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p53 Protects renal inner medullary cells from hypertonic stress by restricting DNA replication

NATALIA DMITRIEVA, LUIS MICHEA, AND MAURICE BURG
National Heart, Lung, and Blood Institute, Bethesda, Maryland, 20892-1603

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Dmitrieva, Natalia, Luis Michea, and Maurice Burg. p53 Protects renal inner medullary cells from hypertonic stress by restricting DNA replication. *Am J Physiol Renal Physiol* 281: F522–F530, 2001.—We previously found that p53 upregulation by hypertonicity protected renal inner medullary collecting duct (mIMCD3) cells from apoptosis. The purpose of the present study was to investigate the mechanism by which p53 protects the cells. We now find that hypertonicity (NaCl added to a total of 500 mosmol) inhibits DNA replication and delays G_1-S transition as concluded from analysis of cell cycle distributions and bromodeoxyuridine (BrDU) incorporation rates. Lowering of p53 with p53 antisense oligonucleotide attenuated such effects of hypertonicity, resulting in an increased number of apoptotic cells in S phase and cells with >4 N DNA. Results with synchronized cells are similar, showing that cells in the early S phase are more sensitive to hypertonicity. Immunocytochemistry revealed that p53 becomes phosphorylated on Ser^{15} and translocates to the nucleus in S both in isotonic and hypertonic conditions. Caffeine (2 mM) greatly reduces the p53 level and Ser^{15} phosphorylation, followed by a remarkable increase of DNA replication rate, by failure of hypertonicity to inhibit it, and by reduction of cell number during hypertonicity. Finally, inhibition of DNA replication by the DNA polymerase inhibitor aphidicolin significantly improves cell survival, confirming that keeping cells in G_1 and decreasing the rate of DNA replication is protective and that these actions of p53 most likely are what normally help protect cells against hypertonicity.

cell cycle arrest; apoptosis; sodium chloride

CELLS OF THE renal inner medulla are normally exposed to variable and often extreme hypertonicity as the result of the renal mechanism for concentrating the urine. This raises questions about the mechanisms that they employ to survive and function under such adverse conditions. p53 Is a tumor suppressor whose loss of function, observed in many types of cancer, contributes to genomic instability and malignancy (2, 15, 28). Numerous stresses increase p53 activity, producing either growth arrest until damage is repaired or apoptosis, eliminating cells that are potentially dangerous to the organism. p53 is induced by DNA damage caused by cytotoxic drugs, free radical formation, or ionizing radiation (25). p53 Can also be induced in the absence of observed DNA damage by growth factor withdrawal, hypoxia, metabolic change, virus infection, cytokines, or deregulated expression of cell cycle genes (25).

In previous studies, we found that hypertonicity increased the amount of total and phosphorylated Ser^{15} p53- and p53-dependent transcription in renal inner medullary collecting duct cells (mIMCD3; see Ref. 11). Under these conditions, reducing p53 with p53 antisense oligonucleotide (p53-AS) increased apoptosis, suggesting that activation of p53 is protective (11). Hypertonicity also arrests growth of mIMCD3 cells (7, 21, 26, 32) and increases levels of GADD45 (21), a growth arrest and DNA damage-inducible protein whose transcription is regulated by p53 (40).

The purpose of present study was to analyze the mechanism by which p53 protects against hypertonicity. We found that phosphorylation of p53 on Ser^{15}, previously noted to be protective during hypertonicity, occurs in the S phase of the cell cycle. Furthermore, reducing p53 expression with p53-AS or caffeine reversed both the G_1-S arrest and reduction of DNA replication that are caused by hypertonicity. Under those conditions, apoptosis increased mainly in the cells in which DNA content had increased. We conclude that p53 protects cells against hypertonic stress by restricting DNA replication.

EXPERIMENTAL PROCEDURES

Cell culture. Subconfluent cultures of mIMCD3 cells (generously provided by S. Gullans; see Ref. 31) were used in passages 13–17. The medium contained 45% DME low glucose, 45% Coon's Improved Medium mF-12 (Irvine Scientific), and 10% FBS (Life Technologies). Osmolality of control ("isotonic") medium was 320 mosmol/kg. Hypertonic media, prepared by adding NaCl, were substituted for the control medium, as indicated. Cells were incubated at 37°C and gassed with 5% CO_2-95% air during growth and during all experiments.

Antisense oligonucleotide experiments. For all experiments with p53-AS (Biognostik), cells were grown on eight-chamber plastic slides (Nalge Nunc International) and preincubated for 16 h with 2 μM of p53-AS (sequence: CGT CAT GTG CTG TGA C) or control (CG-matched randomized-sequence phosphorothioate oligonucleotide: GAC TAC GAC CTA CGT G).

Address for reprint requests and other correspondence: N. Dmitrieva, Bldg. 10, Rm. 6N260, National Institutes of Health, Bethesda, MD 20892-1603 (E-mail: dmitrien@nhlbi.nih.gov).

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Next, the media were changed to iso- or hypertonic ones containing the same oligonucleotides.

Fixation and propidium iodide staining for cell cycle and apoptosis analysis. Cells on the plastic slides were fixed in 100% methanol at −20°C for 15 min. After fixation, the cells were permeabilized with 0.1% Triton X-100, incubated with 1 mg/ml RNase (Sigma) for 15 min, stained with 20 μg/ml propidium iodide (PI) for 5 min, and then mounted with 150 μl of antifade (no. S-7461; Molecular Probes).

The slides were analyzed by a laser-scanning cytometer (LSC; CompuCyte) as previously described (5, 8, 11). In brief, red fluorescence was recorded as a measure of PI binding (DNA content). The data were displayed as histograms showing cell number vs. DNA content or bivariate plots of peak PI fluorescence vs. DNA content. The peak intensity is the highest pixel value within the nucleus, which increases as nuclear chromatin condenses in apoptotic or mitotic cells (5, 8). A gate representing the approximate limit of peak fluorescence in control cells was set by eye. Peak fluorescence exceeding this limit identifies chromatin condensation associated with apoptosis or mitosis. In this study, mitotic cells were only 4–6%, and this number become even less with hypertonicity when apoptosis was stimulated (Fig. 2). That is why maximal pixel PI fluorescence was accepted as a suitable criterion for determination of apoptotic cells.

Analysis of mitosis by immunostaining with anti-phospho-histone H3 and anti-α-tubulin antibody. After fixation in 100% methanol at −20°C for 45 min, the cells were washed three times for 5 min each with 0.1% Triton X-100 in PBS, followed by blocking buffer (3% BSA-0.1% Triton X-100). Next, they were incubated with anti-phospho-histone H3 (mitosis marker, no. 06–570; Upstate Biotechnology) or anti-α-tubulin (no. F2168; Sigma) antibody, washed with 0.1% Triton X-100 in PBS, incubated for 1 h with secondary antibody (Alexa 488 goat anti-mouse or anti-rabbit IgG, nos. A-11029 and A-11034; Molecular Probes), stained with 0.7 μg/ml PI, and mounted with 150 μl of antifade (no. S-7461; Molecular Probes). Cells were observed under a microscope, and the percentage of phospho-histone H3-positive (mitotic) cells or cells with anaphase morphology were calculated. At least 2,000 cells were counted for each point.

