, we carried out unscheduled DNA synthesis assays, incubating neuroblastoma cells with 20 μM cisplatin or 10 μM β-amyloid peptide as damaging agents. Cells transfected with a vector that overexpress p19 incorporated significantly more ^{3}H-thymidine that cells transfected with empty vector in both treatments (Fig. 6A), indicating that there is a more efficient DNA repair. On the other hand, expression of antisense p19 did not affect the basal cellular response to these genotoxics. It suggests that the damage caused with these doses of cisplatin or β-amyloid peptide is not as significant as that provoked by UV. If this was the case the absence of p19 would not represent a disadvantage for the cell. These evidences, confirm the hypothesis that p19 participates directly or indirectly in DNA repair, independently of the genotoxic agent, improving the efficiency of cellular response to confront the damage.

Next, we investigated if p19 intracellular level influences the apoptotic response caused by these genotoxics as occurred with UV. To this end, we determined caspase-3 activity in SH-SY5Y cells, after 20 μM cisplatin o 10 μM β-amyloid peptide for 24 h treatment (Fig. 6C). As expected, both treatments displayed an increase in caspase-3 activity, reflecting the presence of apoptotic cells. Remarkably, p19 overexpressing cells were significantly protected (35% and 31%) against programmed cell death provoked by either genotoxic. Cells with diminished levels of p19 and treated with β-amyloid peptide showed enhanced caspase-3 activity than their untransfected counterparts (21%). Taken together, these findings, enforced the hypothesis that p19 would play a fundamental role protecting cells from apoptosis induced by DNA damage independently of the genotoxic agent.

3.7. p19 overexpression confers increased cellular resistance to different genotoxics

To address if the up or downregulation of p19 has any biological significance and plays any functional role in the cellular responses upon genotoxic treatment, we first examined cell survival on BHK-21p19S or BHK-21p19AS cells. p19 overexpression has been shown to arrest cells in G1 phase, thus to avoid this antiproliferative effect of p19, we induced metallothionein promoter driving sense or antisense p19 cDNA, only 6 h before and 24 h after genotoxic addition and measured cell survival by MTT reduction at different times during the 6 days following the treatment. Control assays showed that the presence of $ZnSO_4$ has no effect on cell proliferation (data not shown). We observed significant differences in the ability of BHK-21p19S or BHK-21p19AS compared to control cells to reduce MTT, in accordance with the initial status of p19 (Fig. 7A). BHK-21p19S cells showed an enhanced cellular growth (37% and 39%) upon cisplatin or β-amyloid peptide treatment compared to BHK-21empty clonal line. Conversely, BHK-21p19AS cells were more sensitive (45% and 42%) to genotoxic insults than empty ones. This protective effect of p19 becomes more relevant if we take into account that, ini-