Analysis of intracellular localization of p53 by immunostaining with anti-p53 and anti-phospho-p53(Ser^{15}) antibodies. The immunostaning procedure was the same as for anti-α-tubulin, except that the primary antibodies were anti-p53 (no. 1 413 147; Boehringer Mannheim) or anti-phospho-p53(Ser^{15}) (no. 9284; New England Biolabs).

We used LSC to analyze intracellular distribution of p53 (its localization in the nucleus versus cytoplasm) and we correlated this with the cell cycle position. This approach has been described in detail previously (9, 10). In brief, green fluorescence was recorded as a measure of p53 or p53(Ser^{15}), and red fluorescence was recorded as a measure of PI binding. The integrals of green fluorescence of p53 or p53(Ser^{15}) antibodies in the nucleus or cytoplasm of each cell and of nuclear PI staining of that cell were measured. The area of the nucleus in each cell was defined on the basis of PI staining. An annulus 1-μm wide around each nucleus was taken to represent cytoplasm. To find nuclear (F_n) and cytoplasmic (F_c) fluorescence intensity, the integrals of green fluorescence were normalized to the areas. To evaluate translocation of p53 or p53(Ser^{15}) during the cell cycle, the ratio of green nuclear to cytoplasmic fluorescence (F_n/F_c) was calculated for each cell, and bivariate distributions showing these ratios vs. DNA content were obtained. Images were taken with a Kodak Digital Science DC120 zoom digital camera.

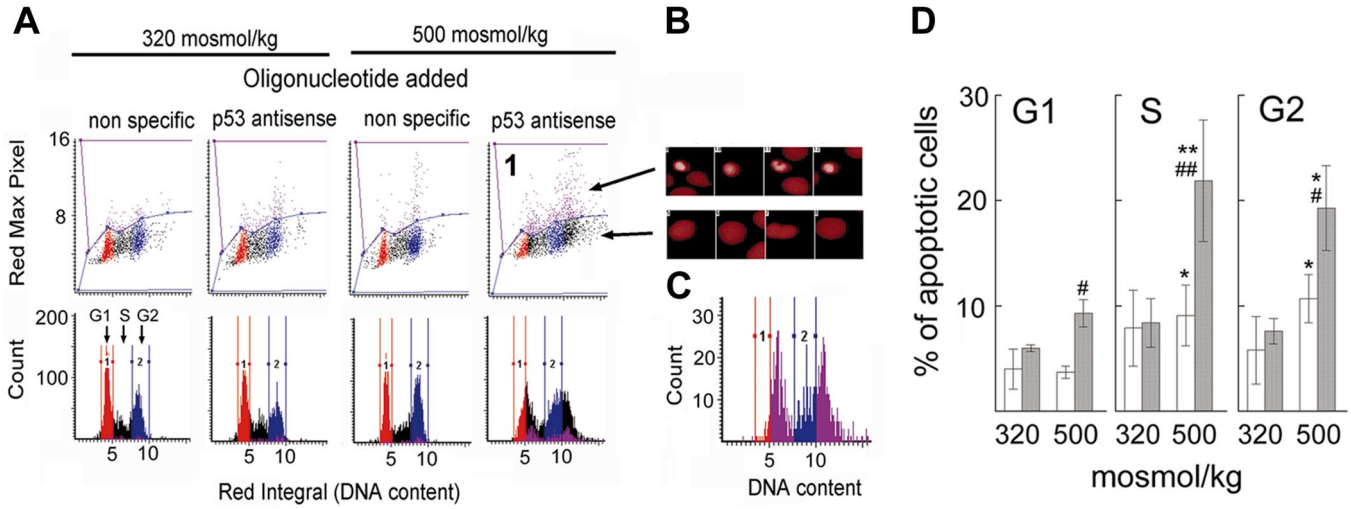

Fig. 1. Effect of p53 antisense (AS) oligonucleotide on cell cycle and survival of inner medullary collecting duct (mIMCD3) cells exposed to hypertonicity. Cells grown on slides at 320 mosmol/kg were incubated for 16 h with p53-AS oligonucleotide or control nonspecific oligonucleotide, and then the medium was replaced with an identical one or with an otherwise identical hypertonic medium (500 mosmol/kg with added NaCl) for the last 6 h. Cells were fixed and stained with propidium iodide (PI) and then analyzed using a laser-scanning cytometer (LSC). *A*: representative cytograms of PI fluorescence peak (PI Max Pixel) vs. PI fluorescence integral (DNA content, *top*) and cell number vs. PI fluorescence integral (DNA content; *bottom*). *B*: cells (PI-stained nuclei) from the regions shown *A* are indicated by arrows. The >4 N DNA content is in individual nuclei, not in doublets. *C*: histogram of cells from *region 1*, showing the DNA content of apoptotic cells (i.e., those with PI staining are bright because their chromatin is condensed). *D*: G_1, S, and G_2 cells were gated based on differences in their DNA content, as shown *A*. Bars indicate the percentage of apoptotic cells (bright cells from *area 1* in *A*) in each phase of the cell cycle. Open bars, nonspecific oligonucleotide added; filled bars, p53-AS. Data are plotted as means ± SE, $n = 3$ experiments. $P < 0.05$ (* and #) and $P < 0.01$ (** and ##) compared with corresponding value for nonspecific oligonucleotide sample (# and ##) or corresponding G_1 value (* and **).

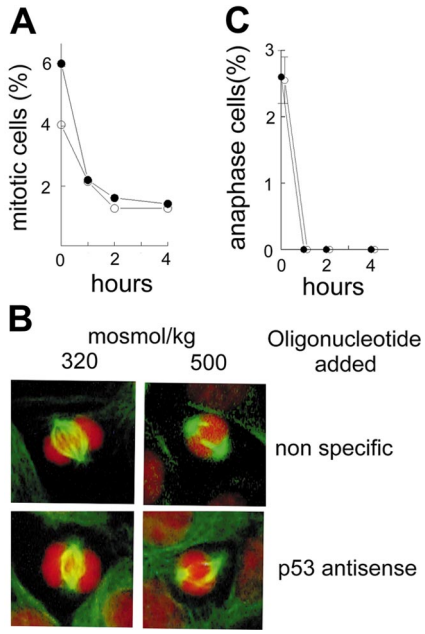

Fig. 2. p53-AS does not abrogate G_2 and mitotic arrest caused by hypertonicity. Cells grown on slides at 320 mosmol/kg were incubated for 16 h with p53-AS oligonucleotide or control nonspecific oligonucleotide, and then cells were exposed for 1, 2, and 4 h to the medium made hypertonic by adding NaCl to 500 mosmol/kg. Cells were stained against phospho-histone H3 (mitosis marker) to find cells in mitosis and against α-tubulin to observe the morphology of the mitotic cells. *A*: percentage of cells in mitosis (phospho-histone H3 positive). Representative of 2 experiments. *B*: representative images of cells with polymerized α-tubulin. Note that anaphase is seen at 320 mosmol/kg but not at 500 mosmol/kg. *C*: percentage of cells that have anaphase morphology. Data are plotted as means ± SE; $n = 3$. ○, Nonspecific oligonucleotide added; ●, p53-AS added.

Protein sample preparation, Western blotting, and immunodetection. Cells were rinsed with PBS adjusted with NaCl to the same osmolality as the medium and then were lysed with 300 μl of lysis buffer (100 mmol/l NaF, 50 mmol/l Tris, 250 μmol/l thimerosal, 1% vol/vol igepal, 16 mmol/l CHAPS, 5 mmol/l activated $NaVO_4$, 50 mg/l Pefabloc, 100 mg/l leupeptin, and 10 mg/l aprotinin). After centrifugation for 20 min at 15,000 *g* and 4°C, the supernatant was separated into aliquots and stored at −80°C. Protein content was measured using the bicinchoninic acid protein assay (Pierce). Proteins were separated by SDS-PAGE. Equal amounts of protein (6 μg) were loaded in each lane of 12% acrylamide-Tris-glycine gels. Immunodetection procedures were carried out using specific antibodies against p53 (no. 1 810944; Boehringer Mannheim) or phospho-p53(Ser^{15}) (no. 9284; New England Biolabs). Densitometry analysis was done with a Molecular Imager FX (Bio-Rad).

Bromodeoxyuridine labeling, immunostaining, and analysis by LSC. Cell cultures were labeled with 10 μM of bromodeoxyuridine (BrDU) for 30 min and processed with a 5-bromo-2-deoxyuridine Labeling and Detection Kit I (catalog no. 1 296 736; Roche) according to the manufacturer's instructions. DNA was stained with 0.7 μg/ml PI.

The slides were analyzed by LSC. Green fluorescence was recorded as a measure of anti-BrDU antibody binding (BrDU incorporation). Red fluorescence was recorded as a measure of PI binding (DNA content). Bivariate distributions of cells showing incorporation of BrDU vs. DNA content were obtained.

Statistical analysis. For statistical analysis, experiments were repeated at least three times and analyzed by ANOVA Parametric Repeated Test followed by the Student-Newman-Keuls Test.

RESULTS

During hypertonic stress caused by high NaCl, p53-AS increases the percentage of cells in early S phase and of cells with >4 N DNA content, resulting in apoptosis. To assess p53-dependent cell cycle changes, mIMCD3 cells stressed by high NaCl were exposed to p53-AS. We have shown previously that the p53-AS decreases abundance of p53 in mIMCD3 cells (11). A representative example of cell cycle analysis is shown in Fig. 1, *A*-*C*. Cells for all experimental conditions were grown on the same eight-chamber slide to ensure that fixation and DNA staining were uniform for all of the samples. Cells were gated in G_1, S, and G_2/M phases of the cell cycle based on DNA content. To establish the gates, an isotonic sample with nonspecific oligonucleotide was used (Fig. 1*A*). For the isotonic sample with no treatment, the position of the gates was

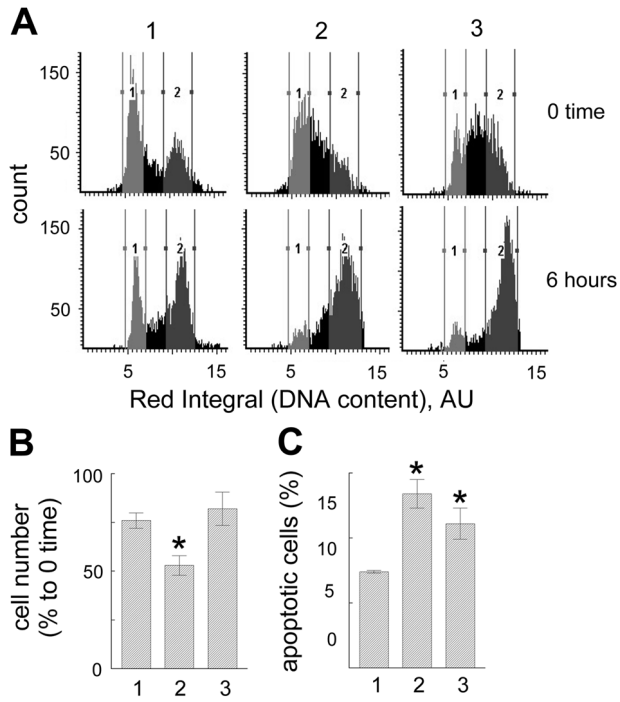

Fig. 3. Effect of cell cycle position on tolerance to hypertonicity. Cells grown on slides at 320 mosmol/kg were synchronized in different phases of the cell cycle by incubation with the reversible DNA polymerase inhibitor aphidicolin (1 μM) for 9 or 14 h. Next, aphidicolin was washed out, and cells were exposed for 6 h to 320 mosmol/kg medium or medium made hypertonic to 500 mosmol/kg with added NaCl. Cells were stained with PI. *A*: cell cycle immediately before (*time 0*) and 6 h after adding NaCl. 1, No synchronization; 2, 9 h preincubation with aphidicolin; 3, 14 h preincubation with aphidicolin. *B*: no. of nonapoptotic cells after 6 h with hypertonicity (PI max pixel not elevated), counted by LSC and normalized to the number at *time 0*. *C*: percentage of apoptotic cells after 6 h with hypertonicity. Apoptotic cells were found based on nuclear brightness, as shown in Fig. 1. *B* and *C*: data plotted as means ± SE; $n = 3$. *$P < 0.05$, compared with corresponding condition for nonsynchronized cells.

the same (data not shown). The same gates were used for analysis of all samples. In isotonic conditions, p53-AS do not affect the cell cycle. Hypertonicity, as previously observed (7, 21, 26, 32), increases the proportion of cells in G_2. In the presence of p53-AS after 6 h of hypertonic stress (500 mosmol/kg with added NaCl), the proportion of cells in early S phase and of cells with >4 N DNA content increases greatly (Fig. 1*A*). Because it is conceivable that cell doublets could be mistaken for cells with >4 N DNA content, representative cells were examined microscopically, which confirmed that they were in fact individual cells (Fig. 1*B*). These changes of the cell cycle caused by p53-AS resulted in an increase in the proportion of apoptotic cells, identified by the high red max pixel values (Fig. 1, *A* and *B*) that are associated with condensed chromatin (5, 11). Cell cycle analysis of these apoptotic cells shows that they are predominantly the cells that have progressed into early S phase or that have >4 N DNA content (Fig. 1*C*). Analysis of the percentage of apoptotic cells confirmed this observation (Fig. 1*D*). In the presence of p53-AS, the increase in the percentage of apoptotic cells is much bigger in the S and G_2 phases compared with the G_1 phase. Among those cells that have >4 N DNA content, 27 ± 7% were apoptotic. Of note even without p53-AS under hypertonicity, there was a higher percentage of apoptotic cells in S and G_2 phases compared with the G_1 phase (Fig. 1*D*).

p53-AS does not abrogate G_2 and mitotic arrest caused by hypertonicity. Appearance of cells with >4 N DNA content and an increased level of apoptosis among these cells suggest some disregulation of G_2-M progression in the presence of p53-AS. Hypertonicity normally induces G_2 arrest (7, 21, 26, 32). Staining of the cells with the mitotic marker phospho-histone H3 (Fig. 2*A*) revealed that p53-AS do not abrogate the G_2 arrest that takes place after hypertonic stress; there is a great decrease in the percentage of mitotic cells both with nonspecific oligonucleotide and with p53-AS. To evaluate morphology of the mitotic cells, we stained the cells with antibody to α-tubulin. As α-tubulin polymerizes to form a mitotic spindle, it becomes concentrated,