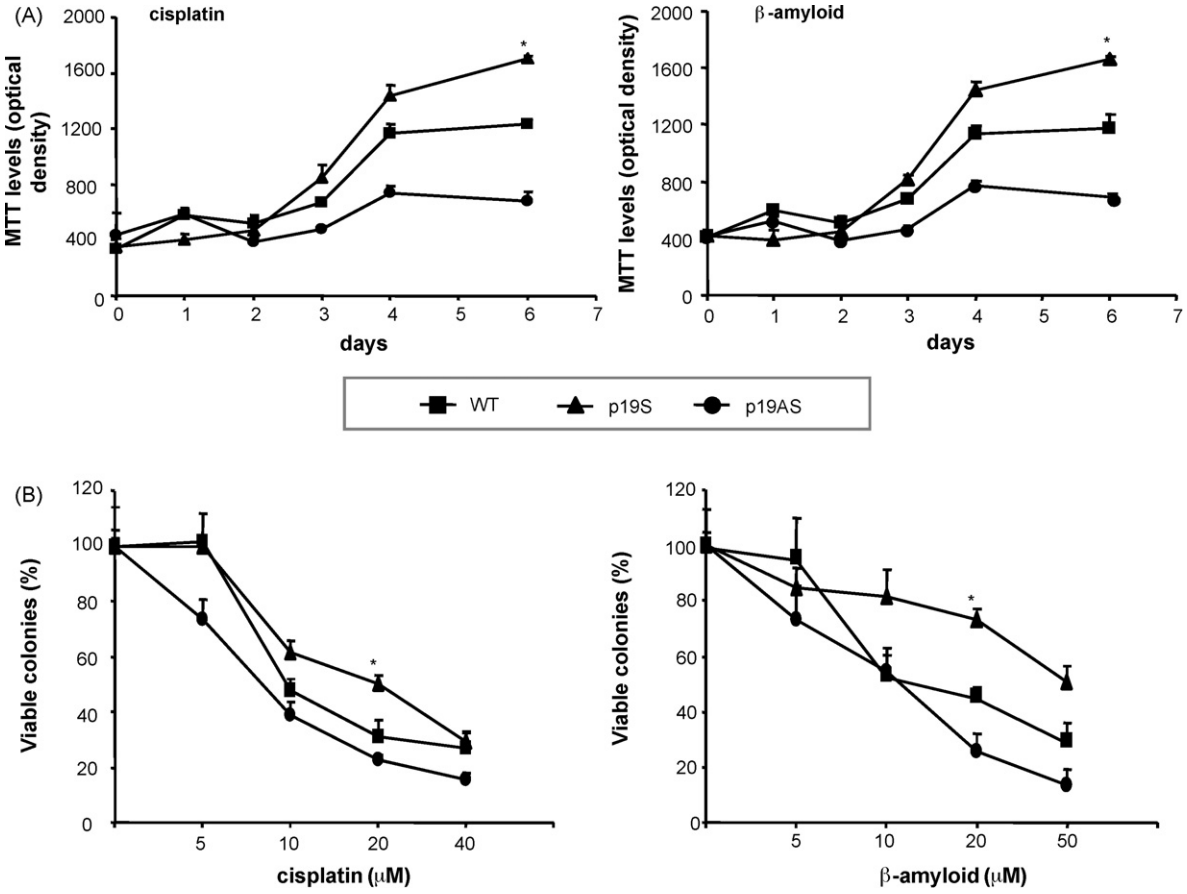

Fig. 7. p19 confers resistance to cisplatin and β-amyloid peptide. (A) Cell viability was assessed MTT reduction assay in BHK-21p19S, BHK-21p19AS or BHK-21empty clonal cell lines during 6 days after 20 μM cisplatin or 10 μM β-amyloid peptide as described under Section 2. (B) Clonogenic survival was assessed in the same BHK-21 derived clonal cell lines 6 days after genotoxic treatment. At this time, colony count range from 50 to 60 for each untreated cell line. This value was set to 100%. In (A) and (B), each point represents the mean ± S.E. of three different experiments performed by sextuplicate. Student's *t*-test was used to compare BHK-21p19S or BHK-21p19AS with BHK-21 empty samples (*$P < 0.05$).

tially, the three clonal lines are endowed with different proliferative potentials (Fig. 7A).

To further analyze the long term effects and physiological relevance of p19 on cell survival, we performed clonogenic assays. Again, we did not observe any significant differences in $ZnSO_4$ treated cells indicating that all cell lines possess similar colony forming capacity despite their variations in their initial proliferative rates (data not shown).

Dose–response curve differed significantly depending on p19 status. We found that 31% of the colonies from BHK-21empty cell line survived at 20 μM cisplatin while 50% of BHK-21p19S stayed alive at the same dose (Fig. 7B). In this assay BHK-21empty and BHK-21p19AS cells yielded similar effects on clonogenicity. β-Amyloid peptide (20 μM) treated cells show to be more protected when overexpressing p19, given that 51% of BHK-21p19S cells formed colonies, while only 29% of BHK-21empty and 14% of BHK-21p19AS cells did. In this case significant differences between BHK-21p19AS and BHK-21empty clonal cells were observed. Collectively, these results contribute to establish a role for p19 in cellular resistance to different genotoxic stress.

Finally, there are some important points to remark: first, the fact that p19 is transcriptionally upregulated by various genotoxics, improving the repair of different types of DNA damage; second, our finding that damaged single strand ODN, double strand ODN or plasmid represent structures sufficient for p21 induction, but not for p19 increase; third, the induction of p19 in presence of NER inhibitor. As a whole, these results open new questions: may p19 be a sensor of DNA damage? Is the basal level of p19 enough to carry out this sensing in time? Some of those damaged structures are generated during stalled replication or NER, however they do not suffice to trigger p19 up-regulation. In absence of exogenous damage, p19 is periodic along cell cycle, peaking at mid G1/S. Global chromatin relaxation constitutes a hallmark of DNA replication, while chromatin distortions are common features of genotoxics action. Thus, it stands to reason that alterations in chromatin may trigger signals towards p19 promoter. Sporadic lesions arising during the fine tuned process of DNA replication need to be removed quickly and efficiently. Thus, an improved repair activity at a precise time point of cell cycle progression may explain, at least in part, the appearance of basal levels of p19 transcript in midG1/S. Conversely, sudden appearance of numerous aberrant DNA structures in a chromatinized context after damage, as occur in vivo, would be accompanied by additional signals responsible for p19 upregulation. Moreover, we could establish a wide role for p19 in cellular resistance to genotoxic stress. In the future, this CDK4/6 inhibitor could represent a target in neurodegenerative diseases or anticancer therapies, so, it is our challenge to elucidate the fine molecular events implicated in its regulation and action.

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