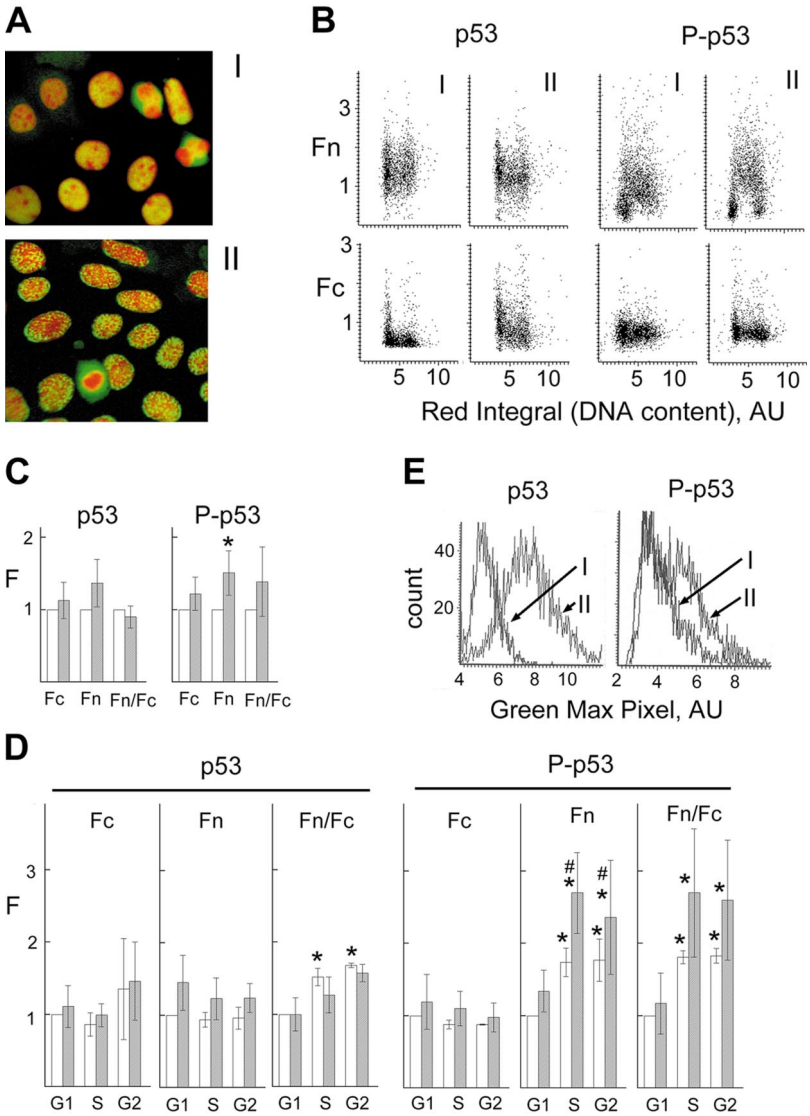

Fig. 4. Immunocytochemical analysis of intracellular localization and levels of p53 and phospho-p53(Ser^{15}). Cells were grown on slides at 320 mosmol/kg, and then the medium was replaced with an identical one of 320 mosmol/kg (I) or with hypertonic medium (II; 600 mosmol/kg with added NaCl) for 1 h. Cells were stained with anti-p53 or anti-phospho-p53(Ser^{15}) antibody (green) and PI (red). *A*: images of cells stained with p53. *B*: representative cytograms plotting green fluorescence intensity [p53 or phospho-p53(Ser^{15}) content] in the nucleus (F_n) or cytoplasm (F_c) vs. red fluorescence integral (DNA content). *C*: changes in F_c, F_n, and their ratio (F_n/F_c) after 1 h with hypertonicity. Bars represent average values for all cells normalized to 320 mosmol/kg control. Open bars, 320 mosmol/kg control; filled bars, hypertonicity. Data are plotted as means ± SE; $n = 3$. $P < 0.05$ compared with 320 mosmol/kg. *D*: F_c, F_n, and F_n/F_c of the cells gated in G_1, S, and G_2/M based on the differences in their PI fluorescence (DNA content), as shown in Fig. 1. Data are normalized to the values for G_1 cells of 320 mosmol/kg control and are plotted as means ± SE; $n = 3$. #$P < 0.05$ compared with corresponding 320 mosmol/kg. *$P < 0.05$ compared with corresponding value for G_1 cells. *E*: cytograms representing cell number vs. maximal brightness of green fluorescence (green max pixel) from the nucleus area of control cells and cells exposed to hypertonicity.

resulting in bright green staining with anti-α-tubulin antibodies. At 320 mosmol/kg, ~2.5% of the cells are in normal anaphase with mitotic spindles partitioning chromosomal DNA (Fig. 2*B*). After 1–4 h of hypertonicity (500 mosmol/kg with added NaCl), none of the cells are in normal anaphase with partitioned DNA, indicating complete mitotic arrest (Fig. 2, *B* and *C*). p53-AS does not change the result; there are normal mitoses at 320 mosmol/kg, but there still are no mitoses at 500 mosmol/kg. Therefore, p53-AS abrogates neither G_2 nor mitotic arrests caused by hypertonicity, suggesting a different mechanism of its action.

Cells in early S phase of the cell cycle are most sensitive to hypertonic stress caused by NaCl. Analysis of p53-AS effects on the cell cycle (Fig. 1) showed that a proportion of cells, which become apoptotic under hypertonicity, is greatest in the S and G_2 phases of the cell cycle, both in control conditions and with p53-AS. p53-AS increases apoptosis in all phases of the cell cycle, but this increase is greatest in S and G_2. This observation suggests that cells in the S and G_2 phases have increased sensitivity to hypertonic stress. To further test this conclusion, we analyzed the survival under hypertonic conditions of cells synchronized in different phases of the cell cycle. We used an inhibitor of DNA polymerase, aphidicolin, to achieve synchronization. After 9 h of incubation with aphidicolin (1 μM), the majority of cells are in late G_1-early S (Fig. 3*A*). After 14 h of incubation with aphidicolin, the cells are mostly in late S-G_2 (Fig. 3*A*). After the synchronization, aphidicolin was washed out, and the medium was made hypertonic (500 mosmol/kg with added NaCl) for 6 h. Next, the number of nonapoptotic ("healthy") and the percentage of apoptotic cells were counted by LSC after PI staining. Hypertonicity decreased the number of healthy cells (Fig. 3*B*). The decrease in cell number and increase in the percentage of apoptotic cells were greater when the cells had been synchronized in early S by 9 h of previous exposure to aphidicolin than when the cells were unsynchronized or synchronized in late S-G_2 (Fig. 3, *B* and *C*). This result confirms the observation from Fig. 1 that cells are more sensitive to hypertonicity in early S. We conclude that increased p53 activity might directly protect them in early S, indirectly protect them by arresting them in G_1, or both.

At the G_1-S interface of the cell cycle, p53 becomes phosphorylated on Ser^{15} and translocates to the nucleus, unaffected by tonicity. Because the effects of p53-AS and hypertonicity by itself appeared to be dependent on cell cycle position, we attempted to relate the expression and localization of p53 and phospho-p53(Ser^{15}) with the position of the cell in the cell cycle. For this analysis, we stained cells for p53 or phospho-p53(Ser^{15}) with specific antibodies and with PI for DNA content and used LSC for the analysis in a way that has been described in several publications (8–10). Images of cells stained against p53 are shown in Fig. 4*A*. It can be seen that the intensity of green fluorescence representing p53 concentration and its localization varies between cells. Figure 4*B* shows representative cytograms plotting green fluorescence intensity from the nuclear area (F_n) or from cytoplasmic area (F_c) vs. DNA content. Hypertonicity only slightly increased F_c and F_n for p53 and phospho-p53 if all cells are taken in analysis together (Fig. 4*C*). This increase was significant only for nuclear fluorescence for phospho-p53(Ser^{15}). Because on the basis of differences in DNA content it was possible to distinguish G_1, S, and G_2/M cells, F_c, F_n, and the ratio F_n/F_c were estimated for cells in each of these phases by gating analysis (as it was done on Fig. 1; see Fig. 4*D*). As is evident, hypertonicity significantly increased F_n for phospho-p53(Ser^{15}) in the S phase. F_n/F_c also have a trend to increase with hypertonicity in S and G_2, meaning that phosphorylated p53 is localized in the nucleus. Moreover, even in isotonic conditions, F_n and F_n/F_c for phospho-p53(Ser^{15}) are higher in S and G_2 cells. The higher level of F_n/F_c is found also in isotonic conditions for total p53. These results suggest that p53 translocates to the nucleus and becomes phosphorylated as

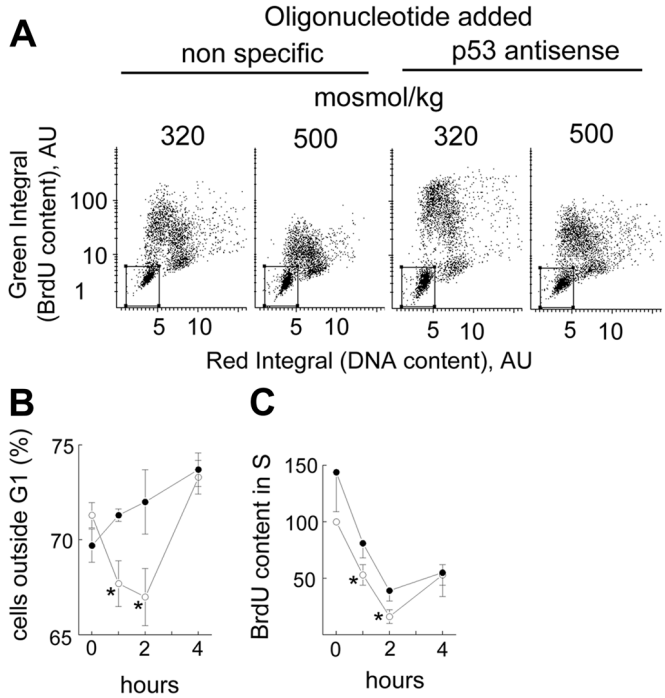

Fig. 5. Effect of p53-AS oligonucleotide on bromodeoxyuridine (BrDU) incorporation by mIMCD3 cells exposed to hypertonicity. Cells grown on slides at 320 mosmol/kg were incubated for 16 h with p53-AS oligonucleotide (●) or control nonspecific oligonucleotide (○), and then the medium was replaced with otherwise identical hypertonic medium (500 mosmol/kg with added NaCl) for 1, 2, or 4 h. BrDU was added during the last 30 min. Cells were fixed, stained with anti-BrDU antibody (green fluorescence) and PI (red fluorescence), and analyzed by LSC. *A*: representative cytograms, obtained for 1-h time point samples, plotting green integral (BrDU content) vs. red integral (DNA content). *B*: percentage of cells outside G_1 phase of the cell cycle (outside of the gate shown in *A*, containing BrDU-negative cells with G_1 DNA content). Data are plotted as means ± SE; $n = 3$. *$P < 0.05$ vs. p53-AS and *time 0*. *C*: BrDU content in S phase cells (average green integral from BrDU-positive cells) plotted as percentage of the value obtained for the isotonic condition with nonspecific oligonucleotide. Data are plotted as means ± SE; $n = 3$. *$P < 0.05$ vs. p53-AS.

cells move from G_1 to the S phase. This approach did not give us a large increase in p53 and phospho-p53(Ser^{15}) levels as seen by Western analysis (6). The possible explanation for such inconsistency is that, under hypertonicity, p53 translocates to very localized spots on DNA (Fig. 4*A*) that could lead to underestimation of integrated fluorescence. Such translocation of p53 is detected by the increase of the maximal pixel of p53 immunofluorescence (Fig. 4*E* and Ref. 8). We previously found by Western analysis that survival of mIMCD3 cells exposed to hypertonicity correlates with the amount of phospho-p53(Ser^{15}) (11). The fact that the p53(Ser^{15}) phosphorylation occurs at the G_1-S interface and during the S phase of the cell cycle suggests that p53 may protect against hypertonicity by affecting DNA replication.

During hypertonicity, p53 slows the G_1-S transition and reduces the rate of DNA synthesis. Hypertonicity slows all phases of the cell cycle, including the G_1-S transition and mitosis (26). To investigate further the role of p53 in these cell cycle changes, we labeled cells with BrDU, which is incorporated in place of thymidine during DNA replication (Fig. 5). Cells were labeled with BrDU during the last 30 min of 1-, 2-, and 4-h periods of hypertonicity. The BrDU content of S phase cells was taken as a measure of DNA replication rate. Hypertonicity decreased the DNA replication rate. p53-AS increased the DNA replication rate both in isotonic and hypertonic conditions (Fig. 5, *A* and *C*). To test the effect of p53-AS on the G_1-S transition, we analyzed the percentage of cells outside the G_1 phase (outside the area of G_1 cells shown in Fig. 5*A*). This parameter was found to be very convenient for the G_1-S transition analysis in hypertonic stress conditions because there is absolute G_2-M arrest (Fig. 2), preventing any cell entering in G_1 from G_2. If progression from G_1 to S were to continue under those conditions, the percentage of cells in G_1 should decrease, and the percentage outside of G_1 should increase. In case of G_1 arrest, the percentage of cells outside G_1 should not increase. As seen from Fig. 5*B*, there is no increase in the percentage of cells outside G_1 during the first 2 h with hypertonicity, meaning that G_1 arrest takes place. The decrease in this percentage is probably caused by predominant death from the S phase (Figs. 1 and 2). The G_1 arrest is transient, and by 4 h with hypertonicity (500 mosmol/kg) cells resume G_1-S transition that leads to their accumulation in G_2 by 6 h with hypertonicity (Fig. 1*A*). However, when the amount of p53 was reduced by a specific antisense oligonucleotide, hypertonicity increased the percentage of cells outside of G_1 (Fig. 5*B*), confirming that p53 is necessary for the hypertonicity to block movement of cells from G_1 into S.

When caffeine reduces p53, hypertonicity-induced cell cycle delay is inhibited, cell number decreases, and apoptosis increases. Caffeine inhibits gamma and ultraviolet radiation-induced phosphorylation of p53 on Ser^{15} by inhibition of ataxia telangiectasia, mutated and ataxia telangiectasia and Rad 3-related kinases (33). Therefore, we used caffeine as an additional way to test the role of p53-induced cell cycle delay in the response to hypertonicity. Caffeine (2 mM) reduces the level of p53 and phospho-p53(Ser^{15}), both under control and hypertonic conditions (Fig. 6*A*). Densitometry analysis showed that the level of p53 phosphorylation was reduced by caffeine both under control and hypertonic conditions (Fig. 6*A*). Associated with the decrease in p53, BrDU incorporation increases both at 320 and 500 mosmol/kg (Fig. 6, *D* and *E*). Also, the number of cells decreases by 6 h with hypertonicity, especially at 500 mosmol/kg (Fig. 6*B*), and many cells in early S and with >4 N DNA content become apoptotic at 500 mosmol/kg (Fig. 6*C*). Thus the effect of caffeine is

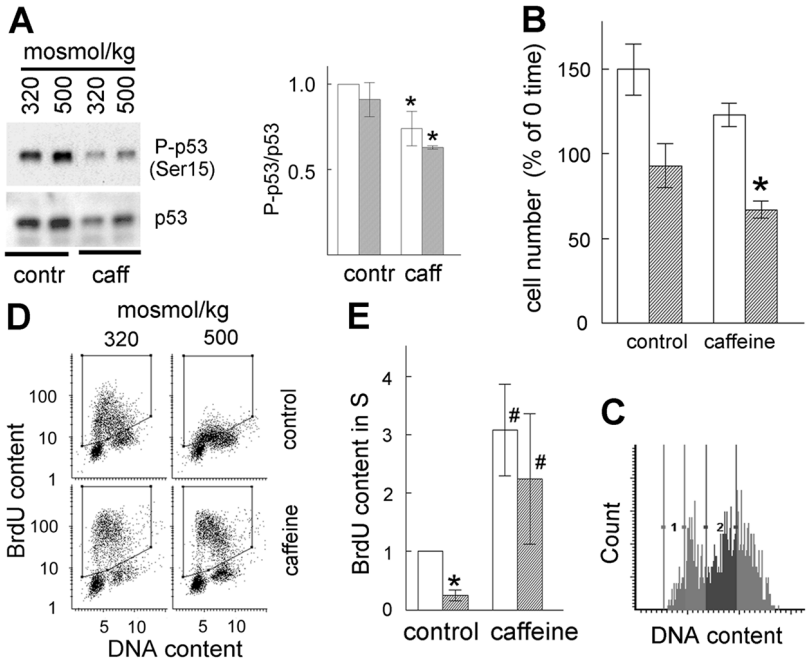

Fig. 6. Effect of caffeine on cells stressed by hypertonicity. Cells grown on slides at 320 mosmol/kg were preincubated with 2 mM of caffeine for 2 h, and then the medium was replaced with an identical one or an otherwise identical one made hypertonic to 500 mosmol/kg with added NaCl. *A*: Western analysis of p53 and phospho-p53 (Ser^{15}) after 1 h (*left*) and densitometry analysis of p53 phosphorylation level (*right*). Data are plotted as means ± SE; $n = 3$. *$P < 0.05$ vs. corresponding control. *B*: no. of nonapoptotic cells (PI max pixel not elevated) after 6 h. Cells were counted by LSC and normalized to *time 0*. Data are means ± SE; $n = 4$. *$P < 0.05$ vs. 320 mosmol/kg. *C*: cell cycle histogram of apoptotic cells with condensed (bright) chromatin, as in Fig. 1*C*. *D*: BrDU was added to the medium for the last 30 min. *E*: average BrDU content of S phase cells (inside the area shown in *D*). Data are normalized to the value at 320 mosmol/kg without caffeine at 1 h and are plotted as means ± SE. *$P <$ 0.05 vs. 320 mosmol/kg. #$P < 0.05$ vs. control.

similar to that of p53-AS, further supporting the hypothesis that p53-dependent cell cycle delay protects the cells against hypertonicity.

Inhibition of DNA replication protects cells from hypertonicity. The results thus far support the hypothesis that ongoing DNA replication sensitizes cells to hypertonicity. As a further test, we used the DNA polymerase inhibitor aphidicolin to test how inhibition of DNA replication affects cell survival under hypertonicity. We added aphidicolin 1 h before the stress to ensure that replication was inhibited at the time of stress application (*time 0*), and then aphidicolin was present in the media during the remainder of the experiment. As seen from Fig. 7*A*, after 6 h with hypertonicity without aphidicolin, cells accumulated in G_2 because, after the initial period of G_1 and S arrest, they resumed DNA replication. In the same conditions with aphidicolin, the cell cycle did not change because DNA synthesis was inhibited. Analysis of changes in cell number showed that aphidicolin prevented the increase in cell number under isotonic conditions and significantly improved survival after hypertonic stress (Fig. 7). Note that, on Fig. 7, the cell number is presented as a percentage of the cell number at *time 0*. Because absolute G_2-M arrest takes place during the first hours with hypertonicity (Fig. 2), cell number should not change in the absence of cell death, which is the case at 500 mosmol/kg aphidicolin. There is no improvement in cell survival at 700 mosmol/kg (the osmolality that cells cannot survive). This might indicate that the mechanism of cell death at 700 mosmol/kg is not related to DNA replication rate, which is consistent with the absence of p53 phosphorylation at Ser^{15} at this osmolality (11).

Thus decreasing entry into S phase and reducing DNA replication protect against hypertonic stress, whether as a result of activation of p53 or because of inhibition by aphidicolin.

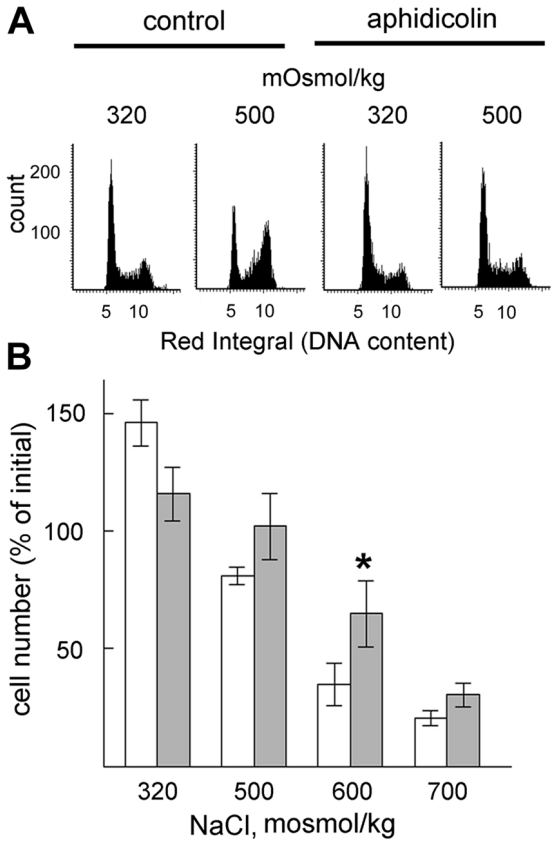

Fig. 7. Effect of aphidicolin on cell survival during hypertonicity. Cells grown on slides at 320 mosmol/kg were incubated with 1 μM aphidicolin for 1 h. Next, the medium was replaced with an identical one or an otherwise identical one in which the osmolality was increased to 500, 600, or 700 mosmol/kg with added NaCl for 6 h. Aphidicolin was present during the entire time of osmotic stress. Cells were fixed and stained with PI. *A*: representative cytograms showing cell cycle distribution at the end of the experiment. *B*: no. of nonapoptotic cells (PI max pixel not elevated) remaining after 6 h of experiment as a percentage of the corresponding cell number at *time 0*. Open bars, control; filled bars, aphidicolin added. Data are plotted as means ± SE; $n = 4$. *$P < 0.05$ vs. corresponding control without aphidicolin.

DISCUSSION

p53 protects cells from hypertonicity by inhibiting DNA replication and transition from G_1 to S. Hypertonicity inhibits cell cycle progression (7, 21, 26, 32). In the present studies, we found that this inhibition was overcome when expression of p53 was reduced by a p53-AS oligonucleotide or by caffeine. With reduced expression of p53, hypertonicity no longer slowed the G_1-S transition or decreased the rate of DNA synthesis. Also, with reduced p53 expression, hypertonicity caused some abnormal DNA synthesis in cells in G_2/M, resulting in >4 N DNA content. We previously found (11) that reduction of p53 expression in the face of hypertonicity causes apoptosis. Now we find that the apoptosis occurs predominantly in the cells with ongoing DNA replication in early S and with >4 N DNA content. We conclude that cells exposed to hypertonicity are protected by p53 in two ways. First, p53 prevents progression from G_1 to S, and, second, it reduces DNA replication rate in S. Similar conclusions were previously reached for other stresses; a number of studies demonstrated that, during S phase and especially at the G_1-S border, cells are particularly vulnerable to DNA damage and consequent mutagenesis upon exposure to genotoxic agents (18, 19).

Cell cycle dependence of nuclear vs. cytoplasmic p53 distribution and p53 phosphorylation on Ser^{15}. We find that the distribution of p53 between the nucleus and cytoplasm and the phosphorylation of p53 on Ser^{15} are cell cycle dependent (Fig. 4). The p53 becomes phosphorylated on Ser^{15} at the G_1-S interface, coincident with its movement into the nucleus, and remains phosphorylated throughout S, independent of hypertonicity. Reduction of the p53 level by p53-AS or caffeine causes an increase in DNA replication rate both under hypertonicity and in control conditions (Figs. 5 and 6). This is consistent with the possibility that p53 participates in coordination of S phase events, most likely related to DNA replication even in the absence of stress. Cell cycle checkpoint genes are essential for cell and organism survival (6, 24, 37), implying that these

pathways are not only surveyors of occasional damage but function in normal cellular physiology. Chromosome structural defects occur during normal cell duplication, even in the absence of exposure of DNA-damaging agents. For example, during DNA replication, errors, such as double-strand breaks, arise from stalled replication forks and require attention by the DNA damage response pathway (42). Dual roles have been proposed for several proteins, such as proliferating cell nuclear antigen (PCNA; see Ref. 23) and p53 (16), in maintaining genomic integrity by checking fidelity of DNA structure both at the basal level of stress that occurs normally and after exposure to external stress.

Experiments with caffeine also demonstrate effects both in unstressed and stressed cells. Among its other effects, caffeine inhibits ATM kinase (33), which is defective in cells from patients with ataxia telangiestasia (AT). In response to DNA damage, AT cells fail to increase phospho-p53(Ser^{15}) (33), fail to reduce both initiation of DNA replication and elongation of DNA (29), and fail to activate G_1 and G_2 checkpoints (17, 30). We find that caffeine reduces phospho-p53(Ser^{15}), even under isotonic conditions, and prevents its increase by hypertonicity (Fig. 6), similar to the effect of caffeine on the responses to ultraviolet radiation (33). Furthermore, we find that caffeine dramatically increases DNA replication rate, even under isotonic conditions, and prevents the inhibition of DNA replication that hypertonicity would otherwise cause.

Thus, p53, in concert with other factors, apparently monitors progression of DNA replication and inhibits replication when DNA is damaged. Hypertonicity causes DNA double-strand breaks (20). The mechanism is unclear, but one possibility is that hypertonicity may induce oxidative stress (41), which can in turn produce single- and double-stranded DNA breaks, DNA base and sugar modification, DNA B protein cross-links, and depurination and depyrimidination (2, 34). p53-Dependent cell cycle arrest presumably promotes cell survival by providing time for accurate repair of damaged DNA.

Recent reports suggest that p53 not only regulates cell cycle checkpoints but also activates DNA repair (42), supporting the proposed dual role for p53 in maintaining genomic integrity (16). According to this model, p53 acts to maintain genomic integrity whether or not it is activated by DNA damage. When p53 is activated, its major function is as a transcription factor for other genes. The target genes include *waf1*, which codes for the kinase inhibitor p21 that directs p53-dependent G_1 arrest (13). p21 Also acts in the S phase to slow DNA replication by binding to PCNA and blocking DNA elongation (38).

In addition to acting as a transcription factor when it is activated, p53 may also be directly involved in DNA repair, even when it is not activated. Cells in which p53 is lacking or mutated are deficient in repair of genomic DNA (35, 14, 39). p53 Interacts specifically with proteins that are components of DNA repair pathways. Most of these proteins are the members of the transcription factor IIH multiprotein complex, which regulates transcription initiation, nucleotide excision repair, and cell cycle progression (4, 12). p53 may be directly involved in DNA repair by binding to single- or double-stranded DNA breaks (36), to ends of double-strand breaks (3), and to DNA mismatches (22). Furthermore, the core domain of p53 has 3′,5′-exonuclease activity (27). The binding and exonuclease activities of p53 exist whether it is activated or not. These findings help put our observations into perspective. Thus we find that p53 translocates to the nucleus at the G_1-S interface, putting it into position to participate in monitoring DNA replication and to delay replication long enough to repair any damaged DNA that may have occurred. Hence, when DNA is damaged by hypertonicity, the increased abundance of phospho-p53(Ser^{15}) in the nucleus may reinforce its role in counteracting the stress.

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REFERENCES

1. **Agarwal ML, Taylor WR, Chernov MV, Chernova OB, and Stark GR.** The p53 network. *J Biol Chem* 273: 1–4, 1998.
2. **Ames BN and Shigenaga MK.** Oxidants are a major contributor to aging. *Ann NY Acad Sci* 663: 85–96, 1992.
3. **Bakalkin G, Selivanova G, Yakovleva T, Kiseleva E, Kashuba E, Magnusson KP, Szekely L, Klein G, Terenius L, and Wiman KG.** p53 binds single-stranded DNA ends through the C-terminal domain and internal DNA segments via the middle domain. *Nucleic Acids Res* 23: 362–369, 1995.
4. **Batty DP and Wood RD.** Damage recognition in nucleotide excision repair of DNA. *Gene* 241: 193–204, 2000.
5. **Bedner E, Li X, Gorczyca W, Melamed MR, and Darzynkiewicz Z.** Analysis of apoptosis by laser scanning cytometry. *Cytometry* 35: 181–195, 1999.
6. **Brown EJ and Baltimore D.** ATR disruption leads to chromosomal fragmentation and early embryonic lethality. *Genes Dev* 14: 397–402, 2000.
7. **Cohen DM and Gullans SR.** Urea induces Egr-1 and c-fos expression in renal epithelial cells. *Am J Physiol Renal Fluid Electrolyte Physiol* 264: F593–F600, 1993.
8. **Darzynkiewicz Z, Bedner E, Li X, Gorczyca W, and Melamed MR.** Laser-scanning cytometry: a new instrumentation with many applications. *Exp Cell Res* 249: 1–12, 1999.
9. **Deptala A, Li X, Bedner E, Chen W, Traganos F, and Darzynkiewicz Z.** Differences in induction of p53, p21-WAF1 and apoptosis in relation to cell cycle phase of MCF-7 cells treated with camptothecin. *Int J Oncol* 15: 861–871, 1999.
10. **Deptala A, Bedner E, Gorczyca W, and Darzynkiewicz Z.** Activation of nuclear factor kappa B (NK-kB) assayed by laser scanning cytometry (LSC). *Cytometry* 33: 376–382, 1998.
11. **Dmitrieva N, Kultz D, Michea L, Ferraris J, and Burg M.** p53 Activation by hypertonicity in renal inner medullary epithelial cells (mIMCD3) protects them from apoptosis. *J Biol Chem* 275: 18243–18247, 2000.
12. **Drapkin R and Reinberg D.** The multifunctional TFIIH complex and transcriptional control. *Trends Biochem Sci* 19: 504–508, 1994.
13. **el Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, and Vogelstein B.** WAF1, a potential mediator of p53 tumor suppression. *Cell* 75: 817–825, 1993.
14. **Ford JM and Hanawalt PC.** Expression of wild-type p53 is required for efficient global genomic nucleotide excision repair in UV-irradiated human fibroblasts. *J Biol Chem* 272: 28073–28080, 1997.
15. **Giaccia AJ and Kastan MB.** The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev* 12: 2973–2983, 1998.

16. **Janus F, Albrechtsen N, Dornreiter I, Wiesmuller L, Grosse F, and Deppert W.** The dual role model for p53 in maintaining genomic integrity. *Cell Mol Life Sci* 55: 12–27, 1999.
17. **Kastan MB, Zhan Q, el Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B, and Fornace AJ Jr.** A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* 71: 587–597, 1992.
18. **Kaufmann WK, Rahija RJ, MacKenzie SA, and Kaufman DG.** Cell cycle-dependent initiation of hepatocarcinogenesis in rats by (+/−)-7r,8t-dihydroxy-9t,10t-epoxy-7,8,9,10-tetrahydrobenzo(a)py rene. *Cancer Res* 47: 3771–3775, 1987.
19. **Kaufmann WK and Wilson SJ.** G1 arrest and cell-cycle-dependent clastogenesis in UV-irradiated human fibroblasts. *Mutat Res* 314: 67–76, 1994.
20. **Kultz D and Chakravarty D.** Hyperosmolality in the form of elevated NaCl but not urea causes DNA damage in murine kidney cells. *Proc Natl Acad Sci USA* 98: 1999–2004, 2001.
21. **Kultz D, Madhany S, and Burg MB.** Hyperosmolality causes growth arrest of murine kidney cells. Induction of GADD45 and GADD153 by osmosensing via stress-activated protein kinase 2. *J Biol Chem* 273: 13645–13651, 1998.
22. **Lee S, Elenbaas B, Levine A, and Griffith J.** p53 And its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches. *Cell* 81: 1013–1020, 1995.
23. **Li R, Waga S, Hannon GJ, Beach D, and Stillman B.** Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. *Nature* 371: 534–537, 1994.
24. **Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, Donehower LA, and Elledge SJ.** Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes Dev* 14: 1448–1459, 2000.
25. **Meek DW.** Post-translational modification of p53 and the integration of stress signals. *Pathol Biol (Paris)* 45: 804–814, 1997.
26. **Michea L, Ferguson DR, Peters EM, Andrews PM, Kirby MR, and Burg MB.** Cell cycle delay and apoptosis are induced by high salt and urea in renal medullary cells. *Am J Physiol Renal Physiol* 278: F209–F218, 2000.
27. **Mummenbrauer T, Janus F, Muller B, Wiesmuller L, Deppert W, and Grosse F.** p53 Protein exhibits 3′-to-5′ exonuclease activity. *Cell* 85: 1089–1099, 1996.
28. **Oren M.** Regulation of the p53 tumor suppressor protein. *J Biol Chem* 274: 36031–36034, 1999.
29. **Painter RB and Young BR.** Radiosensitivity in ataxia-telangiectasia: a new explanation. *Proc Natl Acad Sci USA* 77: 7315–7317, 1980.
30. **Paules RS, Levedakou EN, Wilson SJ, Innes CL, Rhodes N, Tlsty TD, Galloway DA, Donehower LA, Tainsky MA, and Kaufmann WK.** Defective G2 checkpoint function in cells from individuals with familial cancer syndromes. *Cancer Res* 55: 1763–1773, 1995.
31. **Rauchman MI, Nigam SK, Delpire E, and Gullans SR.** An osmotically tolerant inner medullary collecting duct cell line from an SV40 transgenic mouse. *Am J Physiol Renal Fluid Electrolyte Physiol* 265: F416–F424, 1993.
32. **Santos BC, Chevaile A, Hebert MJ, Zagajeski J, and Gullans SR.** A combination of NaCl and urea enhances survival of IMCD cells to hyperosmolality. *Am J Physiol Renal Physiol* 274: F1167–F1173, 1998.
33. **Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM, and Abraham RT.** Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. *Cancer Res* 59: 4375–4382, 1999.
34. **Shackelford RE, Kaufmann WK, and Paules RS.** Oxidative stress and cell cycle checkpoint function. *Free Radic Biol Med* 28: 1387–1404, 2000.
35. **Smith ML, Ford JM, Hollander MC, Bortnick RA, Amundson SA, Seo YR, Deng CX, Hanawalt PC, and Fornace AJ Jr.** p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes. *Mol Cell Biol* 20: 3705–3714, 2000.
36. **Steinmeyer K and Deppert W.** DNA binding properties of murine p53. *Oncogene* 3: 501–507, 1988.
37. **Takai H, Tominaga K, Motoyama N, Minamishima YA, Nagahama H, Tsukiyama T, Ikeda K, Nakayama K, Nakanishi M, and Nakayama K.** Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice. *Genes Dev* 14: 1439–1447, 2000.
38. **Waga S, Hannon GJ, Beach D, and Stillman B.** The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* 369: 574–578, 1994.
39. **Wang XW, Yeh H, Schaeffer L, Roy R, Moncollin V, Egly JM, Wang Z, Freidberg EC, Evans MK, and Taffe BG.** p53 modulation of TFIIH-associated nucleotide excision repair activity. *Nat Genet* 10: 188–195, 1995.
40. **Zhan Q, Chen IT, Antinore MJ, and Fornace AJ Jr.** Tumor suppressor p53 can participate in transcriptional induction of the GADD45 promoter in the absence of direct DNA binding. *Mol Cell Biol 18: 2768–2778, 1998 (published erratum appears in Mol Cell Biol* 18: 5620, 1998).
41. **Zhang Z, Yang XY, and Cohen DM.** Urea-associated oxidative stress and Gadd153/CHOP induction. *Am J Physiol Renal Physiol* 276: F786–F793, 1999.
42. **Zhou BB and Elledge SJ.** The DNA damage response: putting checkpoints in perspective. *Nature* 408: 433–439, 2000.